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Abstract

Perfluoroalkyl substances (PFAS) are a large, diverse group of chemicals and several perfluoroalkyl acids (PFAAs) are known environmental contaminants. Wildlife exposure to PFAAs and precursors has been shown, but less is known regarding replacements such as shorter-chain PFAS. In the present study, exposure to a suite of PFAAs and associations with dietary, biological and ecological factors were investigated in populations of a sentinel apex species – the peregrine falcon (*Falco peregrinus*). Nestling blood (n = 57) and sibling eggs (n = 9) were sampled in 2016 and 2018 from nests in rural and urban regions across the Laurentian Great Lakes Basin, Canada. PFSAs (perfluorinated sulfonic acids) including PFHxS, PFOS, and PFDS were detected in most egg and plasma samples, whereas 11 PFCAs (perfluorinated carboxylic acids; C_5-C_{14}, C_{16}) compared to eight PFCAs (C_8-C_{14}, C_{16}) were detected in most eggs and plasma, respectively. Shorter-chain C_8-C_{11} PFCAs were more dominant in plasma and longer-chain C_{12}-C_{14} PFCAs in eggs, but profiles were similar for PFOS, PFDS, PFUdA and PFHxDA. The exposure to PFAAs in peregrine falcons is likely mediated by dietary factors such as foraging location (δ^{13}C and δ^{34}S) and trophic position (δ^{15}N) given the associations observed in eggs and nestling plasma, respectively. Moreover, significant relationships were observed for circulating ΣPFCAs and region (rural/urban), and nestling body condition after adjusting for sampling year and dietary tracers, suggesting that compared to rural nestlings, urban nestlings may be more exposed to ΣPFCAs and prone to their potential physiological impacts. Our findings highlight the importance of integrating dietary, biological and ecological factors when studying PFAS exposure in birds.

Keywords

Perfluoroalkyl acids and sulfonamides; egg and plasma tissue; nestlings; urbanization; isotope analysis

1. Introduction

Perfluoroalkyl substances (PFAS) are a large and diverse group of synthetic chemicals used in a variety of industrial, commercial and consumer products (Kissa, 2001). Several congeners in the perfluoroalkyl acid (PFAA) sub-grouping, and specifically perfluorinated sulfonic acids (PFSAs) and carboxylic acids (PFCAs), are known to be ubiquitous and persistent in the environment (Buck et al., 2012), including in wildlife and humans (Martin et al., 2004; Sturm and Ahrens, 2010; Houde et al., 2011). A well established and highly bioaccumulative PFAA in the environment is the PFSA, perfluorooctane sulfonic acid (PFOS; Kannan et al., 2001b; Houde et al., 2006). Because of the environmental concern, some PFAAs (e.g. PFOS and PFOA (perfluorooctanoic acid)) have been phased-out in major developed countries (3M, 2000; Vierke et al., 2012). However, their production has been increasing in developing countries, which could potentially result in continued contamination globally (OECD, 2015). Indeed, PFOS levels in biota worldwide do not yet appear to be consistently declining (Land et al., 2018). Moreover, the increasing production and use of the less persistent e.g., shorter-chain PFCAs and PFSAs and their precursors as replacements of long-chain PFAAs (Ritter, 2010), may lead to enhanced exposure, accumulation and/or toxicity in wildlife and humans, as shorter-chain PFAAs have been suggested to have higher bioavailability than long-chain ones (Vongphachan et al., 2011).

The peregrine falcon (*Falco peregrinus*) is an apex bird of prey of the terrestrial food web, and an obligate predator of birds. It is also an established sentinel species useful for characterizing and monitoring a range of environmental contaminants (e.g., Fernie and Letcher, 2010; Smits and Fernie, 2013; Vorkamp et al., 2019). Temporal trends indicate significantly increasing sum(Σ) PFCA concentrations in peregrine eggs from Sweden and Greenland (Holmström et al., 2010; Vorkamp et al., 2019). Such trends in combination with the industrial transition to replacements such as shorter-chain PFAA and precursor production and use, warrant characterization of the exposure to known persistent and, in some cases, bioaccumulative PFAS contaminants, namely PFCAs and PFSAs, of peregrine falcons in other regions.

Diet is a major pathway of contaminant exposure in predators, and variations in bioaccumulative PFAS tissue concentrations may be caused by dietary plasticity (e.g. Gebbink et al., 2011a). Thus, when determining PFAS exposure and accumulation in wildlife, it is important to understand dietary variation relative to differences in foraging sources (stable isotopes: carbon/sulfur) and trophic position (nitrogen; Peterson and Fry, 1987). Other ecological factors, such as urban/rural ecotype, may also contribute to differences in PFSA and/or PFCA exposure, as previously demonstrated in abiotic and biotic samples across Canada (Gewurtz et al., 2013), and in nestling bald eagles (*Haliaeetus leucocephalus*) in the U.S.A. (Route et al., 2014). Such differences in contaminant

exposure could be attributed to anthropogenic influences and/or different dietary habits between rural and urban birds. It has been established that the diet of urban peregrine nestlings had significantly more terrestrial sources (enriched $\delta^{13}C$ values), while rural nestlings had a broader diet which includes aquatic birds (Fernie et al., 2017).

Addled eggs and blood are commonly utilized as non-destructive biomonitoring tools for environmental contaminants (Espín et al., 2016). Nevertheless, PFAAs and precursors may distribute/transfer differently in these two tissues as shown in common guillemots (*Uria aalge*; Holmström and Berger, 2008) and herring gulls (*Larus argentatus*; Gebbink and Letcher, 2012). To better understand the tissue distribution, accumulation and/or exposure pathways, we sampled eggs and nestling plasma of peregrine falcons from rural and urban locations across most of the Laurentian Great Lakes Basin (Province of Ontario, Canada). Our objectives were to: (1) determine the exposure and accumulation (levels and profiles) of short-chain PFAAs (≤ C_7) and long-chain PFAAs (C_8-C_{19}), and their precursors, in peregrine falcon nestlings and eggs; (2) compare PFAA concentrations, profiles and their association with diet (inferred from stable carbon, nitrogen and sulfur isotopes) between nestling plasma (e.g. dietary intake) and sibling eggs (maternal deposition); and finally (3) provide an integrated investigation of the relationships of diet, biology (age, sex and body condition), ecology (year and region) and PFAA exposure in free-living peregrine falcon nestlings.

2. Material and methods

2.1. Study species and sampling

This study was conducted in compliance with the guidelines of the Canadian Council of Animal Care and with all necessary permits. Peregrine falcon nestlings (23 ± 4 d of age) were banded, blood sampled, and measured. Blood samples were collected from up to two sibling nestlings (n = 57) per nest (usually one male and one female) in 2016 and 2018 from 26 active nests across the Laurentian Great Lakes Basin, failed-to-hatch sibling eggs (n = 9) in 2018 from six nests were also collected (Fig. S1). The nests were located in large urban centres in southern part of the Province of Ontario (n = 15 nests), as well as in rural areas approximately 1500 km northwest along Lake Superior in northern Ontario (n = 11 nests). The nesting sites were monitored and detailed sampling and locations have been reported previously (Fernie and Letcher, 2010; Smits and Fernie, 2013; Fernie et al., 2017). Briefly, collected blood samples were centrifuged to separate plasma from red blood cells (RBCs), divided into separate aliquots for each bird, and immediately stored in liquid nitrogen until transfer to a -80 °C freezer. Whole eggs were stored at -20 °C. All samples were shipped frozen to ECCC's National Wildlife Specimen Bank in the National Wildlife Research Centre (NWRC; Ottawa, Ontario, Canada). Egg samples were then processed by removing eggshells, homogenizing the contents, then

separating the homogenate into aliquots for various analyses. All samples were stored at –80 °C until subsequent analyses.

2.2. Perfluoroalkyl acid and sulfonamide analysis

The extraction and instrumental analysis of PFCAs, PFSAs and FASAs (perfluorinated sulphonamides) in peregrine falcon egg and plasma samples were conducted in the Organic Contaminants Research Laboratory, NWRC. The extraction methods for both eggs and plasma and instrumental analysis are reported in detail elsewhere (Chu and Letcher, 2008; Gebbink and Letcher, 2010; Greaves and Letcher, 2013; Letcher et al., 2015; Su et al., 2017). Briefly, each sample of homogenized egg material (0.4 – 0.6 g) or plasma (0.1 – 0.3 g) was weighed into polypropylene tubes and spiked with stable isotope labelled internal standards (ISs; details are listed in Table S1). The mixture was extracted three times in 3 mL of formic acid-acetonitrile solution (0.2 %; *v*:*v*). The extract was cleaned-up on MeOH and water conditioned SPE WAX cartridges (60 mg × 3 mL; Oasis), and washed with 1 mL of formic acid/water solution (2 %; *v*:*v*) and 2 mL of water. Analytes (PFSAs and PFCAs) were collected by eluting the cartridges twice with 1 mL of ammonium hydroxide/methanol solution (1 %; *v*:*v*). The final extract was evaporated to dryness under a gentle nitrogen flow and reconstituted in 500 µL of UPLC grade MeOH, followed by filtration using centrifugal filters (500 µL modified nylon 0.2 µm; VWR) and transferred into polypropylene injection vials. FASAs were analyzed in a subset of samples (*n* = 7 egg; *n* = 8 plasma) by taking a proportion (2 mL) of the initial extract. This fraction was cleaned with active carbon, diluted with 8 mL of water, and subsequently cleaned up on WAX cartridges. Analytes were collected using 1 mL of MeOH. A proportion (500 µL) of the final extract was taken and further cleaned using 0.02 g of active carbon by vortexing for 1 min, filtered, and transferred into injection vials.

A total of 22 PFAAs and FASAs were quantified using UPLC-MS/MS operated in negative electrospray ionization (ESI) mode. The target compounds included four FASAs: FBSA, FOSA, N-MeFOSA and N-EtFOSA, five PFSAs: PFBS, PFHxS, PFEtCHxS, PFOS and PFDS, and 13 PFCAs (C_4-C_{14}, C_{16} and C_{18}): PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUdA, PFDoA, PFTrDA, PFTeDA, PFHxDA and PFODA (full names are provided in Table S1). Quality assurance/quality control (QA/QC) procedures included the analysis of IS spiked samples and duplicates, as well as the concurrent analyses of a procedural blank and an in-house reference material (eggs of double-crested cormorant; *Phalacrocorax auritus*) every 10 samples. Recoveries for the ISs ranged between 26 % – 43 % for FASAs and 73 % – 100 % for PFCAs and PFSAs (Table S1). Relative standard deviation for in-house reference material (*n* = 3) ranged from 0.3 % to 18 % for PFSAs and long-chain PFCAs (C_9 – C_{13}). Relative percent differences inferred from duplicated samples ranged between 4 % – 32 % for all PFAS in eggs (*n* = 9), and within 40 % for compounds with concentrations above 1 ng/g (between 4 % and 17 % for C_9 – C_{14}

PFCAs and PFOS) in plasma (n = 14). The method limit of quantification (MLOQ) and method limit of detection (MLOD; Table S1) were set as the minimum amount of analyte producing a peak with a signal-to-noise ratio (S/N) of 10 and three, respectively. All concentrations were corrected for average blank values (Table S1). Mean concentrations are reported for duplicated samples. All concentrations are expressed in ng/g (*ww*; wet weight).

2.3. Stable isotope analysis

The analysis of stable carbon (^{13}C and ^{12}C), nitrogen (^{15}N and ^{14}N) and sulfur (^{34}S and ^{32}S) isotopes was conducted at the Ján Veizer (formerly G.G. Hatch) Stable Isotope Laboratory (Ottawa, Canada). Detailed analytical procedures of C and N isotopes have been reported elsewhere (Fernie et al., 2017). Briefly, the C and N isotopes in nestling red blood cells and eggs (lipid-extracted and freeze-dried) were determined using an isotope ratio mass spectrometer (IRMS; Delta Advantage, Thermo) coupled to an elemental analyzer (EA; vario EL cube, Elementar) via a Conflo III interface (Thermo). The S isotopes were determined using a Delta^{Plus} XP IRMS (Thermo) coupled to an isotope cube EA (Elementar) via a Conflo IV interface (Thermo). The samples were flash combusted at approximately 1800 ^{o}C (Dumas combustion) and the resulting gas products were carried by helium through the EA to be cleaned and separated, and sent to IRMS via interface. The SI ratios for C, N and S are expressed as δ values (‰) relative to their respective international standards Vienna Pee Dee Belemnite, atmospheric N_2 and Vienna Cañon Diablo Troilite, and normalized to calibrated internal standards. The analytical precision inferred from internal standards showed $\pm SD \leq 0.2$ ‰ for $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$. The relative percent difference (RPD) of duplicates (n = 7) measured every 10 samples was 0.4 %, 0.9 % and 14.0 % for $\delta^{13}C$ $\delta^{15}N$, and $\delta^{34}S$, respectively. The results of a concurrently analyzed in-house reference material (DCCO egg/liver) for $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ were overall within the acceptable range ($\leq \pm 3$ *SD*).

2.4. Data analysis

In the present study, only PFAAs and FASAs with a detection frequency (Table S1) above 80 % were statistically analysed, which for eggs included three PFSAs (PFHxS, PFOS and PFDS) and 11 PFCAs (C_5-C_{14} and C_{16}), and for nestling plasma samples, the same three PFSAs (PFHxS, PFOS and PFDS) and eight PFCAs (C_8-C_{14} and C_{16}). Non-detects were replaced with half of the detection limits or set to zero when calculating sums and ratios. All statistical analyses and plotting of the results were performed using R 3.6.3 (R Core Team, 2020).

Principal component analysis (PCA) was conducted to explore the overall patterns and variations of PFAAs (log-transformed) in eggs and nestling plasma collected in 2018. Analysis of variance (ANOVA) was additionally used to determine the significance of different PFSA and PFCA

compositions between eggs and plasma, and the short-term (2016 vs. 2018) and/or spatial (rural vs. urban) differences in body condition, dietary tracers ($\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$), Σ_3PFSA and Σ_8PFCA concentrations in nestlings. Tukey HSD test was used for post-hoc comparisons when applicable. The spatiotemporal comparisons with eggs were not made since all eggs were collected in 2018 and only one egg was from the rural region. Q-Q plots were used to check the normality of dependent variables, and log-transformation was performed when necessary. In addition, Pearson product-moment correlation was used to analyze possible associations in the concentrations and percentages of individual PFAA, ΣPFSAs and ΣPFCAs between eggs and plasma of sibling nestlings in a subset of samples (averaged from six nests), as well as their associations with dietary tracers. False discovery rate (FDR) adjusted *P*-values were calculated for multiple tests (> 10).

To investigate the relationships among PFAA exposure and biological, ecological and dietary factors, we fitted linear mixed-effect models (Bates et al., 2015) for Σ_3PFSAs and Σ_8PFCAs in nestling plasma (both were log-transformed to meet model assumptions). Fixed effects were set as year and region (rural and urban; spatiotemporal effect); nestling age, sex and body condition (calculated as body weight/tarsus length; biological parameter effect); as well as $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ (dietary effect). Nest site (i.e.,the location of each individual nest) was included as a random effect to control for possible correlations among siblings as multiple blood samples (up to two nestlings) were often collected within the same nest. Full models were fitted with all of the above predictor variables. We checked multi-collinearity using variance inflation factors (VIFs), and all variables were within an acceptable range (< 3; Zuur et al., 2010). Model adequacy was positively evaluated using residual plots. The most parsimonious models (models with the lowest AIC*c* – Akaike Information Criterion corrected for small sample size) were selected as the final models.

3. Results and discussion

3.1. PFAA/FASA exposure concentrations and profiles in eggs and nestling plasma

In the present study, the three most frequently detected PFSAs in the peregrine falcon eggs and nestling plasma were PFHxS (100 %; 98 %), PFOS (100 %, 100 %) and PFDS (100 %, 93 %), whereas there were more PFCAs detected in the same eggs (11 PFCAs: C_5 - C_{14}, C_{16}) than nestling plasma (8 PFCAs: C_8 - C_{14}, C_{16}) with C_5, C_6 and C_7 detected in more than 80 % of the egg samples, but not as frequently in nestling plasma (Table S1).

The PFOS concentrations (mean: 28 ng/g *ww*; 2018; *n* = 9; see Table 1 for further details) in eggs collected across the Laurentian Great Lakes Basin in the present study are considerably lower than PFOS levels reported in eggs collected earlier in Sweden (mean: 83 ng/g *ww*; 2006; *n* = 10; Holmström et al., 2010) and Greenland (mean: 85 ng/g *ww*; 1986 - 2014; *n* = 41; Vorkamp et al., 2019),

192 whereas the ∑PFCA (C_9 - C_{14}) egg concentrations are more comparable (mean: 15, 22, and 26 ng/g *ww*
193 for Ontario, Sweden and Greenland, respectively). This is expected as the PFOS precursor compound,
194 PFOSF (perfluorooctane sulfonyl fluoride), has been phased-out by its main manufacturer since 2000-
195 2002 (3M, 2000). In accordance, PFOS has been found to be decreasing in different taxa including birds
196 breeding in the Laurentian Great Lakes (Gebbink et al., 2011b), the Pacific coast of Canada (Miller et
197 al., 2015), and the coasts of northern Europe (Ullah et al., 2014; Sun et al., 2019). Moreover, plasma
198 concentrations of PFOS in the peregrine nestlings in the present study (18 ng/g *ww*) are well below
199 the levels detected in plasma of double-crested cormorants (185 ng/mL; *n* = 4), herring gulls (315
200 ng/mL; *n* = 2), and bald eagles (21 – 2220 ng/mL; *n* = 33) from the American Great Lakes Basin and
201 immediate regions in 1991 - 1993 (Kannan et al., 2001a) as well as in herring gulls from the Canadian
202 Great Lakes Basin in 2010 (92 ng/g; *n* = 8; Gebbink and Letcher, 2012). In comparison, PFCAs are still
203 largely produced and widely used (Wang et al., 2014; Brendel et al., 2018), which may explain their
204 comparable levels, spatially and temporally, in eggs observed in the present study and those in
205 previous studies of peregrine falcons.
206 PFOS dominated the PFAA profiles in both plasma (≥ 50 %) and eggs (> 60 %) in the present
207 study, followed by PFNA (8.1 %) and PFUdA (7.4 %) in plasma, and PFTrDA (9.3 %) and PFTeDA (7.3 %)
208 in eggs (Fig. S2). The dominance of PFOS in the egg and plasma profiles is in agreement with the PFAS
209 profile generally reported in biota (Houde et al., 2006), in European starling (*Sturnus vulgaris*) eggs
210 from across Canada (Gewurtz et al., 2018), as well as in peregrine eggs from Sweden (Holmström et
211 al., 2010) and Greenland (Vorkamp et al., 2019). Furthermore, the dominance of PFTrDA in the present
212 eggs from Ontario is also consistent with the Swedish PFCA egg profiles (Holmström et al., 2010),
213 although slightly different from the Greenland egg profiles, which were dominated by PFUdA
214 (Vorkamp et al., 2019). Different PFCA contaminant patterns could imply different contaminant
215 sources and pathways, such as long-range transport in Greenland (Bossi et al., 2005) versus more
216 localized contaminant sources in comparatively more industrialized Sweden and Ontario.
217 Nevertheless, in the current study, long-chain PFCAs were generally detected at higher frequencies in
218 both plasma and eggs compared to short-chain PFCAs, consistent with the higher bioaccumulative
219 potential found with increasing fluorinated carbon length (Conder et al., 2008).
220 Although both eggs and nestling plasma in the present study have been analyzed for PFSA
221 precursors (i.e., FBSA, FOSA, N-MeFOSA and N-EtFOSA), they were mostly unquantifiable in eggs and
222 the detection frequencies in plasma were generally low (12 % – 62 %; Table S1) in the peregrines of
223 the current study. Moreover, we observed different PFAA profiles between the peregrine falcon eggs
224 and nestling plasma. The PCA analysis showed clearly separated clusters of egg and nestling plasma
225 PFAAs (Fig. 1): the variations appeared to be driven by shorter-chain PFHxS, PFOA and PFNA in nestling

plasma and longer-chain PFDS, PFTrDA and PFTeDA in eggs. Largely consistent with the PCA results, significant differences were observed in the PFCA and PFSA compositions between eggs and nestling plasma: proportions of all compounds differed except for PFOS, PFDS, PFUdA and PFHxDA (Fig. S3), with significantly higher proportions of C_8 - C_{10} PFCAs in plasma in contrast to the higher proportions of C_{12} - C_{14} PFCAs in eggs. These differences between eggs and nestlings, likely reflect maternal exposure, transfer and deposition into eggs, compared to immediate ingestion by diet for the nestlings (plasma). The PFAA profile in biota may be affected by the bioaccumulation and pharmacokinetics of PFAA in proteins. Previous studies have reported preferential sequestering of PFOS and long-chain PFCAs into eggs of common guillemots (Holmström and Berger, 2008) and herring gulls (Gebbink and Letcher, 2012). The similar proportions of PFDS, PFOA, PFUdA and PFHxDA in eggs and plasma, however, may suggest the retention of these compounds during embryonic development and despite nestling growth in combination with sources of exposure on the breeding territory.

3.2. PFAAs in nestlings and sibling eggs: Dietary intake and/or maternal deposition

Frequently, studies characterizing chemical concentrations and profiles in birds will utilize eggs or occasionally blood, but rarely do studies have the opportunity to evaluate potential associations in chemical concentrations between eggs and their sibling nestlings, valuable for understanding possible implications of chemical exposure and accumulation at different reproductive stages involving the adult (egg) vs nestling peregrine falcons (see also Section 3.3). Chemical concentrations and profiles in eggs and nestling plasma may reflect maternal exposure, deposition and/or dietary intake in the exposure of wild birds to contaminants. In the present study, we did not observe any significant correlations in PFAA concentrations or ratios in eggs and plasma of sibling nestlings (adjusted *P*-values ranged 0.17 – 0.92; Fig. 2). Despite the non-significant adjusted *P*-values, there are potential trends in correlations between eggs and their sibling nestlings in the concentrations of PFDS (r = 0.94), PFOA (r = 0.83) and PFHxDA (r = 0.88), as well as the ratios of PFNA:$\sum$PFAS (r = 0.87) and PFTeDA:$\sum$PFAS (r = 0.81), and significant correlations may have been observed with a large sample size since the present study comprised only opportunistically sourced fail-to-hatch eggs from six nests.

The differences in PFAA levels and profiles between eggs and sibling nestlings are consistent with the general lack of correlation between the two matrices discussed above. Such results are expected: egg concentrations represent the female body burden during egg laying, in this study ca. two months prior to blood sampling nestlings assuming an average incubation period of 33 days (Sherrod, 1983), and could be influenced by the female's diet during spring migration and/or egg formation on her breeding territory. In contrast, blood plasma generally reflects recent dietary intake and metabolism of growing nestlings. Similarly, there were no correlations between eggs and nestlings

of various PFAAs observed in a small terrestrial passerine bird, the great tit (*Parus major*), that nested at and in the vicinity of a fluorochemical plant in Antwerp, Belgium (Lopez-Antia et al., 2019).

3.3. PFAA burdens: Associations with dietary factors

Differences in contaminant exposure and burdens often reflect differences in diet, a major route of contaminant exposure, and hence it was important to identify and characterize possible dietary differences among the present peregrines. Rural and urban peregrine nestlings in the present study differed significantly in their blood stable isotope ratios: urban nestlings had significantly enriched δ^{15}N (trophic position; mean: 8.4 ‰, range: 7.7 – 9.5 ‰) and δ^{13}C (foraging location; mean: -21.1 ‰, range: -22.7 – -19.1 ‰) compared to their rural counterparts (δ^{15}N, δ^{13}C: mean: 8.0 ‰ and -24.4 ‰, range: 6.5 – 9.6 ‰ and -26.7 – -23.6 ‰; $P = 0.04$ and $P < 0.001$, respectively; Table S2 and Fig. S4). These results are generally consistent with that reported in a previous study of the same peregrine falcon populations in 2010 ($P = 0.07$ and $P < 0.001$ for δ^{15}N and δ^{13}C; Fernie et al., 2017), suggesting a consistent difference in foraging patterns in terms of trophic position and foraging location across time (2010 vs. 2016, 2018) of the peregrines in urban versus rural ecotypes. The present study reports for the first time the significant depletion of δ^{34}S (also indicating foraging location) in the urban peregrines (mean: 1.7 ‰, range: -1.3 – 5.4 ‰) compared to the rural peregrines (mean: 3.5 ‰, range: 0.2 – 6.4 ‰; $P < 0.001$). The enrichment of δ^{15}N and δ^{13}C as well as the depletion of δ^{34}S were strongly related to urbanization in Eurasian dippers (*Cinclus cinclus*), potentially due to enriched heavier N isotopes from microbial processing and production of hydrogen sulfide (a form not readily transferred to primary producers) in soil and water in the urban environment (Morrissey et al., 2013). The dietary plasticity reflected in stable nitrogen, carbon and sulfur isotopes in the nestling peregrine falcons may also be a result of the availability of and strategy for resources: a largely terrestrial diet of anthropogenic influences, such as pigeons (*Columba livia*) in the urban peregrines contrasting with a more variable aquatic-based diet in the rural birds (Fernie et al., 2017).

We also investigated the possible influence of dietary factors (i.e., stable isotopes) on the PFAA concentrations measured in eggs and their sibling nestlings. We found marginally significant (all adjusted $P = 0.07$) correlations of δ^{13}C and PFNA ($r = -0.93$), PFDA ($r = -0.95$), PFUdA ($r = -0.94$) and $\sum$PFCAs ($r = -0.95$), and of δ^{34}S and PFPeA ($r = -0.93$) in eggs. However, there was a general lack of significant correlations of egg PFAA concentrations and δ^{15}N (Fig. S5). Our results therefore suggest that foraging location indicated by δ^{13}C and δ^{34}S, not trophic position, of the maternal bird likely contributed the most to these egg burdens. This is in contrast to the lack of significant relationships between these isotopic values and PFAAs in the sibling nestlings (Fig. S5). Adult peregrines may regularly consume prey from a narrow range in trophic position but having migrated immediately prior to egg laying, they likely have consumed prey from an enormous geographic region, possibly

294 explaining the correlations observed here for $\delta^{13}C/\delta^{34}S$ and PFAA levels in eggs. In comparison to adult
295 peregrines, the nestlings, fed by both parents, may consume prey within a smaller geographic area,
296 i.e., the breeding territory closely surrounding their nest, which may explain the lack of correlations.
297 Consistent with the bioaccumulative and biomagnifying characteristics of PFAAs (e.g. Haukås
298 et al., 2007; Xu et al., 2014; Simonnet-Laprade et al., 2019), linear mixed-effect models showed
299 significant associations of $\delta^{15}N$ and both ΣPFSAs and ΣPFCAs while controlling for the possible effects
300 of other factors such as year and region (Fig. 3 and Table 2). Our results suggest peregrine nestlings
301 consuming prey from a higher trophic position, potentially urban peregrines (as discussed above), are
302 more likely to have higher circulating concentrations of circulating ΣPFSAs and ΣPFCAs. The greater
303 regression slope of Σ_3PFSAs versus $\delta^{15}N$ (0.47) compared to Σ_8PFCAs (0.27) may suggest a higher
304 biomagnification rate of PFSAs (of which 91 % is PFOS) in the studied peregrines. Indeed, an
305 experimental study has shown that PFOS generally has greater biota soil accumulation factors than
306 PFCAs with a perfluoroalkyl chain length of 10 or lower (Zhao et al., 2013). There was also a
307 significantly positive relationship between ΣPFSAs and $\delta^{34}S$ (foraging location), as well as a significantly
308 negative relationship between ΣPFCAs and $\delta^{13}C$ (foraging location). These findings indicate that the
309 urban terrestrial-based diet with depleted $\delta^{34}S$ and enriched $\delta^{13}C$ may mediate the exposure and
310 ingestion of PFSAs and PFCAs by nestling peregrine falcons. A similar relationship between $\delta^{13}C$ and
311 flame retardants (i.e., ΣPBDEs) has been observed in the same peregrine populations in 2010 (Fernie
312 et al., 2017).

313 **3.4. PFAA burdens: Associations with biological parameters**

314 We examined the relationship of the nestling peregrine falcons' biological parameters (i.e.,
315 age, sex and body condition) in relation to their exposure to ΣPFSAs and ΣPFCAs. Nestlings' age and
316 sex did not significantly explain the variation in plasma ΣPFSA or ΣPFCA concentrations and were not
317 selected in the final models. There was, however, a significant relationship between body condition
318 and ΣPFCAs (β = –0.06, P = 0.04; Table 2), suggesting that body condition of the peregrine nestlings
319 was negatively associated with higher ΣPFCA burdens that warrants further investigation. As a
320 significant correlation was not observed for ΣPFSAs and body condition of the peregrine falcon
321 nestlings, this may imply different pharmacokinetic characteristics of these compounds in the
322 nestlings. The lack of intersexual differences in PFAA concentrations of the peregrine nestlings in the
323 present study is consistent with previous findings reported in songbirds (Dauwe et al., 2007) and
324 nestling white-tailed eagles (*Haliaeetus albicilla*; Løseth et al., 2019). However, likely as a result of
325 increasing food intake by developing chicks, plasma PFAA concentrations have been found to
326 significantly increase with age in nestling white-tailed eagles (Bustnes et al., 2013; Løseth et al., 2019).
327 The (likely) reason that we did not observe a comparable relationship in the present study could be

that the peregrine nestlings were sampled at similar ages: 17-26 days except for one nestling which was sampled at 33 days (Table S2). In contrast, the nestling white-tailed eagles sampled in the previous studies were aged between 1-3 weeks and 44-87 days.

3.5. Spatial and short-term variations in PFAA concentrations

There were no significant differences in regional (rural versus urban) or short-term (2016 versus 2018) comparisons of PFAA concentrations in plasma of nestling peregrines in the present study (all $P \geq 0.86$; Fig. S6). The plasma PFAA profile also appeared to be similar among regions for both years (Fig. S2). However, the most parsimonious model for both ΣPFCAs and ΣPFSAs included region and/or year while other factors (e.g. stable isotopes) were controlled for (final models; Table 2). Based on our results, we recommend including biological factors (e.g. body condition) and stable C/N/S isotopes as dietary tracers as the preferred approach to the assessment of spatiotemporal comparisons of PFAS exposure.

After controlling for differences among the nestlings in their body condition and/or diet, significantly negative associations between year and both Σ_3PFSAs and Σ_8PFCAs were observed (β = −0.50 and −0.48, P = 0.04 and 0.01, respectively; Table 2, Figs. S7 and S8). Decreasing trends of PFOS have been observed in biota from different parts of the world due largely to the 3M phase-out of PFOSF in 2002 (e.g. Falk et al., 2019; Lynch et al., 2019; Sun et al., 2019). Decreasing trends of long-chain PFCAs such as PFOA, PFNA, PFDA and PFUnDA have also been observed corresponding to the alternative production of short-chain PFCAs (Falk et al., 2019; Lynch et al., 2019). However, the negative year effect observed in the present study should be interpreted with caution due to annual variations in PFAA exposure.

After controlling for body condition, δ^{13}C and δ^{15}N, we observed a significant relationship between region and ΣPFCAs, with urban nestlings showing a trend of higher Σ_8PFCA exposure than rural nestlings (β = 0.75, P = 0.03; Table 2, Fig. S8). A significantly higher exposure to BDE-209 in urban nestlings compared to rural ones associated with possible urban use of Deca-BDE, has previously been observed in these populations (Fernie et al., 2017). Similarly, the positive association observed here in the urban peregrines may also be linked to historical and continued use of Σ_8PFCA in large urban centres that may well serve as a major source of PFCA exposure for breeding peregrines. Previous studies have reported elevated PFSAs in various fish and bird species in urban/industrial sites compared to rural/remote sites in North America (Gewurtz et al., 2013; Route et al., 2014; Gewurtz et al., 2018). The comparable ΣPFSA concentrations in the present rural and urban peregrine nestlings however, may be attributed to the fact that peregrine falcons as obligate apex predators, consume birds throughout the long terrestrial food chain, and thus may be less exposed to the direct influence of major urban sources (e.g., industrial waste) of PFSAs. This could also explain that the trend of higher

362 ΣPFCA levels in urban nestlings was only evident when dietary habits (δ^{13}C and δ^{15}N) were adjusted in
363 the present study. It is worth noting that the most contaminated individuals we observed were two
364 sibling nestlings from the rural region, which may indicate potential source(s) of PFAAs even at the
365 remote northwest side of Lake Superior.

366 It is also important to understand that the rural and urban regions identified in the present
367 study are in fact two distinct geographical regions located at a substantial distance from each other.
368 It is likely that other environmental factors that could not be accounted for in the present study, such
369 as microclimate or food-web characteristics, may have mediated inter-regional differences in the
370 PFAA exposure of the studied peregrine falcons. Thus, the observed regional difference or the lack
371 thereof may not be related to anthropogenic influences alone, and further research to this end would
372 be beneficial caution should be exercised with respect to the simplified rural-urban categorization
373 when interpreting these results. Future research is therefore also desired to further assess the spatial
374 and temporal trends in wildlife exposure to PFAAs in these and other regions.
375

376 **4. Conclusions**

377 We measured a suite of PFAAs and FASAs in eggs and nestling plasma of peregrine falcons
378 collected across the Laurentian Great Lakes Basin. PFAA exposure patterns largely differed between
379 eggs and plasma, indicating different exposure pathways such as dietary intake and maternal transfer,
380 and/or different metabolism, accumulation and pharmacokinetics in eggs and nestlings. Significant
381 associations of ΣPFSAs and ΣPFCAs with δ^{13}C, δ^{34}S and δ^{15}N in nestling plasma suggest the dietary
382 mediation (foraging location and trophic position) of PFAA exposure in peregrine falcons, as well as
383 the biomagnifying potential of some of these PFAAs. Furthermore, our results suggest that body
384 condition (e.g., poorer) of peregrine nestlings may be associated with higher ΣPFCA burdens. Finally,
385 our results imply a higher PFCA exposure in urban nestlings due likely to the historical and continued
386 use of PFCAs in urban centres. Our findings demonstrate the need for continued investigations of the
387 relationships of PFAS exposure and dietary, biological and ecological factors in wildlife species. The
388 association between PFCA exposure and body condition in nestlings also warrants further
389 investigation of the physiological effects of these chemicals to peregrine falcons and other wildlife.
390
391

392 **Acknowledgements**

393 The present study was made possible by funding from Environment and Climate Change
394 Canada and the Chemicals Management Plan of the Government of Canada (KJF, RJL). We also thank

the Marie Skłodowska-Curie Research and Innovation Staff Exchange INTERWASTE project (project number: 734522; funded by European Commission) (KJF, AC, JS) made possible through the coordinator of INTERWASTE, Dr. Stuart Harrad (University of Birmingham), and the University of Antwerp (JS, AC, ME), as INTERWASTE partner for the secondment of JS to ECCC. We are grateful to Brian Ratcliff, Rodney Swatton, Frank Pianka, Mark Heaton, Mark and Marion Nash, Glenn Barrett, Melanie Guigueno, Jeffery Costa, Patrick Gilhooley and many others, for their long-term support during field work, and to France Maisonneuve, Emily Porter, Guy Savard, Julie Biolodeau, and others in the Lab Services unit, and Luke Periard in the Organic Contaminant Research Laboratory, at the National Wildlife Research Centre in Ottawa for their assistance and involvement in the lab analyses.

CRediT authorship contribution statement

Jiachen Sun: Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Writing - Reviewing and Editing, Visualization. **Robert J. Letcher**: Conceptualization, Methodology, Validation, Investigation, Resources, Writing - Reviewing and Editing, Supervision, Funding acquisition. **Marcel Eens**: Writing - Reviewing and Editing. **Adrian Covaci**: Writing - Reviewing and Editing, Supervision, Project administration, Funding acquisition. **Kim J. Fernie**: Conceptualization, Methodology, Validation, Investigation, Resources, Data Curation, Writing - Reviewing and Editing, Supervision, Project administration, Funding acquisition.

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Table legends

Table 1 Summary of concentrations (ng/g *ww*) of all targeted PFAS homologues, Σ_5PFSAs, Σ_{13}PFCAs, Σ_4FASAs, and sums of the most abundant PFSAs (Σ_3PFSAs: sum of PFHxS, PFOS and PFDS) and PFCAs (Σ_8PFCAs: sum of C_8-C_{14} and C_{16}) in eggs and plasma of sibling nestling peregrine falcons sampled from rural and urban areas across the Laurentian Great Lakes Basin, Canada, in 2016 and 2018. Median and *n* (number of samples detected above MLOD), mean (arithmetic mean) **±** *SD*, and range (minimum – maximum) are presented. Non-detects were not included, FASAs (FBSA, FOSA, N-MeFOSA, N-EtFOSA)

554 were only analysed in a subset of samples (a total of 1 and 7 in 2018 plasma from rural and urban
555 regions, respectively and a total of 1 and 6 in 2018 eggs from rural and urban regions, respectively), "-
556 " means not applicable.
557
558 **Table 2** Linear mixed-effect model (LMM) output for plasma Σ_3PFSAs and Σ_8PFCAs (log-transformed)
559 in nestling peregrine falcons from the Laurentian Great Lakes Basin, Canada. Nest identity was
560 included as a random effect in all LMMs. The categorical variables year, region and sex represent 2018,
561 urban and male, respectively. Significant *P* values are bolded. BC: body condition, R^2_m: marginal
562 pseudo R^2, AIC*c*: Akaike information criterion corrected for small sample size. Full models and final
563 models (lowest AIC*c*) are presented.
564
565 **Figure legends**
566 **Figure 1** Biplot of principle component analysis of perfluoroalkyl acids (PFAAs; log-transformed) in
567 eggs and nestling plasma of peregrine falcon collected across the Laurentian Great Lakes Basin,
568 Canada in 2018. Ellipses were drawn at a 68 % confidence interval.
569
570 **Figure 2** Pearson correlation matrix of perfluoroalkyl acid (PFAA) concentrations and ratios (individual
571 PFAA:$\sum$PFAAs) in nestling plasma and sibling eggs (*n* = 6 nests; 2018) of peregrine falcons collected
572 across the Laurentian Great Lakes Basin, Canada. Pearson's correlation coefficients (*r*) are given in the
573 figure, with blue representing positive correlations and red representing negative correlations, and
574 darker colours in conjunction with the shape of the circle becoming sharper representing stronger
575 correlations (*r* values closer to 1/-1 as shown in the gradient bar on the right). FDR adjusted *P*-values
576 of all pairs of concentrations and ratios showed no significant correlations (all $P \geq 0.17$).
577
578 **Figure 3** Positive relationships were identified between trophic position (δ^{15}N; measured in red blood
579 cells) and log concentrations of Σ_3PFSAs and Σ_8PFCAs (measured in plasma) in nestling peregrine
580 falcons from rural and urban areas across the Laurentian Great Lakes Basin, Canada, in 2016 and 2018.
581 Dots are concentrations (log), lines are fitted from the most parsimonious model for each compound
582 family (Table 2).