7-17-2013

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Arch Environ Contam Toxicol (2014) 66:120–138
DOI 10.1007/s00244-013-9934-0

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Received: 7 March 2013 / Accepted: 11 June 2013 / Published online: 17 July 2013

Abstract The exposure and effects of perfluoroalkyl substances (PFASs) were studied at eight locations in Minnesota and Wisconsin between 2007 and 2011 using tree swallows (*Tachycineta bicolor*). Concentrations of PFASs were quantified as were reproductive success end points. The sample egg method was used wherein an egg sample is collected, and the hatching success of the remaining eggs in the nest is assessed. The association between PFAS exposure and reproductive success was assessed by site comparisons, logistic regression analysis, and multistate modeling, a technique not previously used in this context. There was a negative association between concentrations of perfluorooctane sulfonate (PFOS) in eggs and hatching success. The concentration at which effects became evident (150–200 ng/g wet weight) was far lower than effect levels found in laboratory feeding trials or egg-injection studies of other avian species. This discrepancy was likely because behavioral effects and other extrinsic factors are not accounted for in these laboratory studies and the possibility that tree swallows are unusually sensitive to PFASs. The results from multistate modeling and simple logistic regression analyses were nearly identical. Multistate modeling provides a better method to examine possible effects of additional covariates and assessment of models using Akaike information criteria analyses. There was a credible association between PFOS concentrations in plasma and eggs, so extrapolation between these two commonly sampled tissues can be performed.

Electronic supplementary material The online version of this article (doi:10.1007/s00244-013-9934-0) contains supplementary material, which is available to authorized users.

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Perfluoroalkyl substances (PFASs) have been widely used in commercial products, such as water and stain repellents, nonstick cookware, surfactants, polymers, and wetting agents (United States Environmental Protection Agency [USEPA] 2000). The Minnesota Mining and Manufacturing (3M) Company was the primary global producer of perfluorooctane sulfonate (PFOS)–related PFASs and perfluorooctanoate (PFOA), which they manufactured at plants in Cottage Grove, MN, a suburb in the Minneapolis/St. Paul metropolitan area (metro hereafter referred to as Twin Cities [Fig. 1], Oliaei et al. 2006). They produced PFOS-related fluorochemicals during the 1950s and 1960s and continued after that to produce these chemicals in pilot-scale projects. The Cottage Grove plant was also a major producer of PFOA. After evidence of global exposure to PFOS among wildlife and humans (Giesy and Kannan 2001), 3M began phasing out production of PFOA- and PFOS-related products in 2000 (USEPA 2000).

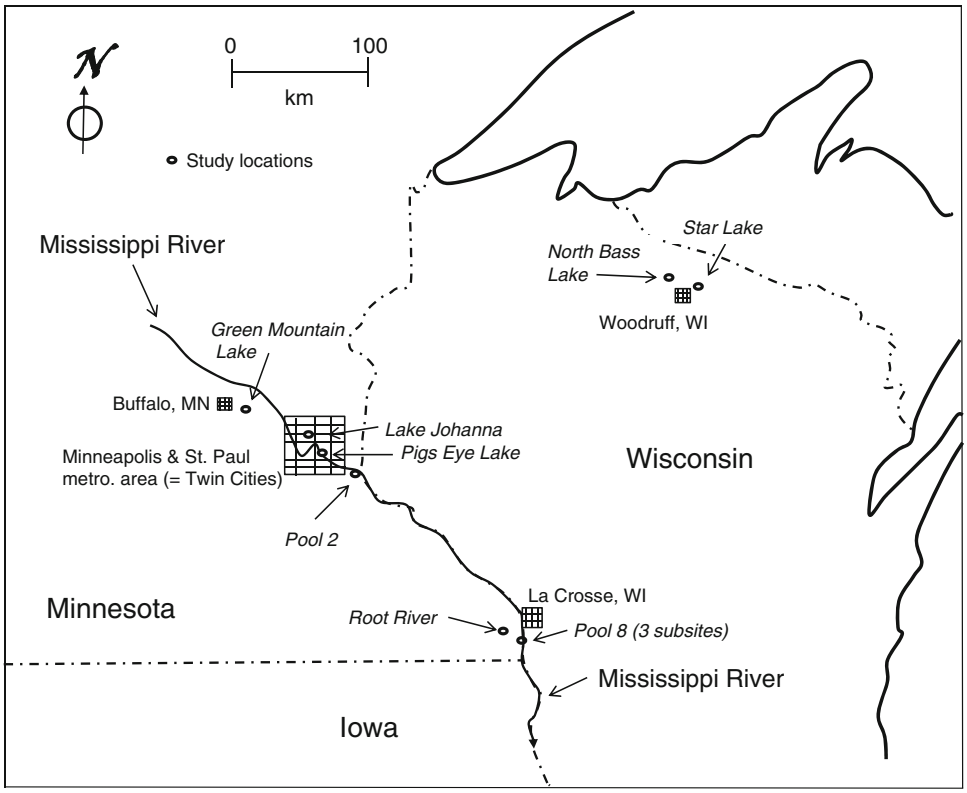

Fig. 1 Map of eight tree swallow study sites in Minnesota and Wisconsin from 2007 to 2011. Pool 8 location had three subsites

Production of many PFASs at Cottage Grove ceased in 2002, although perfluorobutane sulfonate (PFBS) is still being produced there (Oliaei et al. 2006). For a concise overview of PFAS chemistry and production history, see Lindstrom et al. (2011).

The Mississippi River near and downstream of St. Paul, MN, is highly contaminated with PFASs. Contamination of the Mississippi River with PFASs, including the Pigs Eye Lake area, was through direct wastewater discharges, contaminated groundwater, and surface water runoff from the manufacturing plant and various disposal areas (Oliaei et al. 2006; Nakayama et al. 2010; Custer et al. 2013a). A source of PFAS contaminants to Pigs Eye Lake is the Pigs Eye landfill, a site that for years was used by 3M to dispose of incinerator ash containing PFASs (Oliaei et al. 2006). Common carp (*Cyprinus carpio*) fillets had median concentrations of PFOS of 28 and 47 ng/g wet weight (ww) at Pigs Eye Lake and Spring Lake (Ye et al. 2008a). A reference site for that study, located farther upstream on the Mississippi River approximately 100 km downriver at Brainerd, MN, averaged only 8 ng /g ww PFOS. Elevated PFAS concentrations in bald eagle (*Haliaeetus leucocephalus*) nestlings were found in the Mississippi River in the reach below the Twin Cities (Route et al. 2011) as well as in great blue heron (*Ardea herodias*) eggs (Custer et al. 2013a) from a colony near Pigs Eye Lake. High concentrations of PFASs have also been reported in tree swallows (*Tachycineta bicolor*) nesting on a suburban lake in the Twin Cities area (Custer et al. 2012a), although the source for that contamination remains unknown. In that study, a negative association was found between PFAS concentrations and hatching success of eggs at levels much lower than those found in most laboratory studies using birds. Because of small sample size, the investigators suggested that additional studies be performed to further test this association.

Tree swallows have been increasingly used to assess contaminant exposure and effects (see review in Custer 2011) because they can be attracted to a specific location with nest boxes, they feed within a localized area (<1 km; Quinney and Ankney 1985), and they are relatively easy to sample. They are a mid-level consumer and feed on the aerial stage of benthic aquatic insects, so contaminants in swallow tissues are closely tied to sediment contamination levels. Swallows offer a contrast, because of their diet, to the mainly piscivorous colonial water bird and raptor species that have been the predominant study species to date for PFASs. It is also easy to study reproductive success in tree swallows compared with many of these other species. Their nest boxes can be placed to be easily accessible; they can be protected from most predators; and repeated visits to each nest can be made without adversely affecting reproductive outcomes.

The objective of this study was to assess the detection and concentration of PFASs at various types of wetlands, and across urban and industrial locations in Minnesota and Wisconsin. It was postulated that locations in or near the Twin Cities area, with numerous possible connections to wastewater effluent, groundwater connections, and direct industrial inputs, would be one of the most highly contaminated areas with PFASs. Isolated lakes with limited connection to other water bodies should be the least contaminated. Locations on the Mississippi River, downstream of the Twin Cities, should be moderately contaminated. Additional objectives included further testing of whether there is a negative association between PFAS exposure and reproductive success and investigating the distribution of PFASs across tissues types (eggs, nestling plasma, nestling carcasses, and nestling diet). Models for predicting PFAS concentrations in eggs based on concentrations in nestling plasma were then developed. It is important to establish this relation because plasma, rather than egg information, is sometimes the only tissue for which data are available, especially for species of concern, such as eagles (Henny and Elliott 2007; Route et al. 2011). Plasma is the matrix of choice for this group of birds because it can be acquired nondestructively and has the added advantage of better detection limits (C. M. C. personal observation) and can be collected during the course of other work, such as banding and health assessments. Because hatching success is the preferred metric to assess contaminant effects on avian populations, an estimate of egg concentrations is needed.

Materials and Methods

Field Work

Tree swallow nest boxes, between 18 and 37 boxes/site (average 22), were erected at 8 locations in MN and WI between 2007 and 2011 (Fig. 1). Pigs Eye Lake (44°55′38.36″N, 93°01′38.92″W), pool 2 (44°45′26.20″, 92°52′21.83″), and pool 8 (43°40′31.87″, 91°16′35.60″) were located on the Mississippi River. Pool 8 had three subsites that were along a 5-km stretch of the river. Green Mountain Lake (45°09′45.40, 93°47′21.90″), and two lakes in northern Wisconsin—Star Lake (46°01′26.86″, 89°28′12.77″ [pH 7.57] and North Bass Lake (46°11′26.24″, 89°57′29.06″ [pH 5.54])—are geographically isolated lakes. The Root River (43°45′52.86″, 91°21′55.53″) is a lightly populated tributary that runs into the Mississippi River. These four sites served as reference areas. The final site, Lake Johanna (42°02′37.96″, 93°10′15.41″), which is located in the Twin Cities area but is not connected to the Mississippi River, has a history of PFAS contamination (Custer et al. 2012a). Nest boxes were placed on metal fence posts approximately 20 m apart and protected from ground predators using metal cylinders. Each nest box was visited approximately once per week, and the number of eggs and nestlings present were recorded for each nest box during each visit. The nests were visited until the young reached approximately 12 days of age (range 10–13) at which time weekly visits were terminated. During those visits, the age of the female was opportunistically assessed and classified into one of three categories: 1 year old (brown plumage), >1 year old (blue plumage), or not determined. Plumage coloration was categorized according to Hussell (1983). The period when eggs were present in the nest is called the "incubation period," and the "care-of-young period" started when the first egg in the nest hatched.

After egg laying was completed and usually during mid-incubation, 1 egg/clutch (except 2 eggs/clutch in 2011) was randomly collected from nest boxes for chemical analysis. Although there can be variation in contaminant exposure within a clutch, the variation is not consistently associated with egg order (Custer et al. 2010a, 2012a). In addition, within-clutch variation is 5 to 7 times less than among-clutch variation (Custer et al. 1990), so concentrations in a random egg sample are representative of the entire clutch and can be used in the sample egg context as performed here. Between 5 and 20 nests (average 11; Table 1) were sampled/location, with the exact number sampled being dependent on the number of nests present and availability of funds for chemical analysis. The eggs collected for contaminant analyses were weighed on an electronic pan balance (0.01 g), measured (length and width [0.01 mm, two measurements for width]) with calipers, and the contents emptied into chemically clean jars. The egg contents were weighed, and the stage of embryonic development and whether the embryo was alive (viable) or dead was noted. Egg samples were then frozen (<−25 °C) until chemical analysis. Any unhatched eggs were collected and opened, and the stage at which embryo death occurred was noted. Eggs that were addled, i.e., with a variable amount of decomposition and with no obvious embryo visible, were not assigned a stage but rather were classified as infertile eggs. The infertile category could also include unhatched eggs where the embryo died at a very early age (<1–2 days old) before the developing embryo was visible to the naked eye. Either way, infertile or dead, this was a negative outcome. Eggs that disappeared were assumed not to have hatched. Eggs were classified as depredated if shell fragments were found on the ground or in the nest box.

Two nestlings were collected from 5 to 12 nest boxes/location when the nestlings were approximately 12 days old (range 11–14 with >75 % collected at 12 days of age). If only two nestlings were present in the nest, then only one nestling was collected. After being weighed on an electronic pan balance, each nestling was decapitated (Anonymous 2007) and the blood collected in a heparinized tube. The

Table 1 Geometric mean concentrations, 95 % CIs, and samples sizes for total PFASs in tree swallow tissues and diet from locations in Minnesota and Wisconsin from 2007 to 2011

Locations, years, and sample size for each matrix	Geometric means (ng/g ww), significant differences, and 95 % CIs[a]			
	Egg	Plasma	Carcass	Diet
Pigs Eye Lake (2010),[Nb] $N = 10, 10, 8, 2$	227 (181–286)^{ABa}	352 (257–481)^{A}	106 (87.9–127)^{A}	53.6 (3.45–835)^{AB}
Pigs Eye Lake (2011),[N] $N = 10, 10, 8, 2$	418 (289–605)^{A}	437 (341–560)^{A}	123 (69.5–219)^{A}	67.8 (5.55–829)^{A}
Lake Johanna (2008),[A] $N = 5, 3, 3, 2$	170 (106–274)^{BC}	151 (98.1–232)^{B}	44.6 (32.4–61.4)^{B}	−(1 ND–27.8)
Lake Johanna (2009),[A] $N = 13, 12, 12, 12$	180 (134–241)^{BC}	170 (143–201)^{B}	42.3 (35.3–50.6)^{B}	24.4 (15.8–37.6)^{AB}
Pool 2 dam (2009),[N] $N = 8, 6, 6, 6$	59.7 (31.6–113)^{D}	144 (97.7–212)^{BC}	29.5 (19.9–43.7)^{BC}	17.7 (12.2–25.5)^{AB}
Pool 2 dam (2010),[A] $N = 16, 10$	99.2 (80.0–123)^{CD}	133 (93.1–190)^{BC}		
Pool 8, Lawrence (2007),[N] $N = 4$		125 (104–149)^{BCD}		
Pool 8, Schnicks (2007),[N] $N = 4$		173 (119–250)^{B}		
Pool 8, Shellhorn (2007),[N] $N = 5$		129 (102–163)^{BCD}		
Green Mountain Lake (2010),[N] $N = 10, 10, 8, 2$	20.6 (13.6–31.0)^{E}	76.5 (59.6–98.2)^{CDE}	18.4 (13.1–25.8)^{C}	11.5 (7.97–16.5)^{B}
Green Mountain Lake (2011),[N] $N = 10, 10, 8, 2$	6.35 (4.02–10.0)^{F}	26.8 (23.3–30.9)^{FG}	6.80 (6.34–7.28)^{D}	9.89 (2.86–34.2)^{B}
Star Lake (2008),[A] $N = 5$		65.8 (49.9–86.7)^{DE}		
Root River (2007),[N] $N = 5$		51.9 (39.8–67.7)^{EF}		
North Bass Lake (2008),[A] $N = 5$		13.7 (11.5–16.4)^{G}		

[a] Means sharing the same superscript capital letter are not significantly different among locations within each matrix. Significant differences (one-way ANOVA) among locations by matrix type individually. $P < 0.001$ for egg, serum and carcass, and $P = 0.013$ for diet

[b] Superscript letter "A" at site name indicates samples analyzed by Axys Analytical Services, and superscript letter "N" at site name indicates samples analyzed by New York State Department of Health

tubes were centrifuged for ~10 min and the plasma decanted for PFAS analysis. The plasma from the two nestlings was pooled by nest box to provide sufficient volume for chemical analysis. The contents of the stomach (diet) were removed from each nestling carcass, pooled by nest box, and frozen for analysis. Prior to chemical analysis, stomach contents were further pooled into two samples per location so that adequate sample mass (>2 g) was available for PFAS analysis (but see exceptions in Table 1 when individual diet samples were analyzed). At some sites, diet samples were analyzed for other organic contaminants and therefore were not available for PFAS analysis. The carcass remainders were frozen individually, and one carcass from each nest box was analyzed for PFASs.

Chemical Analyses

Samples were analyzed for a suite of PFASs at Wadsworth Center, New York State Department of Health, Albany, NY, or at Axys Analytical Services, Ltd., British Columbia, Canada (Axys) using comparable methods. The samples analyzed by each of the two laboratories are listed in Table 1 and included 10 target PFASs: PFBS, perfluorohexane sulfonate (PFHxS), PFOS, perfloroheptanoate (PFHpA), PFOA, perfluorononanoate (PFNA), perfluorodecanoate (PFDA), perfluoroundecanoate (PFUnDA), perfluorododecanoate (PFDoDA), and perfluorooctane sulfonamide (PFOSA). Perfluorobutane sulfonate (PFBS), PFHxS, PFOS, and PFOSA are collectively referred to as PFSAs; PFHpA, PFOA, PFNA, PFDA, PFUnDA, and PFDoDA are collectively referred to as PFCAs.

Details of the analytical methods used at the Wadsworth Center are presented in Tao et al. (2006). In brief, samples were homogenized with 5 mL of Milli-Q water and then analyzed by an ion-pair extraction method. Analytes were determined by use of a high-performance liquid chromatograph (HPLC) coupled with an electrospray tandem mass spectrometer (HPLC-MS/MS). Analyte separation was performed using an Agilent (Santa Clara, CA) 1100 HPLC. Ten microliters of extract was injected onto a 100 × 2.1-mm (5 μm; Thermo Electron, Bellefonte, PA) Betasil C18 column connected serially with a Javelin guard column (20 × 2.1 mm). Analytes were quantitatively determined by Applied Biosystems (Foster City, CA) API 2000 MS/MS operated in the electrospray negative ionization mode using multiple reaction monitoring. Method blanks and matrix spikes were analyzed throughout the entire analytical procedure. Matrix spikes ($n = 9$) were prepared by spiking known amounts of native PFAS standards into selected samples. Percent recovery of the PFASs averaged 107 % and ranged from 41 % (PFBS) to 160 % (PFDoDA). Concentrations were not corrected for matrix spike recoveries. Reported concentrations of PFASs in eggs were corrected for the recoveries of internal standards and subtracted from mean

blank values if necessary. Internal standards included $^{18}O_2$-PFHxS, $^{13}C_4$-PFOS, $^{13}C_4$-PFOA, $^{13}C_2$-PFNA, $^{13}C_2$-PFDA, $^{13}C_2$-PFUnDA, and $^{13}C_2$-PFDoDA.

The Axys Analytical Services laboratory measured the same 10 PFASs mentioned previously. In brief, surrogate standards of $^{13}C_4$-PFOS, $^{13}C_2$-PFOA, $^{13}C_5$-PFNA, $^{13}C_2$-PFDA, and $^{13}C_2$-PFDoA (Sigma Aldrich and Chiron, Norway) were added and the sample was extracted by shaking with methanolic potassium hydroxide solution. After centrifugation, one aliquot of the supernatant was diluted with water and purified by solid-phase extraction (SPE) containing a weak anion exchange sorbent (Oasis; Waters, Ireland). The eluate was spiked with two recovery standards, $^{13}C_2$-2H-perfluoro-2-decenoicacid and $^{13}C_4$-PFOA (see manufacturers previously mentioned), and analyzed on an HPLC [Waters (Milford, MA) 2690 or Waters 2795] coupled to a triple quadrupole MS-MS (Micromass Quattro Ultima). The LC column was a Waters Xtera C_{18}MS reverse-phase C_{18}, 2.1 × 100-mm with 3.5-μm particles. The MS was operated in negative electrospray ionization ion mode and at unit mass resolution using multiple reaction monitoring. Analyte concentrations were determined by isotope dilution/internal standard method comparing the area of the quantification ion with that of the ^{13}C-labelled standards and correcting for response factors. The 8 carboxylates were quantified against $^{13}C_2$-PFDA, PFOA against $^{13}C_2$-PFOA, and sulfonates against $^{13}C_4$-PFOS. Matrix spikes ($n = 9$) were also analyzed. Percent recovery for the PFASs ($n = 9$) averaged 97.9 % and ranged from 76.8 % (PFOSA) to 110 % (PFBS). Percent recovery averaged 99.9 % for the PFOSs. Concentrations were not corrected for matrix spike recoveries.

Both laboratories had extensive quality control and assurance procedures, used spiked samples to assess recovery, and have participated in extensive round robin exercises, which assure us of comparability of results between the two laboratories. In addition, we tested for differences in concentrations for individual analytes and total PFASs in plasma from Pool 2 where samples were analyzed by both laboratories. There was no significant difference between laboratories in concentrations of 6 of 7 analytes nor for total PFASs. Only PFHxS was greater (2.01 ng/g) as analyzed by Axys compared with New York Department of Health (0.89 ng/g). This congener was only a minor component of total PFASs.

Total PFASs refers to the sum of all of the individual PFAS concentrations greater than the detection limit (DL) for that sample. DLs varied by individual PFAS and tissue type (Table 2). In plasma, nearly all PFCAs had detectable concentrations with DLs between 0.50 and 0.75 ng/mL. The DL for PFOSA was lower, between 0.2 and 0.5 ng/mL, but few samples had detectable concentrations even at those DLs. The DLs for PFSAs in plasma were between 0.2 and 1.0 ng/mL and tended to be greater than the PFCAs because greater concentrations in samples did not require lower DLs. Eggs, carcasses, and diet samples generally had greater DLs than plasma either because of the nature of the tissue or because of smaller sample mass. In general, DLs in carcasses were between 0.5 and 2.5 ng/g ww for PFCAs and between 0.5 and 5.0 ng/g ww for PFSAs and varied by year. In eggs, DLs ranged between 0.8 and 6.0 ng/g ww for PFCAs and between 0.5 and 12 ng/g ww for the PFSAs. The differences between years in DLs were mostly inconsequential, i.e., PFAS congeners were not detected even at the lowest DL. The exception was at Green Mountain Lake where there were some (<50 %) detects at the lowest DL but none at the greater DL. See Table 2 for DL specifics.

Statistical Analyses and Modeling

Exposure

Exposure data were analyzed with a combination of univariate and multivariate statistical assessments. Concentrations of total PFASs were compared first with two-way analysis of variance (ANOVA; year, location, and year × location interaction) for those locations with multiple years to determine whether either factor could be eliminated in subsequent statistical analyses. Because PFAS concentrations commonly differ among tissue types (Custer et al. 2012a), the two-way ANOVAs were performed on each tissue type (egg, plasma, nestling carcass, and diet) separately. Because year did not differ for any location except for Green Mountain, years were combined for subsequent multivariate statistical analyses.

To assess differences in the pattern of the eight PFASs among locations that had >50 % of samples greater than the DL, and to determine which of the PFASs were responsible for location differences, the percent that each PFAS congener comprised of the total PFASs (hereafter referred to as "pattern differences") in plasma samples was compared among locations using the multivariate analysis of similarity (ANOSIM [Clarke and Warwick 2001]), followed by similarity percentage procedures (SIMPER), and visualized with nonmetric multidimensional scaling plots. The R statistic generated from ANOSIM is considered statistically significant based on its P value (*e.g.*, $P < 0.05$). Because R can be significantly different from zero, yet inconsequentially small, the size of R indicates the degree of difference and was the metric used to draw inferences. Clear differences in patterns are evident when R is ≥0.4. There is some support for pattern differences when R is ≥0.3–<0.4, and patterns barely differ when R is <0.3 (adapted from Clarke and Warwick 2001). The SIMPER subroutine calculated which PFAS congener contributed to the differences between

Table 2 Concentrations (geometric mean [ng/g ww] and minimum/maximum) of individual PFASs in tree swallow tissues and diet collected in Minnesota and Wisconsin from 2007 to 2011

Tissue and PFAS congener	Location							
	Pigs Eye Lake	Lake Johanna	Pool 2	Pool 8	Root River	Green Mountain Lake	Star Lake	North Bass Lake
Plasma	$N = 20$	$N = 15$	$N = 16$	$N = 13$	$N = 5$	$N = 20$	$N = 5$	$N = 5$
PFOS	295	137	118	120	40.2	17.3	20.0	6.23
	117–640	75.6–190	60.9–259	82–203	32–52	9.36–41.3	16.1–30.0	5.56–7.00
PFHxS	2.58	10.3	1.48	1.73	1.37	0.13	–	–
	1 ND[a] (0.2)–5.24	5.03–19.2	1 ND (1.50)–4.28	1.04–2.69	1.13–1.79	6 ND (0.20)–0.27	5 ND (1.0)	5 ND (1.0)
PFOA	77.3	2.67	5.90	5.34	4.52	3.79	21.6	2.25
	28.0 – 181	1.52–8.32	1.39–14.9	2.70–10.1	3.66–5.65	0.92–9.70	14.1–31.7	1.93–2.60
PFNA	4.55	3.41	4.82	6.71	2.85	15.3	15.5	2.68
	1.17–7.80	1.81–7.62	1 ND (0.72)[b]–14.0	4.36–9.40	2.29–3.85	4.62–68.0	12.3–20.8	2.18–3.11
PFDA	5.78	6.81	3.66	3.70	1.32	4.29	2.64	0.79
	1.34–8.54	3.43–13.3	1 ND (0.75)–9.28	2.44–5.86	1.13–1.76	1.85–12.2	2.42–2.87	0.51–1.03
PFUnDA	1.71	2.67	1.45	1.86	1.42	2.11	5.07	1.74
	0.62–2.74	1.18–5.63	1 ND (0.75)–2.69	1.14–3.24	0.97–1.76	0.95–3.63	4.57–5.47	1.32–2.18
PFDoDA	0.78	1.63	0.81	–	–	0.18	0.74	–
	1 ND (0.2)–1.25	0.69–4.33	1 ND (0.75)–1.64	10ND (0.50)–1.04	4 ND (0.50)–0.54	3 ND (0.22)–0.32	0.66–0.92	5 ND (0.50)
PFOSA	0.59	–	–	–	–	–	–	–
	1 ND (0.17)–0.93	14 ND (0.50)–0.60	16 ND (0.20)	13 ND (0.20)	5 ND (0.20)	12 ND (0.05)[c]– 0.07	5 ND (0.50)	5 ND (0.50)
Eggs	$N = 20$	$N = 18$	$N = 24$			$N = 20$		
PFOS	270	150	76.4			6.39		
	131–847	57.9–285	16.4–248			1.55–30.0		
PFHxS	0.95	–	–			–		
	0.35–3.18	16 ND (12.0)–13.7	24 ND (0.5)[d]			17 ND (0.05)[e]– 0.12		
PFOA	18.7	–	1.21			0.44		
	4.4–58.8	18 ND (6.0)	4 ND (0.75)–3.05			7 ND (0.40)–1.13		
PFNA	3.10	–	1.02			1.90		
	1.48–6.66	16 ND (6.0)–10.2	5 ND (0.90)–2.94			0.58–30.0		
PFDA	5.47	5.99	2.21			0.97		
	3.03–18.5	7 ND (6.0)–14.0	1 ND (0.90)–5.82			4 ND (0.44)–11.75		
PFUnDA	2.61	–	1.49			1.13		
	0.86–9.63	13 ND (6.0)–7.45	1 ND (0.4)–4.74			0.46–5.05		
PFDoDA	1.96	–	1.05			0.30		
	0.55–4.63	17 ND (6.0)–6.17	4 ND (0.5)–3.97			2 ND (0.2)–1.16		
PFOSA	0.86	–	–			–		
	0.34–3.59	18 ND (0.5)	24 ND (0.7)			13 ND (0.05)[f]–0.058		

Table 2 continued

Tissue and PFAS congener	Location							
	Pigs Eye Lake	Lake Johanna	Pool 2	Pool 8	Root River	Green Mountain Lake	Star Lake	North Bass Lake
Carcass	$N = 16$	$N = 15$	$N = 6$			$N = 16$		
PFOS	90.4	41.8	27.4			4.91		
	33.9–198	23–62.9	15.1–39.0			2.60–11.5		
PFHxS	0.38	–	–			–		
	0.10–1.45	14 ND (4.9)–4.99	6 ND (0.4)			16 ND[g]		
PFOA	18.1	–	0.98			0.85		
	7.92–41.2	15 ND (2.5)	1 ND (0.4)–1.27			4 ND (0.4)–2.41		
PFNA	0.88	–	0.53			3.36		
	1ND (0.3)–1.96	15 ND (2.5)	3 ND (0.6)–1.10			0.77–14.3		
PFDA	1.34	–	0.74			0.89		
	0.60–2.91	11 ND (2.5)–4.01	1 ND (0.7)–1.52			2 ND (0.4)–2.98		
PFUnDA	0.25	–	–			0.46		
	0.14–1.11	15 ND (2.5)	6 ND (0.3)			0.21–0.66		
PFDoDA	0.23	–	–			–		
	1 ND (0.2)–0.66	15 ND (2.5)	6 ND (0.4)			13 ND (0.05)[h]–0.05		
PFOSA	0.63	–	–			–		
	0.32–1.03	15 ND (2.5)	6 ND (0.3)			10 ND (0.05)[i]–0.08		

Sample sizes as provided for each tissue type as are the number lower than the detection limit (ND) when applicable

ND not detected, *PFOS* perfluorooctane sulfonate, *PFHxS* perfluorohexane sulfonate, *PFOA* perfluorooctanoate, *PFNA* perfluorononanoate, *PFDA* perfluorodecanoate, *PFUnDA* perfluoroundecanoate, *PFDoDA* perfluorododecanoate, *PFOSA* perfluorooctane sulfonamide

[a] The number preceding ND is the number lower than the detection limit (DL). The DL is in parentheses

[b] DL = 0.20 in 2009 and 0.72 in 2011

[c] DL = 0.05 in 2010, and 7 of 10 were >DL. DL = 0.17 in 2011, and 0 of 10 were >DL

[d] DL = 0.50 in 2009, and 0 of 8 were >DL. DL = 1.5 in 2010, and 0 of 16 were >DL

[e] DL = 0.05 in 2010, and 3 of 10 were >DL. DL = 0.20 in 2011, and 0 of 10 were >DL

[f] DL = 0.05 in 2010, and 3 of 10 were >DL. DL = 0.17 in 2011, and 0 of 10 were >DL

[g] DL = 0.05 in 2010, and 0 of 8 were >DL. DL = 0.2 in 2011, and 0 of 8 were >DL

[h] DL = 0.05 in 2010, and 3 of 8 were >DL. DL = 0.2 in 2011, and 0 of 8 were >DL

[i] DL = 0.05 in 2010, and 6 of 8 were >DL. DL = 0.17 in 2011, and 0 of 8 were >DL

locations. The pattern analyses factored out the effect of actual concentration. Finally, the effect of tissue type on the proportion of PFOS, the predominant PFAS, to total PFASs was assessed with two-way ANOVA (tissue type and year/location categories) followed by one-way ANOVAs to separate means. All analyses of concentration data were on natural log (ln)-transformed data to more closely meet the homogeneity-of-variance assumption of the ANOVAs. When ≥50 % but <100 % of samples had detectable concentrations, half of the DL was substituted before statistical analysis and calculation of geometric means.

Accumulation rates of PFOS in nestlings were calculated according to the methods of Custer and Custer (1995) at four sites where we collected eggs and sibling nestlings from the same nest boxes. The mass (ng) of PFOS (sample weight × concentration) was calculated for both egg and nestling samples, and then the rate of PFOS accumulated/d was derived from the following equation: $(\text{Mass}_{\text{nestling}} - \text{Mass}_{\text{egg}})$/age of nestling. Because nestlings are only fed from the local environment, and any contribution from the egg was removed, accumulation rate provides a measure of purely local exposure. A correlation coefficient was calculated between accumulation rate and both egg and nestling carcass concentrations ($n = 46$). Because diet samples were pooled across multiple nest boxes, the correlation coefficients were calculated between mean accumulation rate for location/year categories ($n = 7$) and concentrations in diet as well as between diet and egg, and diet and nestling carcass concentrations.

Predictive Exposure Modeling

We developed eight linear models, on an ln log–log scale, to assess the association between PFOS in eggs ($\text{logPFOS}_{\text{egg}}$) and plasma ($\text{logPFOS}_{\text{plasma}}$). We evaluated this association, which included calculation of r^2, between eggs and plasma in concert with three covariates most likely to contribute to observed variation in PFOS concentrations: location, female age, and date of laying (DOL). We allowed intercepts and slopes to vary by location and used information-theoretic methods (Akaike's information criterion [AIC]) to rank models (Burnham and Anderson 2002). Analyses were performed using R 2.15 (R Core Team 2012).

Reproductive Success

Reproductive success, and whether there was an association with chemical contamination or other covariates, was assessed in three ways. At the most basic level, the percent of the eggs hatching of the number of eggs available (did not include the eggs collected for chemical analysis or eggs not followed until hatching) was calculated for each location separately and compared with published information. This calculation used all of the reproductive information regardless of whether PFAS data were available for egg samples. Multistate modeling (Lebreton et al. 2009) was used to determine whether covariates (PFOS concentration, location, female age, or DOL) were associated with hatching success of individual eggs. For both this and the logistic analysis, only sites and samples that had PFAS data for eggs were used. We modeled PFOS concentration rather than total PFAS because of the prominence of this congener in the total PFAS mix (see Results). Models were evaluated using AIC methods. Finally, simple logistic regression analysis was performed to assess the association between hatching success of the clutch and PFOS concentrations mainly for comparative purposes with previously published data analyzed in that manner. The latter two statistical treatments are based on the sample egg method (Blus et al. 1974; Custer & Mitchell 1987) used since the 1970s to assess contaminant effects on hatching success.

The program MCestimate was used for multistate modeling of the daily rate of egg or nestling failure. MCestimate is written in the MATLAB (MathWorks 2012) programming environment and implements the algorithms of Etterson et al. (2007a, 2007b). These algorithms facilitate the estimation of survival and transition probabilities for individual eggs under the assumption that events are described by the transition matrix of a Markov chain (see Appendix S1 for details). Seventeen models (Supplementary Table S2) that were thought to possibly explain the egg survival data were chosen based on published information. We used three pathways of egg failure during the incubation period, i.e., infertility, embryo death, and predation, and one category for nestling death during the care-of-young period. We also had a null model that postulated no covariate association with egg or nestling survival.

Logistic regression analysis was performed using SAS 9.2. Analyses were performed both with and without Lake Johanna data so not to potentially bias the results with the previously published information (Custer et al. 2012a) with a known outcome.

Results

Exposure

Two PFASs, PFHpA and PFBS, were not detected in any tree swallow tissue or diet sample. The other 8 PFASs were detected in >50 % of samples. Nearly all samples had detectable concentrations of total PFASs except for 2 diet samples: 1 of 2 diet samples in 2008 and 1 of 12 diet samples in 2009; both were from Lake Johanna. Two-way ANOVA indicated a significant interaction between year and location ($P < 0.001$ for carcass and plasma), and it approached significance ($P = 0.089$) for eggs, so neither

year nor location were combined in subsequent statistical analyses of total PFAS.

For all three tree swallow tissue types, samples from Pigs Eye Lake had the highest concentrations of total PFASs compared with all other locations, except that Pigs Eye Lake eggs in 2010 did not statistically differ from Lake Johanna egg samples in either year (Table 1). Pigs Eye Lake swallow tissues were between 2.5 and 65 times greater than other locations for all tissue types. For plasma, the upper Mississippi River sites below the Twin Cities (pools 2 and 8) were similar to each other and also similar to Lake Johanna in the Twin Cities. These locations on the upper Mississippi River or the Twin Cites metro lake all had significantly greater plasma concentrations than Green Mountain Lake (2011), the Root River, and the two reference lakes in northern Wisconsin. Plasma from Green Mountain Lake in 2010 was intermediately between, and not significantly different from, the Mississippi River as well as sites below the Twin Cities, nor did it differ from the off-river sites. The two northern Wisconsin lakes differed by a factor of 4× between each other even though they were in relatively close geographic proximity but had different pHs. Years tended to be similar at each location except at Green Mountain Lake where concentrations were significantly lower for each matrix in 2011 compared with 2010. There were few significant differences among locations in diet concentrations. Diet samples did not statistically differ in part because of the availability of fewer samples for analysis, generally two pooled diet samples per location, and because of greater variation in individual diet concentrations; note the large 95 % confidence intervals (CIs) especially at Pigs Eye Lake. For concentrations of individual PFAS congeners by locations, and numbers with detectable concentrations, see Table 2.

The pattern of individual PFAS congeners in plasma strongly differed (global $R = 0.719$) among all locations (Fig. 2), except that three locations in pool 8 and one location in pool 2 did not statistically differ from one another (all P values were >0.10, and all R values were <0.23), thus indicating little separation among the Mississippi River locations downstream of the Twin Cities. The pattern at the three remote lake locations were distinct (all R values were >0.90) from the Mississippi River locations (Pigs Eye Lake, pools 2 and 8), the Root River, and Lake Johanna. These pattern differences were driven primarily by PFOS (primary contributor to dissimilarity [SIMPER]) in 31 of 45 pairwise comparisons and accounted for an average of 43.5 % of the dissimilarity). The PFAS pattern was secondarily driven by PFOA (primary contributor in 9 of 45 and accounting for 44.3 % of the dissimilarity) and PFHxS (primary contributor in 4 of 45 and accounting for 30.7 %). PFNA was the primary driver once and accounted for 36.5 % of the dissimilarity. Lake Johanna was the location where PFHxS was the primary driver in differences.

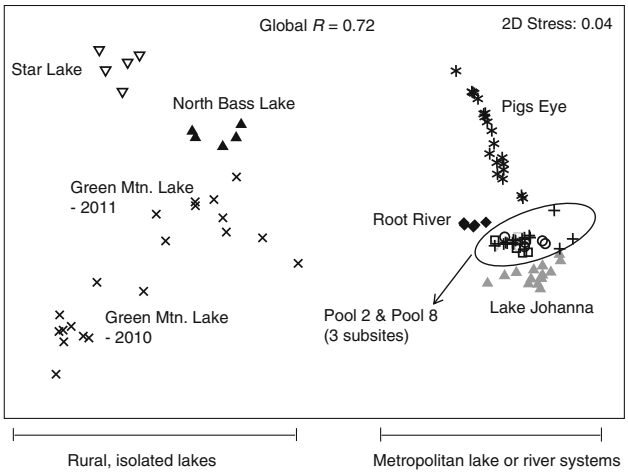

Fig. 2 Nonmetric multidimensional scaling plot for the pattern (percent that each individual perfluorochemical congener [PFASs] comprised of total PFASs) for eight PFASs in blood plasma of tree swallows nesting in Minnesota and Wisconsin from 2007 to 2011

Because PFOS was the primary driver in the multivariate analyses, the proportion that PFOS comprised of the total PFASs was calculated and analyzed for differences among locations, years, and tissues. There was a significant year × location interaction of the portion that PFOS comprised of total PFASs ($P < 0.001$ for carcass and plasma), although not for eggs ($P = 0.320$), so subsequent analyses were performed using one-way ANOVAs with year/location category, e.g., Pigs Eye 2010, Pigs Eye 2011, etc. The proportion of PFOS to total PFASs was graded from ≥94 % in Lake Johanna eggs and carcasses, to 86 and 93 % on the Mississippi River near the Twin Cities, and to <60 % at lakes not associated with the Mississippi or Root Rivers (Fig. 3). Lake Johanna and the Mississippi River sites had significantly greater proportion of PFOS in the PFAS mixture for all three tissue types than isolated nonriver sites. At each location, the proportion of PFOS to total PFASs in plasma tended to be less than in either eggs or carcasses, although the magnitude of this difference varied among locations.

The accumulation rate (ng of PFOS accumulated/d in nestlings) ranged from a high of 203 ng/d (Pigs Eye 2011) to a low of 5.29 ng/d (Green Mountain 2011 [Table 3]). Lake Johanna and pool 2 accumulated PFOS at approximately half or less the rate at Pigs Eye. There was a high correlation between PFOS accumulation rate and concentrations in eggs ($r = 0.85$, $n = 46$), nestling carcass ($r = 0.99$), and diet ($r = 0.98$, $n = 7$ pooled diet sample/location/y). The correlation coefficient between egg and nestling concentration was $r = 0.90$. There was also a similarly high association between diet and egg concentrations ($r = 0.96$) and diet and carcass ($r = 0.99$) on a mean location-per-year basis. This basis had to be used because diet samples were pooled across nest boxes at a location to have enough sample mass for chemical analysis.

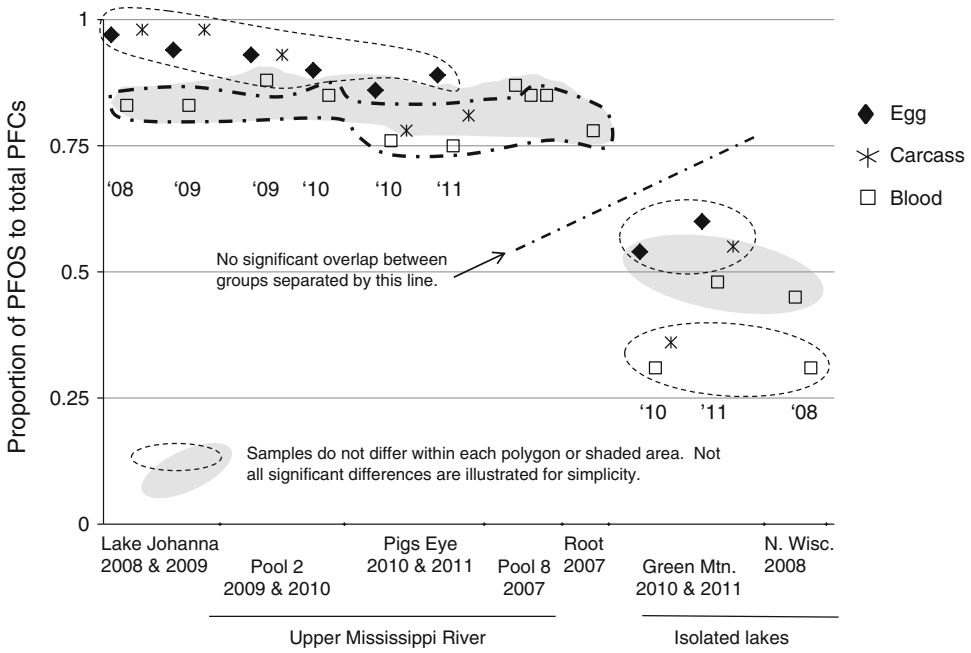

Fig. 3 Proportion of PFOS to total PFASs in three tree swallow tissues from birds nesting in Minnesota and Wisconsin from 2007 to 2011

Table 3 Mean accumulation rate (± 1 SE) of perfluorooctane sulfonate (PFOS) in 12-day old nestling tree swallows at four locations in Minnesota and Wisconsin from 2008 to 2011

Location	Year	*N*	PFOS (ng/d)	SE	Minimum–maximum rates
Pigs Eye Lake	2010	8	119	15.2	73.4–216
	2011	6	203	39.4	41.6–296
Lake Johanna	2008	2	40.6	0.92	39.7–41.6
	2009	11	48.2	5.20	16.7–73.0
Pool 2	2009	6	42.2	6.30	19.8–59.6
Green Mtn. Lake	2010	8	10.1	1.44	5.91–18.6
	2011	5	5.29	0.68	3.51–7.35

Exposure Models

There was a good relationship ($r^2 > 0.90$) between PFOS concentration in plasma and eggs with the best-supported model being $\log(\text{PFOS}_{\text{egg}}) \approx \log(\text{PFOS}_{\text{plasma}}) + \text{location}$ (Table 4, model no. 1). The best model allowed the intercept, but not the slope, to vary among location. The model with different slopes for each location (model no. 4 only) was not as well supported ($\Delta\text{AIC}_c = 4.53$) as the constant slope models (nos. 1 through 3), meaning that the relationship between

Table 4 AIC and correlation of determination describing the strength of the association of ln log-transformed plasma and egg concentrations of PFOS for tree swallows nesting in Minnesota and Wisconsin from 2007 to 2011

No.	Terms	AICc	ΔAICc	ω	r^{2a}
1	$\text{Log}(\text{PFOS}_{\text{plasma}}) + \text{location}$	97.35	0	0.53	0.90
2	$\text{Log}(\text{PFOS}_{\text{plasma}}) + \text{location} + \text{age}$	98.96	1.61	0.24	0.91
3	$\text{Log}(\text{PFOS}_{\text{plasma}}) + \text{location} + \text{DOL}$	99.62	2.27	0.17	0.90
4	$\text{Log}(\text{PFOS}_{\text{plasma}}) + \text{location} + \log(\text{PFOS}_{\text{plasma}}) \times \text{location}$	101.88	4.53	0.06	0.91
5	Location only	109.65	12.3	0	0.87
6	$\text{Log}(\text{PFOS}_{\text{plasma}})$	111.20	13.85	0	0.86
7	$\text{Log}(\text{PFOS}_{\text{plasma}}) + \text{age}^b$	111.81	14.46	0	0.87
8	$\text{Log}(\text{PFOS}_{\text{plasma}}) + \text{DOL}^b$	113.31	15.96	0	0.86

DOL date of laying

[a] r^2 indicates the strength of the association between PFOS in plasma and eggs given the other parameter(s) in the model

[b] The covariates, female age and DOL, were separately added to Model 6

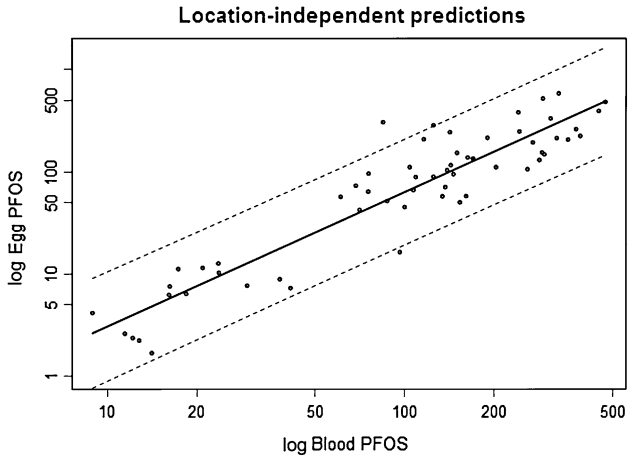

Fig. 4 Location-independent predictions for log concentrations of PFOS in blood to egg from tree swallows nesting in Minnesota and Wisconsin from 2007 to 2011. Formula for the line is ln log ($PFOS_{egg}$) = −1.9 + 1.31 × ln log ($PFOS_{plasma}$)

log($PFOS_{plasma}$) and log($PFOS_{egg}$) remained the same regardless of location. Models with location only, plasma concentrations only, plasma concentrations + female age, or plasma concentrations + DOL were also not well supported ($\Delta AIC_c > 12.0$) by the data. We found for every 1-unit increase in the log($PFOS_{plasma}$), there was a corresponding increase in the log($PFOS_{egg}$) of 0.67 (95 % CI = 0.33, 1.01; log[$PFOS_{plasma}$] = 0.67 × log[$PFOS_{egg}$]). On the observation scale, for every 10 % increase in the concentration of PFOS in plasma, there was a corresponding increase of 6.6 % in PFOS concentration in egg (95 % CI = 3.2–10.1 %). For a more general predictive model on the log–log scale that is location-independent (model no. 6, [r^2 = 86 %; Fig. 4]), the model estimated that for every 1-unit increase in log PFOS in plasma, there was a corresponding increase of 1.31 (95 % CI = 1.17, 1.45) in log PFOS in eggs; on the observation scale, for every 10 % increase in the concentration of PFOS in plasma, there was a corresponding increase of 13.3 % in egg (95 % CI = 11.8, 14.9 %). To calculate PFOS concentrations in eggs based on the location-independent model, the equation is as follows: log($PFOS_{egg}$) = −1.9 + 1.31 × log($PFOS_{plasma}$), where log is the natural logarithm and the CIs are −1.25 and −2.56 for the intercept and 1.45 and 1.17 for the slope. The parameter estimates and confidence intervals for all models (Table S2) have the intercept represented by location "Green Mountain," and the location parameter estimates describe differences relative to "Green Mountain." The estimates for age describe the difference relative to >1-year-old females.

Reproduction Effects

The percent of eggs hatching was less than the nationwide average (~85 %, Robertson et al. 1992) at Pigs Eye (68 %), pool 2 (69 %), and Lake Johanna (71 %, Table 5); all were sites with the highest PFAS egg concentrations (Table 2). The percent hatching was at or greater than the nationwide average at pool 8 (85 %), Green Mountain Lake (92 %), Star Lake (89 %), North Bass Lake (93 %), and Root River (90 %). There were seven egg-loss categories; human disturbance and flooding categories were restricted to only two locations. The other egg loss categories were relatively evenly distributed among locations, although "missing" seemed to be more pronounced at Pigs Eye, pool 2, and Lake Johanna compared with the other five locations. There were few nestling losses except at Green Mountain Lake where 10 nestlings disappeared from nest boxes. At all other locations, nestling survival, as a percentage of the hatched eggs, was >90 %.

Multistate modeling showed a negative association between hatching success and PFOS concentration for the 397 eggs that had PFOS values. The null model had a ΔAIC value of 17.7 clearly indicating there was an association between reproductive success and at least one of the covariates (Table 6). The best model associated PFOS exposure with embryo death, and that model received 83 % of the Akaike weight among all models considered. PFOS exposure was not associated with infertility (ΔAIC = 19.4 for $m_p(.)m_e(.)m_i(\text{PFOS})m_n(.)$), nor was PFOS associated with nestling mortality (ΔAIC = 19.5 for $(m_p(.)m_e(.)m_i(.)m_n(\text{PFOS}))$ or predation (ΔAIC = 19.7 for $(m_p(\text{PFOS})m_e(.)m_i(.)m_n(.))$. Four other models, all associated with DOL, also had little support with ΔAIC >18.0 and model weights of 0.000 and included $m_p(\text{Date})m_e(.)m_i(.)m_n(.)$; $m_p(.)m_e(\text{Date})m_i(.)m_n(.)$; $m_p(.)m_e(.)m_i(\text{Date})m_n(.)$; and $m_p(.)m_e(.)m_i(.)m_n(\text{Date})$.

When the fitted daily rate of embryo death from the best model was extrapolated over the developmental life span of an egg through hatching, the model predicted a substantial decrease in survival probability with increasing exposure to PFOS (Fig. 5a [upper panel]). The concentration at which hatching was decreased by >20 % was 283 ng/g ww in eggs (Fig. 5). The estimated egg concentration at which hatching would be decreased by 50 % was [lower panel] 494 ng/g. There was some support for an association between age of female and reproductive success (ΔAIC = 4.3) and less support for an association between location and reproductive success for both embryo death (ΔAIC = 5.5) and infertility (ΔAIC = 9.1, Table 6). An association between nest predation and female age had little support (ΔAIC = 10.2). All five of these models had significant likelihood ratios compared with the null model (Table 6). All other ΔAIC values were ≥11.0. The average rates of failure (failure/d) for eggs and nestlings were highest for infertile eggs (0.0377 [SE and 95 % CI = 0.0092, 0.0230–0.0606]) followed by dead embryo (0.0101, 0.0014, 0.0076–0.0133). The lowest probabilities were predation on eggs (0.0017, 0.0005, 0.0009–0.0029) or death of nestlings (0.0016, 0.0007, 0.0007–0.0040).

Using logistic regression, there was also a significant negative association between PFOS concentration in eggs

Table 5 Reproductive data for tree swallows nesting at eight locations in Minnesota and Wisconsin from 2007 to 2011

Reproductive data	Location							
	Pigs Eye	Pool 2	Lake Johanna	Pool 8	Green Mtn. Lake	Star Lake	North Bass Lake	Root River
No. of eggs laid	164	141	106	118	226	89	95	80
No. of eggs collected	44	29	20	21	51	10	9	0
No. of eggs remaining	120	112	86	97	175	79	86	80
No. of losses								
Infertile	1	10	6	3	3	0	4	3
Dead embryo	7	5	0	2	1	3	1	3
Abandoned	3	0	4	0	3	0	0	1
Missing	11	14	8	4	2	0	1	1
Human disturbance	0	0	4	0	0	0	0	0
Depredated	13	5	3	4	5	6	0	1
Flooded	2	0	0	0	0	0	0	0
Total egg losses	37	34	25	13	14	9	6	9
No. of not followed until hatch	3	0	0	11	4	0	0	6
No. of (%[a]) eggs hatched	80 (68)	78 (69)	61 (71)	73 (85)	157 (92)	70 (89)	80 (93)	65 (90)
No. of (%[b]) nestlings surviving to 11–12 days	80 (100)	77 (99)	61 (100)	73 (100)	142 (90)	70 (100)	75 (94)	63 (97)
Losses								
Dead	0	0	0	0	5	0	5	0
Missing	0	1	0	0	10	0	0	2
Total nestling losses	0	1	0	0	15	0	5	2
Total no. of nests with eggs (nestlings)	34 (23)	28 (23)	20 (15)	22 (18)	39 (35)	16 (14)	16 (16)	17 (14)

[a] Percent hatched is the number hatched divided by the number remaining after egg collections minus the number not followed until they hatched

[b] Percent of nestlings that survived divided by the number of eggs that hatched

Table 6 AIC results for multistate models of covariate associations and tree swallow egg and nestling survival at locations in Minnesota and Wisconsin from 2007 to 2011

Model	Negative-log likelihood	AIC	ΔAIC	Wt	Parameters	P[a]
$m_p(.)m_e(\text{PFOS})m_i(.)m_n(.)$	368.8	747.6	0.0	0.832	5	5.73×10^{-4}
$m_p(.)m_e(\text{FemAge})m_i(.)m_n(.)$	369.9	751.9	4.3	0.096	6	0.0038
$m_p(.)m_e(\text{site})m_i(.)m_n(.)$	369.5	753.0	5.5	0.054	7	0.0057
$m_p(.)m_e(.)m_i(\text{site})m_n(.)$	371.3	756.7	9.1	0.009	7	0.0239
$m_p(\text{FemAge})m_e(.)m_i(.)m_n(.)$	372.9	757.7	10.2	0.005	6	0.0416
$m_p(.)m_e(.)m_i(\text{FemAge})m_n(.)$	373.3	758.5	11.0	0.003	6	0.0572
$m_p(.)m_e(.)m_i(.)m_n(\text{FemAge})$	376.1	764.1	16.5	0.000	6	0.3978
$m_p(.)m_e(.)m_i(.)m_n(\text{site})$	375.2	764.5	16.9	0.000	7	0.3400
$m_p(.)m_e(.)m_i(.)m_n(.)$	378.6	765.3	17.7	0.000	4	

m_p probability of egg predation/d, m_e probability of dead embryo/d, m_i probability of an infertile egg/d, m_n probability of nestling death/d

[a] P values are from a likelihood ratio test against the null model

Note one model, $m_p(\text{site})m_e(.)m_i(.)m_n(.)$ was inestimable due to rarity of predation events and is not included in the table

and the percent of the clutch that hatched ($P = 0.019$, Fig. 6). The point at which percent hatching decreased below the nationwide average ($\sim$85 % hatching) was 148 ng/g ww in eggs. The formula for the logistic regression describing this association is as follows: $y = 1/e^{-(\beta_0+\beta_1 X)} + 1$ where $\beta_0 = 2.206$ and $\beta_1 = -0.0035$. When Lake Johanna data from 2008 and 2009 were added into the logistic regression analysis, the parameters were $P = 0.0042$ and $\beta_0 = 2.197$ and $\beta_1 = -0.0044$, thus moving the line slightly more negative.

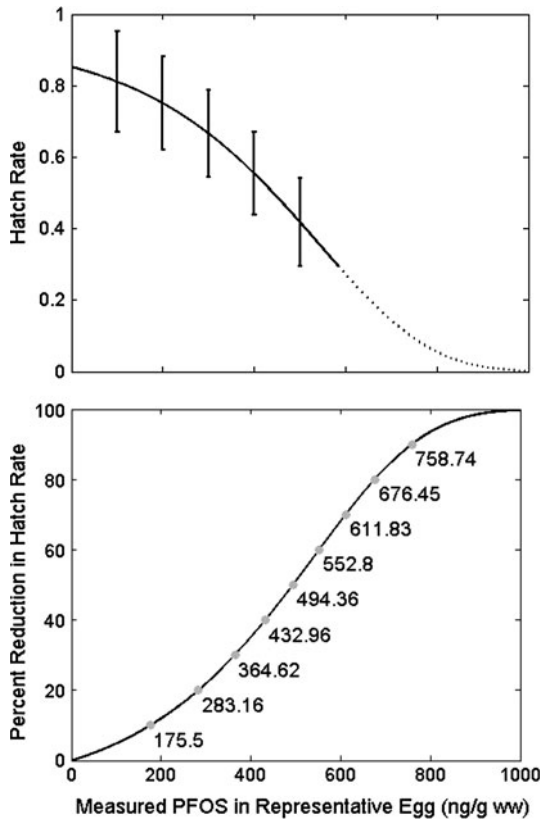

Fig. 5 Predicted relationship [±1 SE (*upper panel*)] between PFOS and survival of an egg from laying to hatching based on the best multistate model, $m_p(.)m_e(\text{PFOS})m_i(.)m_n$. Percent decrease (*lower panel*) in hatching success and associated PFOS concentrations in eggs

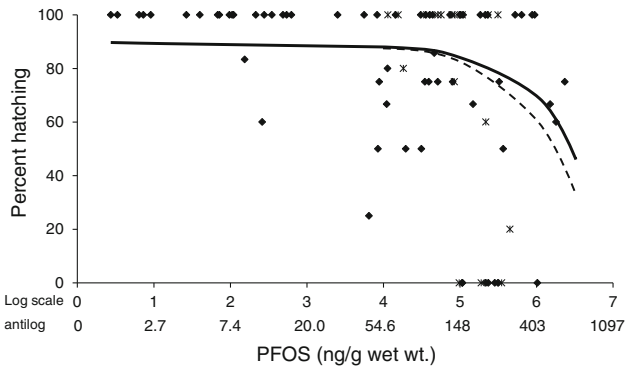

Fig. 6 Logistic regressions of ln log PFOS in eggs and the percent of the clutch that hatched for tree swallows nesting in Minnesota and Wisconsin from 2009 to 2011. Solid line is generated from the following formula: $y = 1/e^{-(\beta_0+\beta_1 X)} + 1$ where $\beta_0 = 2.206$ and $\beta_1 = -0.0035$. Data from Lake Johanna are indicated by the stars, and the dashed regression line includes the Lake Johanna data

Discussion

Exposure–Concentration and Composition

Pigs Eye Lake, the location on the upper Mississippi River adjacent to a major historic PFAS disposal site, had the highest concentrations of both total PFASs and PFOS compared with the other seven locations in the upper Midwest. Pigs Eye was more than twice as contaminated as Lake Johanna, the next most contaminated location, for eggs (2011 only), plasma, or nestling carcasses. Although the putative sources for PFAS contamination at Pigs Eye and pool 2 are probably obvious, being adjacent to a historic landfill and downstream of large municipal wastewater-treatment plant (WWTP) and manufacturing plant, the source(s) of PFASs in Lake Johanna remain unknown. The similar composition of the eight PFAS congeners at Lake Johanna to the pool 2 and Pigs Eye locations points to a similar source.

Concentrations of PFOS in tree swallow eggs at Pigs Eye (geometric mean = 270 ng/g) were similar to PFOS in great blue heron (*Ardea herodias*) eggs (288 ng/g ww [in 2010]) and slightly lower than in heron eggs in 2011 (396 ng/g ww) collected from the nearby rookery on Pigs Eye Lake (Custer et al. 2013a). All concentrations provided later in the text are on a wet-weight basis in whole egg contents or plasma unless specified otherwise. This similarity was unexpected because herons are a piscivore species, and piscivores tend to have greater exposure to PFASs than birds, such as an insectivorous swallow, which feed at lower trophic levels (Kannan et al. 2005). Great blue herons have a much larger feeding range, e.g., ≤20 km from a rookery site (Custer and Galli 2002; Custer et al. 2004), so they may have been foraging, at least some of the time, in areas such as lakes and ponds off the Mississippi River, nearby tributaries, and wetlands with less PFAS contamination. The feeding radius of swallows is usually within 1 km of their nest box (Quinney and Ankney 1985), so they are most likely feeding in PFAS-contaminated locations at all times. Geometric mean concentrations of PFOS in bald eagle (*H. leucocephalus*) nestling plasma ranged from 541 to 1,250 ng/ml (2006–2009, Route et al. 2011) from a 123-km long stretch of the Mississippi River that included both the Pigs Eye and pool 2 tree swallow locations. These concentrations were greater than found in tree swallow nestling plasma (295 ng/g ww). The eaglets were ~35–63 days old, whereas the tree swallow nestlings were only 12 days of age, so the eaglets had an additional 20–40 days to accumulate PFASs, which could partially account for the greater plasma concentrations. In addition, eaglets are fed fish and carrion and hence are fed from a higher trophic level than tree swallows (aquatic insectivores), which might also contribute to greater exposures in eaglets. There was considerable variation among eaglet exposure, further indicating that the specific feeding location is an important variable in explaining exposure patterns.

Concentration of various PFASs in tree swallow eggs and nestling plasma at Pigs Eye (geometric mean PFOS plasma = 295 ng/mL, egg = 270 ng/g) were considerably greater than concentrations at most other locations

worldwide other than the Great Lakes of North America, the Baltic Sea in Europe, and a few other, often point-source locations (Dauwe et al. 2007). Concentrations in swallow eggs even exceeded concentrations found in top predatory birds at these other locations, thus clearly showing the high level of PFAS contamination along the Upper Mississippi River. Mean egg or plasma concentrations of PFOS in other locations were as follows: 10 ng/g in tawny owl (*Strix aluco*) eggs from Norway (Ahrens et al. 2011); means from 4.96 to 26.8 ng/g in osprey (*Pandion haliaetus*) eggs from Delaware Bay, USA (Toschik et al. 2005); 14.6 to 32.1 ng/g in common eider (*Somateria mollissima*) and European shag (*Phalacrocorax aristotelis*) eggs in Norway (Herzke et al. 2009); 17.1 to 22 ng/g in great skua (*Stercorarius skua*) eggs from Scotland (Leat et al. 2013); 9.6 to 39.8 ng/g in eggs of five species of arctic seabirds in 2008 from Prince Leopold Island, Nunavut, Canada (Braune and Letcher 2013); arithmetic means of 15 to 85 ng/g in guillemot (*Uria aalge*) eggs from Iceland, Faroe Islands, and Norway (Löfstrand et al. 2008); means of ~40 ng/g (Norway, Verreault et al. 2007) and 60 to 80 ng/g ww in herring gull (*Larus argentatus*, North and Baltic Seas, Rüdel et al. 2010) eggs, although exposure in herring gulls from the Baltic Sea seems to be increasing to a high of 150 ng/g ww in 2008 (Rüdel et al. 2011); median of 48 ng/g in cormorants (*P. carbo*) from the Baltic sea (Rüdel et al. 2011); 83 ng/g in peregrine falcon (*Falco peregrinus*) eggs from Sweden (Holmström et al. 2010); 100 ng/g in glaucous gull (*Larus hyperboreus*) eggs from Norway (Verreault et al. 2005); arithmetic mean concentrations <70 ng/g in two heron species from China but as high as 142 ng/g in black-crowned night herons (*Nycticorax nycticorax*, Wang et al. 2008); and 67.6 ng/g from tree swallow eggs in MI, USA (Giesy et al. 2006). In nestling plasma, mean values were 6 ng/ml in black-tailed gulls (*Larus crassirostris)* from Japan (Giesy and Kannan 2001); 6.2 to 14 ng/ml in albatrosses from Midway Atoll (Kannan et al. 2001); 29.8 ng/ml in adult shag plasma from Norway (Herzke et al. 2009); and 40 (adult male) and 22 ng/g ww (adult female) in great skua plasma from Scotland (Leat et al. 2013).

Locations where PFAS contamination exceeded that of tree swallows included herring gulls at 15 locations across the Great Lakes, which had arithmetic mean concentrations of total PFOS in eggs between 99 and 586 ng/g with 7 of 15 sites having mean concentrations >300 ng/g (Gebbink and Letcher 2010). The greater level of contamination in herring gulls, compared with swallows, at some locations may be because they feed at a higher trophic level (Kannan et al. 2005)—see further discussion later in the text—or perhaps more importantly because of their longer residency time feeding in contaminated areas. Herring gulls can be year-round residents in some parts of the Great Lakes and may feed in contaminated locations year round. This contrasts with many migratory species, such as the tree swallow, which leave contaminated breeding locations for part of the year. Similarly to the herring gull, guillemots, a resident species in the Baltic Sea, had mean PFOS in eggs >200 ng/g beginning in 1981 (Holmström et al. 2005); peak concentrations (1,324 ng/g) occurred in the late 1990s, and may have been decreasing since then. Likewise, guillemots nesting in Stora Karlsö, Sweden, had 400 ng/g PFOS (Löfstrand et al. 2008) in eggs. Migratory cormorants nesting in the Elbe estuary (Germany) had median egg concentrations of 400 ng/g (Rüdel et al. 2011) with maximum concentrations of 1,451 ng/g. Migratory piscivorous species nesting in the Great Lakes also had increased PFOS exposure depending on location. Nestling double-crested cormorant (*P. auritus*) and bald eagles had maximum plasma concentrations of 372 (cormorant) and 2,220 ng/ml (eaglet) with much lower concentrations (34 ng/ml cormorants and 13 ng/ml in eaglets) found in more remote areas and greater concentrations near population centers (Kannan et al. 2001).

Another location with high PFAS exposure is a fluorochemical plant in Antwerp, Belgium, near which nesting great tits (*Parus major*) had high whole blood concentrations (172–1,625 ng/ml at Vliegenbos, Dauwe et al. 2007). These concentrations would be 2× to 5× greater if expressed as plasma concentrations (Kannan et al. 2001) because of the dilution factor provided by red blood cells, which contain ≥5 times less PFASs than plasma (Gebbink and Letcher 2012) and far exceeded serum concentrations in the current tree swallow study. Liver PFOS concentrations were also extraordinarily high (approximately 1,000 ng/g ww) in both great and blue tits (*P. caeruleus,* Hoff et al. 2005) nesting near that plant. Although liver tissue was not assessed in the current study, the liver concentrations in Belgian tits were ~15 times greater than in liver tissue at Lake Johanna from an earlier study (Custer et al. 2012a). Unfortunately, no information was provided on reproductive success of the tits.

Although the previous discussion has been predicated on the standard food chain biomagnification paradigm for birds, intriguing data indicate that this may not be as straightforward for PFASs as commonly assumed. Martin et al. (2004) found *Diporeia*, a planktonic crustacean that occupies the lowest trophic level in the Lake Ontario food chain, had the highest total PFCAs (sum of homologous perfluoroalkyl carboxylates) and those concentrations exceeded by a factor of ~8.5 the concentration found in lake trout (*Salvelinus namaycush*), the top aquatic predator in that system. *Diporeia* also had the highest ΣPFOS, except for slimy sculpin (*Cottus cognatus*) whose diet is primarily *Diporeia*. These data demonstrate a divergence of the benthic *versus* pelagic food chains and should serve as a cautionary tale against

blanket application of the food chain paradigm without a thorough understanding of food chain linkages. In some situations, therefore, tree swallows may be equally, or more exposed, to some PFASs depending on the benthic aquatic insects they consume compared with higher trophic level piscivorous avian predators. This may partially explain the situation at Pigs Eye Lake where exposure of tree swallows was similar to that of great blue herons (Custer et al. 2013a). This was also found for dioxins and furans (PCDD-F) where the total PCDD-F and PCDD-F toxic-equivalent exposures were 15 and 2.4 times greater in tree swallows compared with the piscivorous belted kingfisher (*Ceryle alcyon*, Custer et al. 2010b). Polychlorinated biphenyl (PCB) toxic-equivalents were also greater in swallows than kingfishers, although total PCBs were not.

It has been shown that WWTPs generally do not remove, or only partially remove, PFASs, and the type of PFAS affects the rate of removal (Becker et al. 2008). Approximately half of the PFOS was retained in the sludge and thus prevented from entering the aquatic environment, whereas PFOA passed through practically undiminished. The size of the human community and the degree of industrial inputs affect the total amount of PFASs entering the aquatic environment. For instance, a WWTP in Germany serving 11,000 inhabitants with mostly domestic water inputs contributed only 2.4 ng/L PFOS in wastewater effluent compared with a plant serving 72,000 inhabitants as well as various industrial sources that contributed between 50 and 195 mg/L in wastewater (Becker et al. 2008). These data are consistent with the current study, in which concentrations of PFOS and total PFASs in tree swallow tissues seemed to reflect the size of the sewer shed, including point sources. Pigs Eye had the highest concentrations and was adjacent to a historic landfill and large WWTP facility serving the Twin Cities metro area. This area was in the top 10 (of 177) sites along the upper Mississippi River and large tributaries for PFOS concentrations in water (Nakayama et al. 2010). There was no difference in exposure of tree swallows at pools 2 and 8; both had significantly less exposure than the Pigs Eye location, and both of these locations are on the Mississippi River with multiple WWTP effluent sources from cities of various sizes. Consistent with the biological data, water concentrations at sampling points in this downstream stretch averaged 15.6 ng/L (Nakayama et al. 2010), which was approximately half of the water concentration found in the Pigs Eye stretch (28–37 ng/L PFOS). Finally, the Root River site is located in a small watershed with no known point sources of PFASs, has relatively few people inhabiting small cities and towns, has WWTPs discharging to the river, and had exposure similar to, or only slightly greater than, isolated lakes in Minnesota and Wisconsin. The exposure levels of PFOS in swallow plasma on the Root River (40.2 ng/ml) was also on the upper end of the range found in albatross plasma (3.0–34 ng/ml) nesting on Midway Atoll in the Pacific Ocean (Kannan et al. 2001), one of the most remote locations sampled, further indicating only a slight increase compared with background concentrations. This pattern of exposure relative to population centers is consistent across the globe. Median egg concentrations of PFOS in common eider eggs from Norway were approximately twice as high (28.8–32.1 ng/g ww) near urban areas compared with a remote location (14.6, Herzke et al. 2009). The same pattern is also present in the Great Lakes (Kannan et al. 2001) and along the Mississippi River (Route et al. 2011).

The proportion of various PFASs to total PFASs separated locations, and the largest separation was based on connectivity to wastewater treatment or point sources. The three isolated lakes were completely separated from the riverine sites (Figs. 2 and 3). The lack of differences in both the multivariate context and for total PFASs in plasma between pool 2 and the three subsites in pool 8, as well as the more similar composition of those locations to Pigs Eye, probably indicate not only a downstream movement of PFASs originating in the Twin Cities area but also continuous contributions of PFASs from cities all along the upper Mississippi River. The proportion of the various PFASs in swallow plasma at the Root River site also seems indicative of human sources by way of WWTPs rather than atmospheric deposition, although total concentrations were more similar to background levels present at the three isolated lakes. If the source on the Root River was atmospheric, then the congener profile should look like that of the three isolated lakes rather than the Mississippi River sites, all of which obtain wastewater treatment inputs. Terrestrial birds in Germany (rook [*Corvus frugilegus*] and feral pigeons [*Columbia livia*]), where the PFAS source was most likely atmospheric, had a different distribution of congeners compared with the aquatic cormorant in that study (Rüdel et al. 2011); PFOS was much less dominant in the terrestrial species compared with the aquatic species. This was similar to the different composition and lack of dominance by PFOS in swallows at the three lakes in Minnesota and Wisconsin that only had atmospheric deposition. In addition, as pointed out by Rüdel et al. (2011), care must be taken when comparing PFAS patterns when overall exposure is low because many of the congeners will be lower than the DL, thus making PFOS appear more dominant than it might actually be if DLs for all other congeners were sufficiently low.

The influence of WWTPs on the distribution of PFAS congeners holds true for fish data as well. In the upper, sparsely populated stretches of the Missouri River (in North and South Dakota and Nebraska north of Omaha), PFOS contributed <50 % in the majority of samples (~60 % of samples), whereas PFOS was less dominant in

a smaller percentage (~25 % of samples) in the highly populated stretches downstream of Kansas City (Ye et al. 2008b). On the Mississippi River, percent PFOS exceeded 80 % of the total PFASs at most sampling locations, all of which were downstream of the Twin Cities area. In Germany PFOS in bream (*Abramis brama*) livers from an isolated lake constituted 66 % of the total PFASs compared with 86–93 % of total PFSAs from industrial rivers, such as the Elbe and Rhine rivers (Rüdel et al. 2011).

There was a high correlation between accumulation rate in 12-day-old nestlings, which only reflects local contamination, and the concentrations found in eggs, nestling carcasses, and food. This is strong evidence that even although some contaminant exposure present in eggs could have come from migration or wintering areas, the large majority in eggs can be attributed to the local environment. The natural history of the tree swallow also supports this assertion. They are income breeders, and they forage in the local area for 4–6 weeks before egg-laying. It is not surprising that there was a high correlation between accumulation rate and nestling carcass concentrations and accumulation rate and diet.

Reproductive Success

There was a significant negative association between concentrations of PFOS in eggs and percent hatching success using both multistate modeling on the survival of each egg independently and logistic regression, which used the percent of the clutch that hatched. This result was consistent with earlier studies (Custer et al. 2012a). The best multistate model indicated that embryo mortality increased with increasing PFOS exposure. The concentration where hatch rate decreased to <80 % began at 283 ng/g ww in eggs (Fig. 5). From the logistic equation, the concentration at which the percent hatching decreased below the nationwide average (85 %) was 148 ng/g (Fig. 6). This was similar to the 120 ng/g found at Lake Johanna in an earlier tree swallow study (Custer et al. 2012a). In addition, the point at which the first total failure appeared was at approximately 150 ng/g at Lake Johanna compared with 147 ng/g in the current study. The current study, with a larger dataset from three different PFAS-contaminated locations, validated the earlier conclusion about a negative association between PFAS exposure and reproductive failures. Both logistic regression curves were similar whether Lake Johanna was included or excluded in the current study (Fig. 6).

Although it is possible that other contaminants could have caused or contributed to the reproductive effects seen, these other, better-known contaminants were not at levels of concern at the locations in the current study. Mercury was far below levels of concern in swallows at the northern Wisconsin lakes and on the Mississippi River near La Crosse, WI (Custer et al 2007, 2012b, 2013b). Polychlorinated biphenyls were likewise at low concentrations in tree swallow eggs on the Mississippi River near La Crosse (Custer et al. 2007, 2013b) and at background concentrations at both Pigs Eye Lake (<0.5 μg/g ww, unpublished data) and Star Lake (<0.1 μg/g ww, unpublished data). Polybrominated diphenyl ether (PBDE) flame retardants were also not elevated (Custer et al. 2010c, total PBDEs = 49.3 ng/g ww [Pigs Eye Lake] and 15.5 ng/g ww [Star Lake], unpublished data) to levels of concern in swallows at these study sites.

The available literature data on reproductive effect levels in birds for PFASs are sparse, and there is only one field study (Custer et al. 2012a). Data from laboratory experiments (Gallagher et al. 2004; Newsted et al. 2007) and egg-injection studies (Molina et al. 2006; O'Brien et al. 2009) should be used with caution because neither are necessarily directly applicable to field studies. Egg-injection studies in particular are difficult to relate to field situations because they do not incorporate extrinsic factors that affect hatching success in wild bird populations (Custer et al. 2005) and often only report the amount injected, not the amount biologically incorporated into the whole egg. In a laboratory feeding trial of northern bobwhite quail (*Colinus virginianus*) and mallard ducks (*Anas platyrhynchos*), the eggs were artificially incubated to examine primarily embryo toxicity. There was no effect on percent of eggs that hatched with biologically incorporated egg concentrations of 17,700 ng/g ww (quail) and 15,100 ng/g ww PFOS (mallard, both converted from yolk concentrations using a 29:71 % yolk albumin mass ratio in herring gulls [Gebbink and Letcher 2012]). In an unpublished report (Gallagher et al. 2004), an even larger dietary exposure group also had no hatching success impairment with whole egg concentrations of a similar magnitude to that described previously. Newsted et al. (2005) set a predicted no effect concentration of 1.0 μg/mL in egg yolk (= approximately 2,900 ng/g ww in whole egg using the above-mentioned conversion factors). These effect concentrations were ~20–100 times greater than effect levels seen in the field with tree swallows. Similarly high thresholds (>90,000 ng/g ww) for no reproductive effects were found for PFBS in a laboratory study of quail and mallards (Newsted et al. 2008). Assuming that the amount of the chemical injected into the egg is equal to the concentration actually present in the egg tissue (thus making it equivalent to field egg concentrations), then the calculated LD_{50} value for domestic chicken (*Gallus gallus domesticus*) is 4,900 ng/g ww (Molina et al. 2006) and 93,000 ng/g (O'Brien *et al.* 2009), both one to two orders of magnitude greater than we found for tree swallows in the current study. The lower CI value in Molina et al. (2006) was 280 ng/g, similar to that of tree swallow values derived from both multistate and logistic models.

Several things may explain these radically different effect levels compared with those of the current field study. In the feeding trials by Newsted and co workers (2006, 2008) and Gallagher et al. (2004), the eggs were artificially incubated, so any adult behavioral effects would not have been assessed. They also used single-chemical treatments, whereas tree swallows in the current study were exposed to a mixture of PFASs, although PFOS dominated the spectrum. There is always the possibility that tree swallows are unusually sensitive to PFAS exposure. Although this is unlikely, it is not unprecedented. For example, chickens are extremely sensitive to a variety of organic contaminants compared with wild birds (Karchner et al. 2006), and Old World vultures are unusually sensitivity to diclofenac compared with New World vultures (Rattner et al. 2008). It was probably because of these high effect thresholds from laboratory studies that few field studies have been performed to date. It would be useful, based on the results of the current tree swallow study, to conduct field studies on other avian species to determine whether PFAS exposure is associated with reproductive impairment.

Exposure Modeling

The predictive equations for estimating egg concentrations from plasma concentrations should be used with caution because they were developed with limited data from a single geographic region. This region most likely had a common source for PFOS, so they may not be applicable to new data from other areas. On the positive side, however, the ratio of plasma to eggs was 1:1.3 which was similar to the one-to-one ratio (eggs to serum) found in a laboratory study of quail (Newsted et al. 2008). It also must be recognized that there is uncertainty around both the intercept and slope estimates from the current study; however we should expect in any new location a positive correspondence between PFOS concentration in plasma and egg. Additional data from a larger collection of individuals might help determine whether female age and seasonality influence relations between blood and egg concentrations, but if our data are any indication, the effects should be moderate relative to the effect of location.

Conclusion

This is the second field study in which a negative association was found between hatching success and PFAS contaminant concentrations in tree swallow eggs. The concentration at which effects became evident (150–200 ng/g ww) was far lower than effect levels for other avian species found in laboratory feeding trials or egg-injection studies. This discrepancy was likely because behavioral effects and other extrinsic factors are not accounted for in these laboratory studies; there is a mixture of PFASs in field studies rather than a single-contaminant exposure as is common in laboratory studies; and there is the outside possibility that tree swallows are unusually sensitive to PFASs. Until more field effect studies are performed on other avian species, the answer to that question will remain unanswered. The results from multistate modeling and simple logistic regression analyses were nearly identical. Multistate modeling may provide a better method to examine possible effects of additional covariates and assessment of models using AIC analyses, but this requires more sophisticated programming skills. There was a credible association between PFOS concentrations in plasma and eggs, so extrapolation between these two commonly sampled matrices can be performed. Finally, there was a significant difference in both exposure and PFAS congener pattern in two isolated lakes with different pHs. This is the first time this has been reported, so additional lakes should be sampled to the increase the sample size and either validate or invalidate this finding.

Acknowledgments We thank John Moriarty, Parks and Recreation Department, Ramsey Co., MN; Matt Brown, Northwestern College, St. Paul, MN; Steve Lenhart, United States Army Corp of Engineers, Hastings, MN; Helen C. Nelson, Buffalo, MN; City of South St. Paul; MN Department of Transportation; Jim Nissen, Upper Mississippi River Fish and Wildlife Refuge; the Iowa, Chicago and Eastern Railroad; and other landowners for allowing us to have nest boxes on their properties. We also thank MaryAnn Starus, Sandra Raimondo, L. Kirk Lohman, and two anonymous reviewers for comments on earlier drafts of the manuscript. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the United States Government. The views expressed in this paper are those of the authors and do not necessarily reflect the views or policies of the USEPA.

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