Perfluoroalkyl acid (PFAA) profile and concentrations in two co-occurring tit species : distinct differences indicate non-generalizable results across passerines

Reference:
Lasters Robin, Groffen Thimo, Bervoets Lieven, Eens Marcel.- Perfluoroalkyl acid (PFAA) profile and concentrations in two co-occurring tit species : distinct differences indicate non-generalizable results across passerines
The science of the total environment - ISSN 0048-9697 - 761(2021), 143301
Full text (Publisher's DOI): https://doi.org/10.1016/J.SCITOTENV.2020.143301
To cite this reference: https://hdl.handle.net/10067/1748920151162165141

Perfluoroalkyl acid (PFAA) profile and concentrations in two co-occurring tit species: distinct differences indicate non-generalizable results across passerines

Robin Lasters [a,b,*,1], Thimo Groffen [a,b,1], Lieven Bervoets[a], Marcel Eens[b]

[a]Systemic Physiological and Ecotoxicological Research, Department of Biology, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerp, Belgium.

Robin.Lasters@uantwerpen.be

Thimo.Groffen@uantwerpen.be

Lieven.Bervoets@uantwerpen.be

[b]Behavioural Ecology and Ecophysiology Group, Department of Biology, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk, Belgium.

Marcel.Eens@uantwerpen.be

*Corresponding author

[1]Both authors contributed equally to this work

Abstract

Eggs of terrestrial bird species have often been used to biomonitor both legacy and emerging anthropogenic contaminants, such as perfluoroalkyl acids (PFAAs). However, few, if any, studies have examined whether results obtained in a given model species can be generalized across bird species. Therefore, we compared potential differences in egg PFAA profile and concentrations between two widely studied passerine species, great tit (*Parus major*) and blue tit (*Cyanistes caeruleus*), which are similar in many aspects of their ecology and life history. Whole clutches of both species were collected from the same breeding season and at the same place (Antwerp, Belgium), enabling us to study laying order effects. Additionally, we evaluated how egg PFAA concentrations for both species changed along a distance gradient from a PFAA point source. Although the sum PFAA concentrations did not significantly differ between great tits and blue tits, large differences in PFAA profile and laying order effects were observed. Great tits showed a more diverse PFAA detection profile, including perfluorooctane sulfonic acid (PFOS) and various long-chain perfluorocarboxylic acids (PFCAs) but no short-chain compounds. Contrarily, short-chain PFCAs (perfluorobutanoic acid (PFBA) and perfluorohexanoic acid (PFHxA)) were only detected in blue tit eggs. The variation of perfluorooctanoic acid (PFOA) concentrations within clutches was large in both species, although laying order effects on PFOA concentrations were only found in blue tits. Although egg PFOA concentrations of both species decreased similarly from the fluorochemical point source onwards, more variation in egg PFOA concentrations could be explained by distance from the fluorochemical plant in great tits (60%) than in blue tits (15%). Results showed that both species markedly differed in terms of egg PFAA profile and concentrations, most likely reflecting differences in diet, foraging habits and egg protein composition. Finally, biomonitoring results of PFAAs in eggs are likely not generalizable across bird species.

Keywords: Eggs, PFAAs, great tit, blue tit, clutch variation

Introduction

Perfluoroalkyl acids (PFAAs) are a diverse group of man-made organic compounds that consist of a fully fluorinated carbon chain and a functional acid group (Buck et al., 2011). The strength of the carbon-fluorine bond makes them extremely resistant to both abiotic and biotic degradation (Beach et al., 2006; Surma et al., 2017). The hydrophobic and lipophobic characteristics of these compounds result in distinctive physicochemical properties, including oil and water repellence (Surma et al., 2017). In addition, the hydrophobic alkyl chain of PFAAs allows them to bind with proteins, by forming hydrophobic interactions, which increase with increasing alkyl chain length, within the hydrophobic cavities and on the surface of protein molecules (Fedorenko et al., 2021). This can explain their accumulation in protein-rich tissues, such as liver and bird eggs (Vicente et al., 2015). Previous biomonitoring studies have demonstrated the bioaccumulation and biomagnification of PFAAs throughout the food chain (Conder et al., 2008; Fang et al., 2014). Hence, PFAAs have been reported globally from 2000 onwards in the environment, wildlife and humans (Giesy and Kannan, 2001, 2002; Butt et al., 2010; Rodriguez-Jorquera et al., 2016).

Bird eggs have been frequently used as a less-invasive matrix for biomonitoring of PFAAs (Hoff et al., 2005; Gebbink and Letcher, 2012; Custer et al., 2014; Groffen et al., 2017, 2019a). The majority of these biomonitoring studies measured PFAA concentrations in one randomly collected egg per clutch. However, little is known on the variation of PFAA deposition both within and among clutches (Custer et al., 2012; Vicente et al., 2015; Lasters et al., 2019). Within-clutch variation (WCV) of PFAA concentrations was much larger than the among-clutch variation (ACV) in Audouin gulls (*Larus audouini*) (Vicente et al., 2015) and great tits (Lasters et al., 2019). In the latter study, laying order effects were found to be an important driver of the large WCV in PFAA concentrations (Lasters et al., 2019). Therefore, it is unlikely that random collection of one egg will result in a representative measure of PFAA concentrations on the whole clutch level.

Biomonitoring studies along a distance gradient emanating from an active fluorochemical plant (3M) in Antwerp have revealed the highest concentrations of perfluorohexane sulfonic acid (PFHxS), perfluorooctane sulfonic acid (PFOS), perfluorodecane sulfonic acid (PFDS) and perfluorooctanoic acid (PFOA) ever detected in wild bird eggs (Lopez-Antia et al., 2017; Groffen et al., 2017, 2019a), which highlights that the fluorochemical plant in Antwerp is a PFAA hotspot. However, very few studies have examined possible interspecific differences in PFAA exposure among birds (Lopez-Antia et al., 2017; Su et al., 2017). Lopez-Antia et al. (2017) measured PFOS concentrations in eggs of three bird species from different trophic levels and found no significant differences among the species in terms of PFOS concentrations. On the other hand, Su et al. (2017) observed significant differences between an obligate piscivorous species (Caspian tern) and a facultative piscivore (herring gull). However, the eggs were collected on very different spatial gradients and within a large timeframe, which hinders the species comparability of these results. This emphasizes the need of a standardized monitoring design, along the same gradient and time period. By focusing on two widely studied species, the ability exists to identify broad intra-specific patterns in PFAA exposure that may be generalizable to other organisms.

To meet this end, passerines from the tit family (Paridae), specifically the blue tit (*Cyanistes caeruleus*) and great tit (*Parus major*), may be promising candidate birds to study potential differences in PFAA profile and concentrations in relation with clutch variation and laying order effects. Both tit species have been frequently used as biomonitoring species for POPs and metals and often share the same habitat (Dauwe et al., 2002; Van den Steen et al., 2009a; 2009b; Groffen et al., 2017). Nevertheless, they differ from each other in some life-history traits, such as clutch size, dispersal behaviour, diet, lifespan and metabolism (Cramp and Perrins, 1993). Together, this may result in accumulation differences to PFAAs and hence egg deposition of these pollutants between both species. Consequently, it is relevant to conduct a comparative analysis between these species in terms of PFAA concentrations and profile as well as to examine their suitability for biomonitoring of PFAAs.

Therefore, the central objective of this study was to investigate potential differences in egg PFAA profile and concentrations between two co-occurring passerines, using data of whole clutches that originate from the same area and breeding season. Secondly, variation patterns (WCV and ACV) of PFAA concentrations in clutches were assessed and potential influences of laying order effects on these patterns were examined. Then, we evaluated how egg PFAA concentrations for both species changed along a distance gradient from a known PFAA point source (3M). Lastly, we evaluated both species with respect to their relevance and suitability as biomonitor of PFAAs.

We hypothesize that great tits will show a more diverse PFAA detection profile compared to blue tits as great tits can spend up to 31% of their total foraging time on ground level and blue tits forage almost exclusively arboreal (Cramp and Perrins, 1993; Grzędzicka, 2018). Generally, we predict that great tits may have higher egg PFAA concentrations than blue tits due to their longer lifespan (Cramp and Perrins, 1993) and hence larger bioaccumulation potential. Typically, endogenous resources from the maternal reserves are used for the first eggs and may contain higher PFAA concentrations than the exogenous resources from the diet, due to the longer accumulation time within the mother bird. Consequently, a general decrease of PFAA concentrations throughout the laying order is expected, as the origin of resources used for the production of eggs might differ among eggs. Additionally, blue tits also invest more nutrients in egg production, relative to their body weight, than great tits (Cramp and Perrins, 1993; Van den Steen et al., 2009a). In general, WCV of PFAA concentrations is expected to be larger than ACV in both species, but even more profound in blue tits.

Materials and methods

Study area and data collection

During the autumn of 2015, nest boxes (diameter entrance: 32 mm) originally designed to allow nesting of great tits, were placed at five sites in the vicinity of Antwerp (Belgium), representing a distance gradient (0 – 11 km) starting from the fluorochemical plant 3M (Fig. 1). Besides the fluorochemical plant (28 nest boxes), Vlietbos (24 nest boxes; 1 km SE from the plant), Rot-

Middenvijver (further called 'Rot'; 20 nest boxes, 2.3 km ESE from the plant), Burchtse Weel (21 nest boxes; 3 km SE from the plant) and Fort 4 in Mortsel (58 nest boxes; 11 km SE from the plant) were selected as study areas. The selection of these sites was based on previous monitoring studies throughout the same area (Dauwe et al., 2007; D'Hollander et al., 2014; Lopez-Antia et al., 2017).

During the breeding season of 2016, many nest boxes ($N > 10$) got occupied by blue tits at Fort 4, which enabled a comparative analysis between both species based on whole clutch data. For the other study areas, the number of nest boxes occupied by blue tits was too limited ($N < 4$) to enable a proper species comparison (Table S1). Therefore, in accordance with the egg sampling design for great tits in Groffen et al. (2019a), only the third egg was collected from clutches in these study areas. In this way, we could test in a standardized way how PFAA concentrations changed along the distance gradient between both species.

At the onset of the breeding season, the nest-building phases of each nest were followed up every two to three days. In order to determine the egg laying order, advanced nests were checked daily to determine the egg laying date and to identify individual eggs. Each egg was then numbered with a non-toxic marker according to the laying order. Prior to the start of incubation, third eggs were collected from the nest boxes of all study areas (Table S1). In addition, complete clutches from Fort 4 with a known laying order were collected and eggs were individually stored in 50 mL polypropylene (PP) tubes in a freezer (-20 °C) for further analyses. In total eight clutches of great tit (clutch size: 4-8 eggs ± 1.3 (min - max ± SD); $N = 47$) and blue tit (clutch size: 7-14 eggs ± 1.7, $N = 81$) were collected. In Table S1, a schematic overview is provided of all the clutch sample sizes of blue tits (present study) and great tits (see Groffen et al., 2019a and Lasters et al., 2019) that were used for the analyses in the present study.

Chemical analysis

All used abbreviations of PFAAs are according to Buck et al. (2011). All target analytes and the isotopically mass-labelled internal standards (ISTDs; Wellington Laboratories, Canada) used in the quantification of these analytes are illustrated in Table S2. Samples were analyzed for four target perfluoroalkyl sulfonic acids (PFSAs): perfluorobutane sulfonic acid (PFBS), PFHxS, PFOS and PFDS. Moreover, 11 perfluorocarboxylic acids (PFCAs) were added as target analytes, including pentafluorobenzoic acid (PFBA), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), PFOA, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnDA), perfluorododecanoic acid (PFDoDA), perfluorotridecanoic acid (PFTrDA) and perfluorotetradecanoic acid (PFTeDA). The ISTDs included $^{18}O_2$-PFHxS, [1,2,3,4-$^{13}C_4$]PFOS, $^{13}C_4$-PFBA, [1,2-$^{13}C_2$]PFHxA, [1,2,3,4-$^{13}C_4$]PFOA, [1,2,3,4,5-$^{13}C_5$]PFNA, [1,2-$^{13}C_2$]PFDA, [1,2-$^{13}C_2$]PFUnDA and [1,2-$^{13}C_2$]PFDoDA. The stock ISTD solution was diluted in a mixture of 50:50 (v:v) of HPLC grade acetonitrile (ACN; LiChrosolv. Merck Chemicals, Belgium) and MQ water (18.2 mΩ, TOC: 2.0 ppb, Merck Millipore, Belgium) at a concentration of 125 pg/μL to spike the samples.

Chemical extraction

Whole egg content was transferred into a polypropylene (PP) tube and homogenized by repeatedly sonicating and vortex-mixing. Approximately 0.2 g of homogenized sample was weighed (± 0.01 mg, Mettler Toledo, Zaventem, Belgium) and used for the analysis. The extraction procedure was described and validated by Groffen et al. (2019c). Homogenates were spiked with 80 μL of 125 pg μL^{-1} of each ISTD (in 50:50 (v:v) of ACN:HPLC grade water). After adding 10 mL of ACN, the samples were sonicated three times (with vortex-mixing in between periods) and left overnight on a shaking plate (135 rpm, 20°C, GFL 3020, VWR International, Leuven, Belgium). Afterwards the samples were centrifuged (4°C, 10 min, 2400 rpm at 1037 g, Eppendorf centrifuge 5804R, rotor A-4-44) and the supernatant was stored in a 14 mL PP tube. After conditioning and equilibration of the Chromabond HR-XAW SPE cartridges (Application No 305200, SPE department, Macherey-Nagel, Germany, 2009) with 5 mL of ACN and 5 mL of MQ, respectively, the samples were loaded onto the cartridges. Hereafter, the cartridges were washed with 5 mL of 25 mM ammonium acetate and 2 mL of ACN. The elution was performed using 2 x 1 mL of 2% ammonium hydroxide in ACN and the purified extract was completely dried with an

Eppendorf rotational-vacuum-concentrator (30°C, type 5301, Hamburg, Germany). The dried extract was reconstituted in 200 μL of 2% ammonium hydroxide in ACN and filtered through a 13 mm Acrodisc Ion Chromatography Syringe Filter with 0.2 μm Supor polyethersulfone membrane (VWR International, Leuven, Belgium) into a PP injector vial prior to instrumental analysis.

UPLC-TQD analysis

The target analytes were analyzed using an ACQUITY Ultrahigh Performance Liquid Chromatography (ACQUITY, TQD, Waters, Milford, MA, USA) coupled to a TQD tandem quadrupole mass spectrometer (UPLC-MS/MS) with negative electrospray ionization. To separate the different target analytes, an ACQUITY UPLC BEH C18 VanGuard Pre-column (2.1 x 50 mm; 1.7 μm, Waters, USA) was used. The mobile phase was composed of ACN, HPLC grade water and 0.1% HPLC grade formic acid. The solvent gradient started at 65% to 0% water in 3.4 min and back to 65% water at 4.7 min. The flow rate was set to 450 μL/min and the injection volume was 10 μL. PFAA contamination that might originate from the system was delayed by insertion of an ACQUITY BEH C18 pre-column (2.1 x 30 mm; 1.7 μm, Waters, USA) between the solvent mixer and the injector. Each target analyte was identified and quantified based on multiple reaction monitoring (MRM) of the diagnostic transitions that are displayed in Table S2.

Quality control and assurance

Per batch of ten samples, one procedural blank (10 mL of ACN) was included to detect any contamination. To prevent cross-over contamination between samples during detection in the UPLC-MS/MS, ACN was regularly injected to rinse the columns. Limits of quantification (LOQs) were calculated for each analyte based on instrumental LOQ considering a signal-to-noise ratio of 10 and are displayed in Table 1. Individual PFAAs were quantified using their corresponding ISTD with exception of PFPeA, PFHpA, PFTrDA, PFTeDA, PFBS and PFDS for which no ISTD were present. According to Groffen et al. (2019c), these analytes were all quantified using the ISTD of the compound closest in terms of functional group and size (Table S2). The quantification of the target analytes was based on an internal standard calibration curve, which is detailed in Lasters et al. (2019). For calibration verification, we regularly evaluated a mid-level calibration point which was a 1:1 (125 pg/µL PFAAs:125 pg/µL mPFAAs) spiked non-extracted solution of ACN. For samples in which the peak response area of the target analyte exceeded those of the internal standard with a factor 1000 (= highest calibration point), diluted sample duplicates were performed in order to fit within the linear range and hence to reliably quantify the sample. The recovery ranges (min-max) for each PFAAs were calculated by comparing the peak signal areas of the internal standard for each target compound in the spiked egg samples with those of the procedural blanks (Table S3).

Statistical analyses

The PFAA composition profile in great and blue tit eggs was calculated as the contribution of single compounds to the total ∑PFAA, ∑PFSA and ∑PFCA concentrations in the eggs. Only eggs originating from clutches with ≥ 50% detection frequency for a certain PFAS compound were included for the analyses, while compounds with <50% overall detection frequency were excluded. The boundary of 50% detection frequency was developed in order to prevent that the distribution of the data would be left-skewed due to overleverage of left-censored data (i.e. <LOQ values). For quantifications below LOQ, replacement concentration values were calculated following the maximum likelihood estimation method (de Solla et al., 2012). The iterative Solver function in Excel (Microsoft corp, version 16.0) was used to fit all the data values for each compound along a log-normal probability plot based on the estimated mean and variance of all measurements (Villanueva, 2005). Assumptions of the used statistical models were examined with Shapiro-Wilks test and data were log-transformed when needed to meet normality assumptions. All statistical analyses were done in R (version 3.5.2) and $P < 0.05$ was set as the basic level of significance. Adjusted *P*-values were obtained after Bonferroni correction.

The package lmerTest was used to fit linear mixed-effect models (LMMs) with Gaussian error distribution to test for significant differences in egg PFAA profile and concentrations between blue tits and great tits. The influence of egg laying order was tested by adding this variable as predictor to the

LMM and by standardizing the concentration values of the eggs in function of each clutch mean and standard deviation. In this way, there is no confounding factor of lesser or greater contaminated clutches due to varying clutch sizes that can bias possible laying order effects. Hereby, the nest box identity was included as random intercept to take into account the interdependency of eggs that originate from the same clutch/mother bird. The egg laying order was included as random slope in the model to adjust for the nested structure of the dataset (i.e. eggs nested in their respective clutch cluster). Then, the clutch variation patterns in PFAA concentrations were compared between both species by estimating variance components (ACV and WCV) from an intercept-only LMM, with nest box identity as random intercept. All these analyses were restricted to PFOA and the ∑PFAAs, as only these variables resulted in ≥50% detection of the samples from both species at Fort 4. All the reported estimates of the effect sizes are shown in figures as empirical means ± standard error.

Finally, ANCOVA was used with quadratic terms to model egg PFAA concentrations in function of the two following predictors: species and distance from the PFAA point source (3M). A two-way interaction term was added to test for species-specific differences in possible concentration changes along the distance gradient. For these analyses, data of the third eggs from blue tits and great tits were used due to the limited number of breeding blue tits in nest boxes at study areas other than Fort 4. In this way, a standardized comparison could be made of the PFAA pattern and concentrations blue tits and great tits. This analysis was restricted to PFOA and PFOS as these were the only compounds detected in ≥50% of the samples from both species along the gradient.

Results

Species comparison: PFAA profile and concentrations (whole clutch data)

An overview of the median and mean concentrations, ranges, detection frequencies and relative contribution to the ΣPFAAs for all detected PFAAs in eggs of the blue tits and great tits from clutches at Fort 4 is displayed in Table 1. PFOS was the major contributor to the ΣPFAA concentrations in the eggs of great tits (74%) with concentrations ranging from 6.7 – 55 ng/g wet weight (ww). By contrast, PFBA contributed most to the ΣPFAA concentrations in blue tit eggs (66%) with concentrations ranging from <LOQ to 216 ng/g ww (Table 1). For PFOA, the egg concentrations were significantly higher in blue tits compared to great tits (Fig. 2; P < 0.01, χ^2_6 = 10.5), although this compound was detected in similar frequencies for both species. The ΣPFAA concentrations did not significantly differ between both species (Fig. 2; P > 0.05).

Table 1 provides a comparative overview of the PFAA detection frequencies between both species and shows large differences between them. Strikingly, PFOS was never detected in any blue tit egg while this compound was detected in all of the eggs in great tits (Table 1). Short-chain PFCAs (PFBA and PFHxA) were only detected in blue tit eggs, while various long-chain PFCAs (PFNA, PFDoDA, PFTrDA and PFTeDA) were only found in great tit eggs (Table 1). For blue tits, the highest detection frequency was observed for PFOA (90%), followed by PFBA (63%) (Table 1). In great tits, PFOS and PFOA were both mostly detected and in equal frequencies. With exception of PFOS, none of the target PFSAs (PFHxS, PFBS and PFDS) was detected in both species. In addition, some of the target short-chain PFCAs (PFPeA and PFHpA) were not detected in any of the egg samples as well. Notably, PFHxS and PFHxA were detected in 35% and 5% of the blue tit clutches at the fluorochemical plant site, respectively, while these compounds were never detected in any of the great tit eggs.

Clutch variation patterns in PFAA concentrations (whole clutch data)

For both tit species, a large variation in PFOA egg concentrations was observed throughout the laying order (Fig. 3). The within-clutch variation (WCV) explained most of the total variation in PFAA concentrations and was much larger than the among-clutch variation (ACV) (Fig. 4). The WCV contributed for 71% and 97% to the total variation in ΣPFAA concentrations of great tit and blue tit clutches, respectively. This pattern was reflected in an overall significant decline of PFOA concentrations in blue tit clutches (-0.017 ± 0.012; χ^2_6 = 0.86, P < 0.05), while these concentrations followed no pattern in great tit clutches (Fig. 3; P > 0.05).

PFOS and PFOA concentrations along the distance gradient (using 3^rd^ egg data)

The change of egg PFAA concentrations along the distance gradient is depicted in Fig. 5. The egg PFOS concentrations decreased significantly in both species from the fluorochemical plant onwards (Fig. 5; $F_{1,125} = 63$, $P < 0.001$), with a more steep decline in great tits compared to blue tits (significant interaction term: $F_{1,125} = 5.9$, $P < 0.05$). The linear model showed that distance from the fluorochemical plant explained 66% and 32% of the total variation in egg PFOS concentrations of the great tit and blue tit, respectively (Fig. 5; R^2 great tit = 0.66 and R^2 blue tit = 0.32). Egg PFOS concentrations were significantly higher in great tits than blue tits along the whole gradient (Fig. 5; $F_{1,125} = 25$, $P < 0.001$) with concentrations ranging from <LOQ to 187000 ng/g ww and from <LOQ to 6743 ng/g ww, respectively.

Similarly, PFOA concentrations in eggs of blue tits and great tits declined with distance from the fluorochemical plant (Fig. 5; $F_{1,125} = 23$, $P < 0.001$). However, PFOA concentrations decreased in the same way along the distance gradient as the interaction term between species and distance from the fluorochemical plant was not significant ($P > 0.05$). The linear model estimated that 60% and 15% of the total variation in PFOA concentrations could be explained by the distance from the fluorochemical plant (Fig. 5; R^2 great tit = 0.60 and R^2 blue tit = 0.15). The egg PFOA concentrations in blue tits were significantly higher compared to great tits (Fig. 5; $F_{1,125} = 12$, $P < 0.01$) and concentrations ranged from <LOQ to 359 ng/g ww and from <LOQ to 34 ng/g ww along the gradient.

Discussion

Species comparison: PFAA profile and concentrations (whole clutch data)

Compared to previous biomonitoring studies on bird eggs near Antwerp, the PFAA concentrations in whole clutches of both tit species were relatively low (Groffen et al., 2017, 2019a; Lopez-Antia et al., 2017, 2019). The study area in which whole clutches were collected for the species comparison, i.e. Fort 4, is located around 11 km from the nearest known PFAA point source (3M). Earlier monitoring studies in birds showed that egg PFAA concentrations decreased rapidly from the point source onwards (Groffen et al., 2017, 2019a), which was also confirmed in the present study for PFOS and

PFOA (Fig. 5). This finding is also in agreement with monitoring studies in birds near other fluorochemical hotspots (Custer et al. 2012, Russell et al., 2019).

The hypothesis that great tits would show a more diverse detection profile compared to blue tits was confirmed (Table 1). The profile of both species was unexpectedly divergent from each other: the PFAA profile of great tits was characterized by domination of PFOS along with frequent detections of various long-chain PFCAs, including the ubiquitous compound PFOA. In blue tits, on the other hand, PFOA was most frequently observed and target short-chain PFCAs (PFBA and PFHxA) were only detected in this species. Importantly, PFOS was not detected in any of the blue tit clutches at Fort 4 and only a few long-chain PFCAs (PFDA and PFUnDA) were found compared to great tits. The dominant exposure source of PFAAs in terrestrial animals is considered to be the diet (D'Hollander et al., 2015; Gebbink et al., 2015). Therefore, it is likely that these contrasting results reflect differences in diet and foraging habits between both species.

Both species are income breeders that mainly use recently incorporated exogenous resources for the formation of their eggs (Ward and Bryant, 2006; Van den Steen et al., 2009a) and they are primarily insectivorous birds that preferentially feed on caterpillars throughout the year (Pollock et al., 2017; Grzędzicka, 2018). However, great tits are more ground-feeding birds compared to blue tits, who feed almost exclusively arboreal in the canopy during the breeding season (Krebs, 1971; Cramp and Perrins, 1993). Consequently, blue tits feed almost solely on herbivorous prey, such as aphids and caterpillars (Cowie and Hinsley, 1988), which are expected to mostly accumulate short-chain PFAAs. These short-chain compounds are highly water soluble and are known to be dominantly present in contaminated plant tissues, especially the leaf parts (Blaine et al., 2013, Brendel et al., 2018). In this way, short-chain PFAAs can be transferred through this particular food chain to blue tits and may ultimately be deposited in the eggs. On the other hand, great tits may be exposed to more different PFAA compounds through the intake of additional food sources when foraging on the ground, such as spiders and beetles (Cramp and Perrins, 1993; Van den Steen et al., 2010). Several studies suggest that long-chain PFAAs (e.g. PFOS and C_{10}-C_{14} PFCA analogues) can accumulate in potential food sources of the great tit (D'Hollander et al., 2014; Groffen et al., 2019b).

Spatial or temporal variation in egg sampling of both species could also affect the PFAA profiles between great tits and blue tits. However, the whole clutch data were collected in the same area (Fort 4) and time period (i.e. March-April 2016) and this was further evidenced by the fact that the laying date of the 1st egg did not significantly differ between the species (Table S4). Consequently, these diverging results are not likely a result of differences in spatial or temporal variation.

Importantly, it cannot be ruled out that the diverging detection and accumulation profiles between both species are also caused by differences in egg nutrient composition. From all nutrient classes, proteins are considered the most important carriers transferring PFAAs from one biological matrix (e.g. liver mother bird) to the other (e.g. eggs) (Jones et al, 2003; Lau et al., 2007; Wang et al., 2019). Although very little is known about egg nutrient allocation in tit species, recent proteome analysis of blue tits demonstrated that concentrations of abundant egg proteins can differ to great extent with those in great tits (Valcu et al., 2019). For instance, ovotransferrin, an abundant egg protein, can be present in more than 10 times higher concentrations in yolk of blue tits compared to great tit yolk (Valcu et al., 2019). Furthermore, egg nutrient deposition may also vary among individuals due to differences in age or clutch size (Bourgault et al., 2007; Valcu et al., 2019). Unfortunately, no data of the mother bird, such as age or reproductive status, could be obtained.

Higher protein concentrations in blue tit eggs may have resulted in increased matrix effects during the extraction process. This is supported by the fact that LOQ values for some of the detected PFAAs were often much larger in blue tits compared to great tits, especially for PFOS and some long-chain PFCAs that were absent in the detection profile of blue tits (Table 1). Moreover, extraction recoveries of some compounds (e.g. PFOS) were considerably lower for blue tit eggs compared to those of great tits (Table S3). This indicates that larger matrix effects, due to presumably higher protein residue concentrations in the blue tit eggs, may have caused larger suppression of the ion signal leading to lower peak signal resolutions. Therefore, it is plausible that the absence of particular PFAAs in blue tit eggs is also a consequence of analytical difficulties as a result of the varying egg protein composition between both species.

Clutch variation patterns in PFAA concentrations (whole clutch data)

The WCV for all PFAS was higher than the ACV in both species, which is supported by other studies on PFAA clutch variation (Custer et al., 2012; Vicente et al., 2015; Lasters et al., 2019). As was described previously, the production of eggs is energetically costly and tits use current rather than stored nutrients for this process (Williams, 2005; Ward and Bryant, 2006; Van den Steen et al., 2009a). The WCV pattern was even larger in blue tit clutches than in those of great tits (Fig. 4), which can best be explained by the larger clutch sizes of blue tits (Table S4) between both species. Blue tits can have clutches up to 16 eggs, having among the largest clutch sizes ever reported in songbirds (Cramp and Perrins, 1993). Furthermore, blue tits invest a larger amount of resources into their eggs than great tits, relative to their body weight (Van den Steen et al., 2009a). Finally, variation in food preferences of the mother bird or shifting availability of prey types throughout the breeding season can also be contributing mechanisms to increase WCV in passerines (Longcore et al., 2007; Custer et al., 2010; Valcu et al., 2019).

The PFOA concentrations decreased significantly throughout the laying order in blue tits which may be caused by physiological exhaustion of the mother bird during the egg laying period. The formation of eggs is a very energy-demanding process, during which females experience constraints in nutrient mobilization from the liver to the eggs as the egg laying period proceeds (Bourgault et al., 2007; Valcu et al., 2019). In addition, females rely on daily replenishment of their endogenous maternal resources with dietary resources during the laying period (Ward and Bryant, 2006; Bourgault et al., 2007; Van den Steen et al., 2009b). Nutrients stored in maternal tissue usually hold higher concentrations of bioaccumulative pollutants than those in dietary resources (Braune and Norstrom, 1989; Van den Steen et al., 2009b). For the production of the first eggs, the females will mainly use maternal resources, whereas dietary resources, which might contain lower PFOA concentrations, are used for the later eggs (Van den Steen et al., 2009a).

This finding is in agreement with those reported for other organic pollutants (Van den Steen et al., 2006, 2009a, 2009b), PFOS in gulls (Vicente et al., 2015) and for most other PFAAs in great tits, but not PFOA (see Fig. S1; Lasters et al., 2019). Although speculative, it is plausible that metabolic differences between both species in assimilation efficiency of specific nutrients to which PFOA binds, might underpin this result. Lastly, it should be noticed that the declining pattern of PFAA concentrations in blue tits is not very evident, whereas more declining trends could be observed for various PFAA compounds in great tits. Although speculative, It may be that a large part of the blue tit mother birds overwintered in a relatively low PFAA exposure region prior to the breeding season and consequently built up relatively low concentrations of PFAAs in their maternal reserves prior to the breeding season. If they then disperse for breeding purposes to Fort 4, which is also a relatively low PFAA exposure region, the difference between the concentrations in the maternal reserves and those in the diet may not be too large. This may result in a more homogeneous distribution of PFAA concentrations in the eggs and hence absence of clear laying order effects. Furthermore, the sample size is relatively small for our clutch dataset and, to the best of our knowledge, no other studies exist that examined laying order effects of PFAAs in passerines, which also makes it difficult to compare our study results properly and to disentangle possible explanations from each other.

PFOS and PFOA concentrations along the distance gradient (3rd egg data)

Using the sub-dataset of the third eggs, PFOS concentrations in great tits were significantly higher than in eggs of blue tits along the whole distance gradient (Fig. 5). On average, great tits have a longer lifespan and larger body size (± 20%) than blue tits (Cramp and Perrins, 1993). In theory, this should result in a larger bioaccumulation and biomagnification potential of PFOS (Conder et al., 2008, Yoo et al., 2008) in great tits compared to blue tits. However, the reverse pattern was true for PFOA and also the general PFAA detection profile vastly differed between both tit species, as mentioned in the earlier species comparison based on whole clutch data. This suggests that both species experience different PFAA exposure sources via food, have different foraging habits or different egg protein composition, as discussed earlier. Unfortunately, no data of the diet or age could be obtained to validate these hypotheses.

The distance to the fluorochemical plant site in the linear model explained less of the total variation in PFOA and PFOS concentrations in eggs of blue tits, compared to great tits (Fig. 5). Furthermore, PFOS concentrations significantly decreased more rapidly in great tits eggs and the contribution of PFOA along the gradient remained constantly low (<3%), while this was more variable in blue tits. Together, these findings indicate that exposure sources other than the fluorochemical plant site and biological differences play an important role in the contribution to the total PFAA burden of blue tits. Females of the blue tits disperse more frequently than those of the great tit, over distances up to several kilometers, which results in less recruitment of local offspring compared to great tits (Greenwood et al., 1979; Cramp and Perrins, 1993; Van den Steen et al., 2010). Therefore, it could be that some of the breeding blue tit females at the considered study areas along the pollution gradient are immigrants that originate from sites with another PFAA exposure background. Hence, the egg PFAA concentrations in blue tits show a more variable pattern along the pollution gradient than in great tits.

Based on the present whole clutch data of the blue tit and great tit, which are both important ecotoxicological model species, large WCV of PFAA concentrations was found as well as clear differences in terms of PFAA profile and concentrations between both species. Therefore, our results strongly suggest that even co-occurring bird species may have different exposure pathways to PFAAs and/or different egg nutrient composition. This implies that biomonitoring results cannot be generalized across species, but rather, species have their own specific relevance with respect to biomonitoring of PFAAs and provide complementary information. In this regard, the great tit may foresee rather qualitative information about the various types of PFAAs present in a given environment compared to the blue tit. On the other hand, the blue tit may provide more quantitative information than the great tit on the extent of contamination for some dominant PFAAs, for instance PFOA.

Conclusion

To the best of our knowledge, we have presented the first comparative study in PFAA profile and concentrations between two passerines from egg data of the same study area and same breeding season, which provided novel insights in the ecotoxicology of PFAAs. Distinct differences were found between two co-occurring passerine species, the great tit and blue tit, in terms of PFAA profile and laying order effects. Great tits showed a much more diverse egg PFAA detection profile than blue tits and egg concentrations of individual compounds were divergent, suggesting differences in diet and foraging habits. Moreover, egg protein composition differences between both species may also explain some of the observed differences between the species, as analytical difficulties were experienced with the blue tit egg extraction, presumably due to larger matrix effects in this species. For both species, variation of PFAA concentrations within clutches was much larger than among clutches. On the other hand, patterns of laying order effects on PFAA concentrations were different between great tits and blue tits. Although egg PFOA concentrations of both species similarly decreased from the fluorochemical point source onwards, much more variation in egg PFOA concentrations could be explained by distance from the fluorochemical plant in great tits than in blue tits. Based on our results, biomonitoring results of PFAAs in eggs are most likely not generalizable across bird species.

Acknowledgements

The authors are very thankful to the Fund for Scientific Research Flanders (FWO-Flanders) and the University of Antwerp for funding this research (FWO nr: G038615N). In addition, we would like to express our sincere thanks to 3M for the opportunity to conduct this study at their site. Furthermore, we would like to acknowledge Ana Lopez-Antia, Peter Scheys and Geert Eens for their help during the fieldwork and Tim Willems for performing the UPLC analysis. We are extremely thankful to Wouter Melens, Koen Maes and the Agency for Nature and Forest (ANB) for providing us access to the different sampling sites and for the possibility to store field materials in these areas. Finally, we would like to thank E. Matthysen, V. Jaspers, P. de Voogt and R. Scheifler for proofreading the manuscript and providing helpful comments and suggestions that improved the quality of this study.

References

Beach, S. A., Beach, J. L., Newsted, K., Coady, K. and Giesy, J. P. (2006). Ecotoxicological evaluation of perfluorooctanesulfonate (PFOS). Reviews of Environmental Contamination and Toxicology 186: 133 – 174.

Blaine, A. C., Rich, C. D., Hundal, L. S., Lau, C., Mills, M. A., Harris, K. M. and Higgins, C. P. (2013). Uptake of perfluoroalkyl acids into edible crops via land applied biosolids: field and greenhouse studies. Environmental Science and Technology. 47: 14062 – 14069.

Bourgault, P., Thomas, D. W., Blondel, J., Perret, P. and Lambrechts, M. M. (2007). Between-population differences in egg composition in blue tits (*Cyanistes caeruleus*). Canadian Journal of Zoology. 85: 71 – 80.

Braune, B. M. and Norstrom, R. J. (1989). Dynamics of organochlorine compounds in herring gulls: III. Tissue distribution and bioaccumulation in Lake Ontario gulls. Environmental Toxicology and Chemistry. 8: 957 – 968.

Brendel, S., Fetter, E., Staude, C., Vierke, L. and Annegret, B. (2018). Short-chain perfluoroalkyl acids: environmental concerns and a regulatory strategy under REACH. Environmental Sciences Europe 30: 9 – 20.

Buck, R. C., Franklin, J., Berger, U., Conder, J. M., Cousins, I. T., De Voogt, P., Jensen, A. A., Kannan, K., Mabury, S. A. and Van Leeuwen, S. P. J. (2011). Perfluoroalkyl and polyfluoroalkyl substances in the environment: terminology, classification, and origins. Integrated Environmental Assessment and Management 7: 513 – 541.

Butt, C. M., Berger, U., Bossi, R., and Tomy, G. T. (2010). Levels and trends of poly- and perfluorinated compounds in the arctic environment. Science of the Total Environment 408: 2936 – 2965.

Conder, J. M., Hoke, R. A., De Wolf, W., Russell, M. H. and Buck, R. C. (2008). Are PFCAs bioaccumulative? A critical review and comparison with regulatory criteria and persistent lipophilic compounds. Environmental Science and Technology 42: 995 – 1003.

Cowie, R. J. and Hinsley, S. A. (1988). Feeding ecology of great tits (*Parus major*) and blue tits (*Parus caeruleus*), breeding in suburban gardens. Journal of Animal Ecology 57: 611 – 626.

Cramp, S. and Perrins, C. M. (1993). Handbook of the Birds of Europe the Middle East and North Africa. The Birds of the Western Palearctic. Volume VII – Flycatchers to Shrikes. Chapter Paridae: pp. 145 – 281. Oxford University Press, Oxford: New York.

Custer, C. M., Gray, B. R. and Custer, T. W. (2010). Effects of egg order on organic and inorganic element concentrations and egg characteristics in tree swallows, *Tachycineta bicolor*. Environmental Toxicology and Chemistry 29: 909 – 921.

Custer, C. M., Custer, T. W., Schoenfuss, H. L., Poganski, B. H. and Solem, L. (2012). Exposure and effects of perfluoroalkyl compounds on tree swallows nesting at Lake Johanna in east central Minnesota, USA. Reproductive Toxicology 33: 556 – 562.

Custer, C. M., Custer, T. W., Dummer, P. M., Etterson, M. A., Thogmartin, W. E., Wu, Q., Kannan, K., Trowbridge, A. and McKann, P. C. (2014). Exposure and effects of perfluoroalkyl substances in tree swallows nesting in Minnesota and Wisconsin, USA. Archives of Environmental Contamination and Toxicology 66: 120 – 138.

Dauwe, T., Bervoets, L., Janssens, E., Pinxten, R., Blust, R. and Eens, M. (2002). Great and blue tit feathers as biomonitors for heavy metal pollution. Ecological Indicators 1: 227 – 234.

Dauwe, T., Van de Vijver, K., de Coen, W. and Eens, M. (2007). PFOS levels in the blood and liver of a small insectivorous songbird near a fluorochemical plant. Environment International 33: 357 – 361.

De Solla, S. R., de Silva, A. O. and Letcher, R. J. (2012). Highly elevated levels of perfluorooctane sulfonate and other perfluorinated acids found in biota and surface water downstream of and international airport, Hamilton, Ontario, Canada. Environment International. 39: 19 – 26.

D'Hollander, W., De Bruyn, L., Hagenaars, A., de Voogt, P. and Bervoets, L. (2014). Characterisation of perfluorooctane sulfonate (PFOS) in a terrestrial ecosystem near a fluorochemical plant in Flanders, Belgium. Environmental Science and Pollution Research 21: 11856 – 11866.

D'Hollander, W., Herzke, D., Huber, S., Hajslova, J., Pulkrabova, J., Brambilla, G., De Filippis, S. P., Bervoets, L. and de Voogt, P. (2015). Occurrence of perfluorinated alkylated substances in cereals, salt, sweets, and fruit items collected in four European countries. Chemosphere 129: 179 – 185.

Fang, S., Chen, X., Zhao, S., Zhang, Y., Jiang, W., Yang, L. and Zhu, L. (2014). Trophic magnification and isomer fractionation of perfluoroalkyl substances in the food web of taihu lake, China. Environmental Science and Technology 48: 2173 – 2182.

Fedorenko, M., Alesio, J., Fedorenko, A., Slitt, A. and Bothun, G. D. (2021) Dominant entropic binding of perfluoroalkyl substances (PFASs) to albumin protein revealed by ^{19}F NMR. Chemosphere 263: 1 – 9.

Gebbink, W. A. and Letcher, R. J. (2012). Comparative tissue and body compartment accumulation and maternal transfer to eggs of perfluoroalkyl sulfonates and carboxylates in Great Lakes herring gulls. Environmental Pollution 162: 40 – 47.

Gebbink, W. A., Berger, U. and Cousins, I. T. (2015). Estimating human exposure to PFOS isomers and PFCA homologues: the relative importance of direct and indirect (precursor) exposure. Environment International 74: 160 – 169.

Giesy, J. P. and Kannan, K. (2001). Global distribution of perfluorooctane sulfonate in wildlife. Environmental Science and Technology 35: 1339 – 1342.

Giesy, J. P. and Kannan, K. (2002). Perfluorochemical surfactants in the environment: These bioaccumulative compounds occur globally, warranting further study. Environmental Science and Technology 36: 146A – 152A.

Greenwood, P. J., Harvey, P. H. and Perrins, C. M. (1979). The Role of Dispersal in the Great Tit (*Parus major*): The Causes, Consequences and Heritability of Natal Dispersal. Journal of Animal Ecology. 48: 123 – 142.

Groffen, T., Lopez-Antia, A., D'Hollander, W., Prinsen, E., Eens, M. and Bervoets, L. (2017). Perfluoroalkylated acids in the eggs of great tits (*Parus major*) near a fluorochemical plant in Flanders, Belgium. Environmental Pollution 228: 140 – 148.

Groffen, T., Lasters, R., Lopez-Antia, A., Prinsen, E., Bervoets, L. and Eens, M. (2019a). Limited reproductive impairment in a passerine bird species exposed along a perfluoroalkyl acid (PFAA) pollution gradient. Science of the Total Environment 652: 718 – 728.

Groffen, T., Eens, M. and Bervoets, L. (2019b). Do concentrations of perfluoroalkylated acids (PFAAs) in isopods reflect concentrations in soil and songbirds? A study using a distance gradient from a fluorochemical plant. Science of the Total Environment 657: 111 – 123.

Groffen, T., Lasters, R., Lemière, F., Willems, T., Eens, M., Bervoets, L. and Prinsen, E. (2019c). Development and validation of an extraction method for the analysis of perfluoroalkyl substances (PFASs) in environmental and biotic matrices. Journal of Chromatography B 1116: 30 – 37.

Grzędzicka, E. (2018). Habitat and diet variability of two coexisting tit species in central European forests. Bird Study 65: 52 – 61.

Hoff, P. T., Van de Vijver, K., Dauwe, T., Covaci, A., Marevoet, J., Eens, M., Blust, R. and De Coen, W. (2005). Evaluation of biochemical effects related to perfluorooctane sulfonic acid exposure in organohalogen-contaminated great tit (*Parus major*) and blue tit (*Parus caeruleus*) nestlings. Chemosphere 61: 1558 – 1569.

Jones, P. D., Hu, W., De Coen, W., Newsted, J. L. and Giesy, J. P. (2003). Binding of perfluorinated fatty acids to serum proteins. Environmental Toxicology and Chemistry 22: 2639 – 2649.

Krebs, J. R. (1971). Territory and breeding density in the great tit, *Parus major* L. Ecology 52: 2 – 22.

Lasters, R., Groffen, T., Lopez-Antia, A., Bervoets, L. and Eens, M. (2019). Variation in PFAA concentrations and egg parameters throughout the egg-laying sequence in a free-living songbird (the great tit, *Parus major*): implications for biomonitoring studies. Environmental Pollution 246: 237 – 248.

Lau, C., Anitole, K., Hodes, C., Lai, D., Pfahles-Hutchens, A. and Seed, J. (2007). Perfluoroalkyl acids: a review of monitoring and toxicological findings. Toxicological Sciences 99: 366 – 394.

Longcore, J. R., Haines, T. A. and Halteman, W. A. (2007). Mercury in tree swallow food, eggs, bodies, and feathers at Acadia National Park, Maine, and an EPA Superfund site, Ayer, Massachusetts. Environmental Monitoring and Assessment 126: 129 – 143.

Lopez-Antia, A., Dauwe, T., Meyer, J., Maes, K., Bervoets, L. and Eens, M. (2017). High levels of PFOS in eggs of three bird species in the neighbourhood of a fluoro-chemical plant. Ecotoxicology and Environmental Safety 139: 165 – 171.

Lopez-Antia, A., Groffen, T., Lasters, R., AbdElgawad, H., Sun, J., Asard, H., Bervoets, L. and Eens, M. (2019). Perfluoroalkyl acids (PFAAs) concentrations and oxidative status in two generation of great tits inhabiting a contamination hotspot. Environmental Science and Technology 53: 1617 – 1626.

Pollock, C. J., Capilla-Lasheras, P., McGill, R. A. R., Helm, B. and Dominoni, D. M. (2017). Integrated behavioural and stable isotope data reveal altered diet linked to low breeding success in urban-dwelling blue tits (*Cyanistes caeruleus*). Scientific Reports 7: 5014.

Rodriguez-Jorquere, I. A., Silva-Sanchez, C., Strynar, M., Denslow, N. D. and Toor, G. S. (2016). Footprints of urban micro-pollution in protected areas: investigating the longitudinal distribution of perfluoroalkyl acids in wildlife preservers. PLoS One 11: 1 – 18.

Russell, M. C., Newton, S. R., McClure, K. M., Levine, R. S., Phelps, L. P., Lindstrom, A. B. and Strynar, M. J. (2019). Per- and polyfluoroalkyl substances in two different populations of northern cardinals. Chemosphere 222: 295 – 304.

Su, G., Letcher, R. J., Moore, J. N., Williams, L. L. and Grasman, K. A. (2017). Contaminants of emerging concern in Caspian tern compared to herring gull eggs from Michigan colonies in the Great Lakes of North America. Environmental Pollution 222: 154 – 164.

Surma, M., Piskula, M., Wiczkowski, W. and Zielinski, H. (2017). The perfluoroalkyl carboxylic acids (PFCAs) and perfluoroalkane sulfonates (PFSAs) contamination level in spices. European Food Research and Technology 243: 297 – 307.

Valcu, C-M., Scheltema R. A., Schweiggert, R. M., Valcu, M., Teltscher, K., Walther, D. M., Carle, R. and Kempenaers, B. (2019). Life history shapes variation in egg composition in the blue tit *Cyanistes caeruleus*. Communications Biology 2: 1 – 14.

Van den Steen, E., Dauwe, T., Covaci, A., Jaspers, V. L. B., Pinxten, R. and Eens, M. (2006). Within- and among-clutch variation of organohalogenated contaminants in eggs of great tits (*Parus major*). Environmental Pollution 144: 355 – 359.

Van den Steen, E., Eens, M., Jaspers, V. L. B., Covaci, A. and Pinxten, A. (2009a). Effects of laying order and experimentally increased egg production on organic pollutants in eggs of a terrestrial songbird species, the great tit (*Parus major*). Science of the Total Environment 407: 4764 – 4770.

Van den Steen, E., Jaspers, V. L. B., Covaci, A., Neels, H., Eens, M. and Pinxten, R. (2009b). Maternal transfer of organochlorines and brominated flame retardants in blue tits (*Cyanistes caeruleus*). Environment International 35: 69 – 75.

Van den Steen, E., Pinxten, R., Covaci, A., Carere, C., Eeva, T., Heeb, P., Kempenaers, B., Lifjeld, J. T., Massa, B., Norte, A. C., Orell, M., Sanz, J. J., Senar, J. C., Sorace, A. and Eens, M. (2010). The use of blue tit eggs as a biomonitoring tool for organohalogenated pollutants in the European environment. Science of the Total Environment 408: 1451 – 1457.

Vicente, J., Sanpera, C., García-Tarrasón, M., Pérez, A. and Lacorte, S. (2015). Perfluoroalkyl and polyfluoroalkyl substances in entire clutches of Audouin's gulls from the Ebro delta. Chemosphere 119: 62 – 69.

Villanueva, P. (2005). MLE-based procedure for left-censored data excel spreadsheet. Office of Pesticide Programs. U.S. Environmental Protection Agency, Washington, DC.

Wang, F., Zhao, C., Gao, Y., Fu, J., Gao, K., Lv, K., Wang, K., Yue, H., Lan, X., Liang, Y. et al. (2019). Protein-specific distribution patterns of perfluoroalkyl acids in egg yolk and albumen samples around a fluorochemical facility. Science of the Total Environment 650: 2697 – 2704.

Ward, S. and Bryant, D. M. (2006). Barn swallows *Hirundo rustica* form eggs mainly from current food intake. Journal of Avian Biology 37: 179 – 189.

Williams, T. D. (2005). Mechanisms underlying the costs of egg production. Bioscience 55: 39 – 48.

Yoo, H., Kannan, K., Kim, K. S., Lee, K. T., Newsted, J. L. and Giesy, J. P. (2008). Perfluoroalkyl acids in the egg yolk of birds from Lake Shihwa, Korea. Environmental Science and Technology 42: 5821 – 5827.

Tables and figures

Tables

Table 1: Limits of quantification (LOQs; ng/g ww, determined as 10x the S/N ratio), median and mean concentrations (ng/g ww), min-max ranges (ng/g ww), detection frequencies (Freq. (%)) and relative contribution (Contr. (%) to the ∑PFAAs) of the target PFAA analytes in pooled blue tit eggs and pooled great tit eggs from whole clutches of Fort 4 near Antwerp (Belgium) in 2016. [1] Great tit data were adopted from Lasters et al. (2019). <LOQ = below the limit of quantification.

SPECIES	**PFCAs**									**PFSAs**	**∑PFAAs**
Blue tit (*N* = 81)	PFBA	PFHxA	PFOA	PFNA	PFDA	PFUnDA	PFDoDA	PFTrDA	PFTeDA	PFOS	/
LOQ	0.12	0.30	0.04	3.0	0.78	0.78	2.6	0.28	1.3	11	/
Median	0.29	<LOQ	4.30	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	20
Mean	13	<LOQ	5.7	<LOQ	<LOQ	0.87	<LOQ	<LOQ	<LOQ	<LOQ	24
Range	<LOQ – 85	<LOQ – 1.2	<LOQ – 58	<LOQ	<LOQ – 7.2	<LOQ – 11	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ-59
Freq.	63	5	90	0	6	26	0	0	0	0	100%
Contr.	66	0.18	30	0	0.90	2.9	0	0	0	0	/
Great tit [1] (*N* = 47)											
LOQ	0.26	0.30	0.05	0.59	0.43	0.78	0.44	0.26	0.36	2.6	/
Median	<LOQ	<LOQ	1.4	1.0	1.4	<LOQ	1.8	1.1	<LOQ	24	25
Mean	<LOQ	<LOQ	2.0	1.0	1.5	<LOQ	2.1	1.0	0.43	26	22
Range	<LOQ	<LOQ	0.72 – 3.7	<LOQ – 2.4	<LOQ – 3.5	<LOQ	0.90 – 4.8	<LOQ – 5.7	<LOQ – 2.0	6.7 – 55	8.4-32
Freq.	<LOQ	<LOQ	100	81	96	<LOQ	95	94	40	100	100%
Contr.	0	0	7	3.1	4.6	0	7	3.7	0.95	74	/

Figures

Fig. 1: Overview of the study area in Antwerp, Belgium. Sampling locations are indicated as letters: A. Fluorochemical plant 3M, B. Vlietbos, C. Middenvijver-Rot, D. Burchtse Weel, E. Fort 4. Figure was adopted from Groffen et al. (2019a).

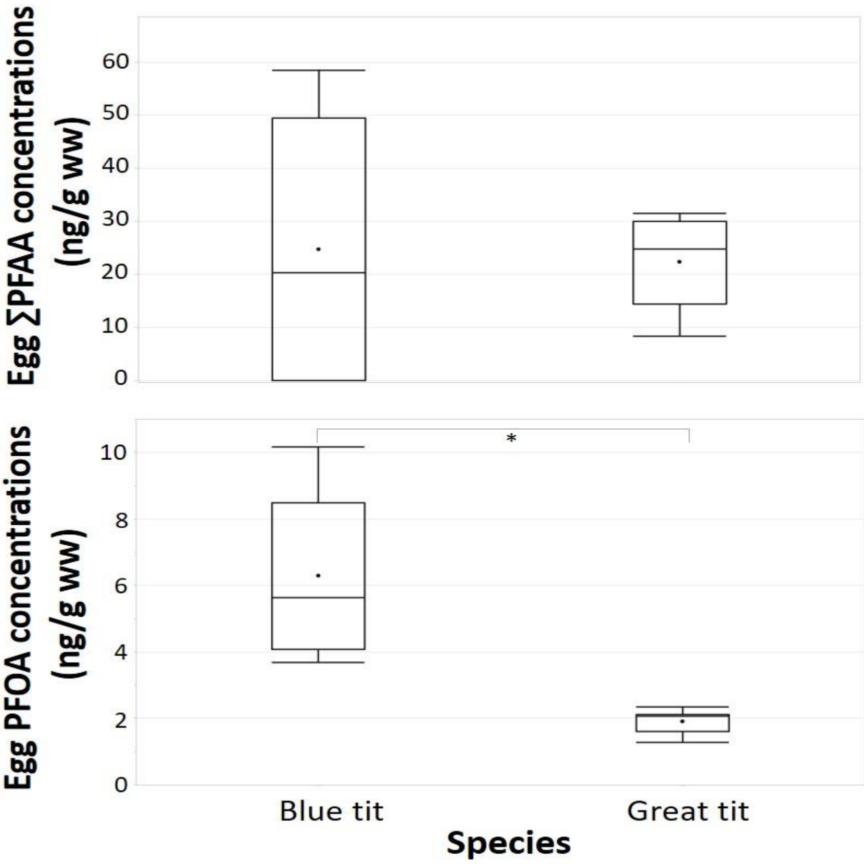

Fig. 2: Comparison of egg PFAA concentrations, expressed in ng/g wet weight (ww), between blue tit and great tit eggs from clutches of Fort 4 (Antwerp) in 2016. The filled circle within each boxplot represents the empirical mean. Top graph: no significant differences in egg ∑PFAA concentrations between both species; lower graph: significantly higher egg PFOA concentrations in blue tits compared to great tits ($P < 0.01$). Great tit data were adopted from Lasters et al. (2019).

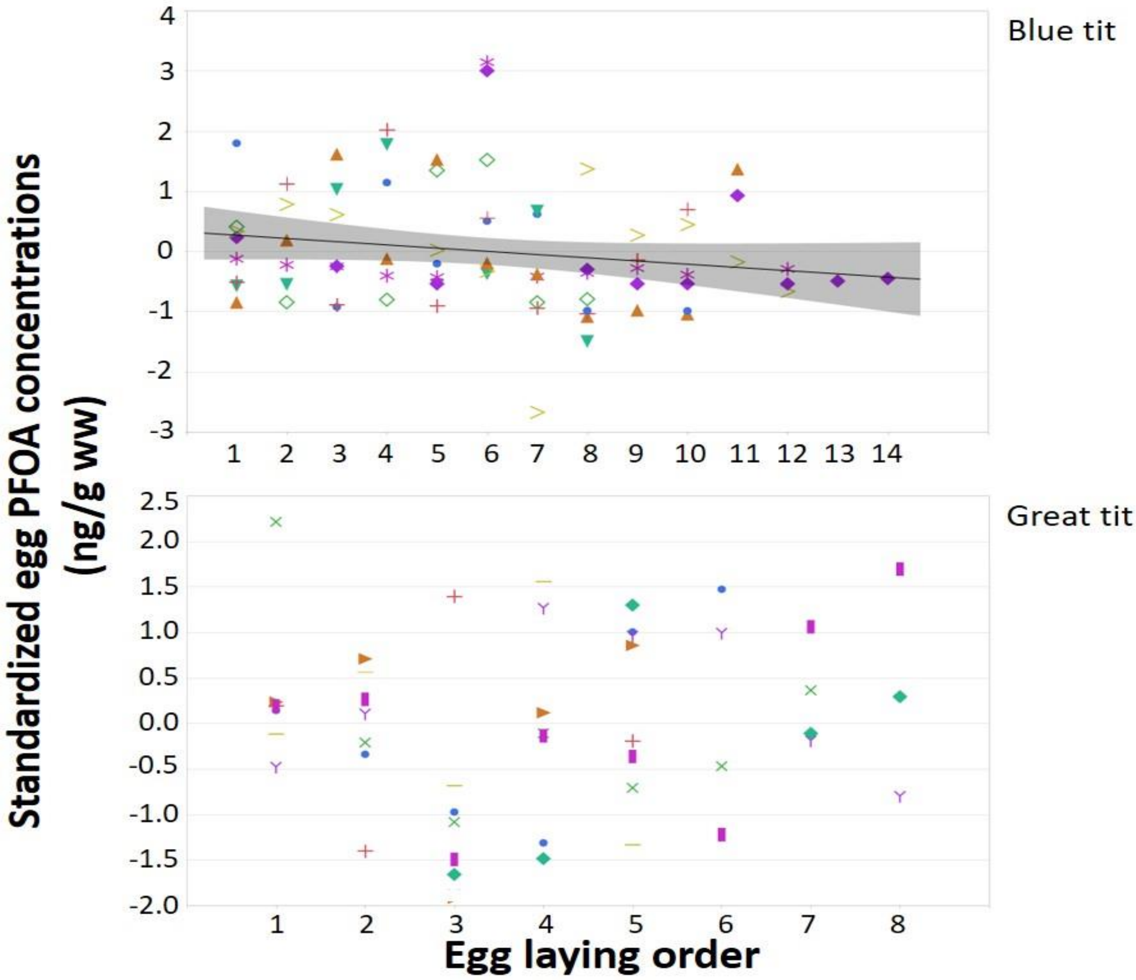

Fig. 3: Comparison of egg laying order associations with log egg PFOA concentrations (ng/g wet weight) between blue tit (top graph) and great tit (lower graph) clutches from Fort 4 (Antwerp) in 2016. Standardized egg PFOA concentrations for blue tits significantly declined ($P < 0.05$) along the laying order while no significant association was found for the great tit. The gray band denotes the 95% confidence interval for the regression estimates of the slope. Each symbol represents an individual clutch. Great tit data were adopted from Lasters et al. (2019).

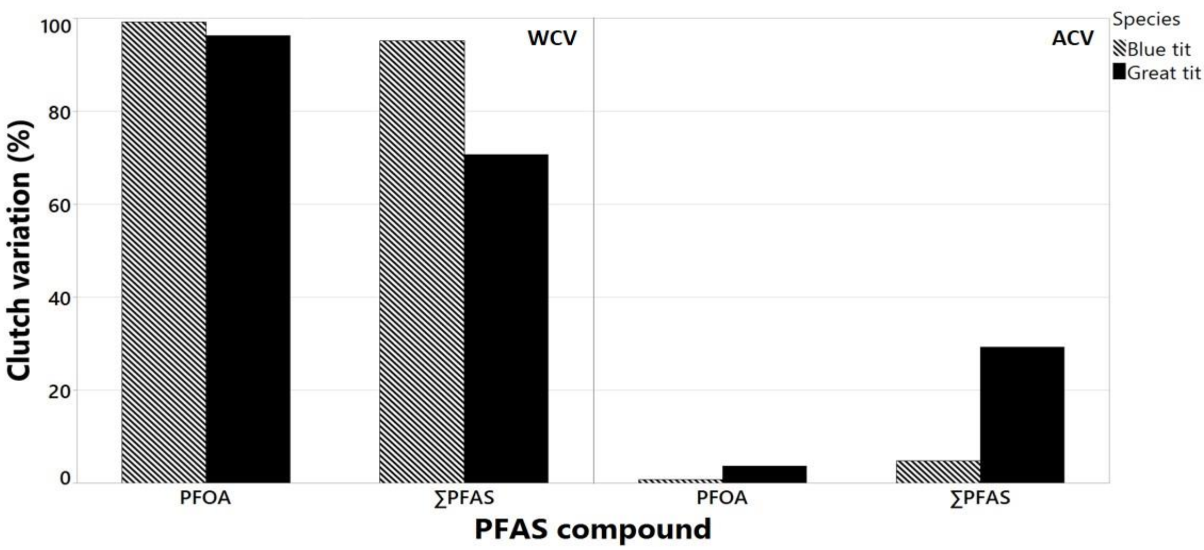

Fig. 4: Comparison of variation within clutches (WCV; left figure) and variation among clutches (ACV; right figure) of PFOA and ∑PFAA concentrations between blue tit (shaded bar) and great tit (black bar) eggs from Fort 4 (Antwerp) in 2016. Great tit data were adopted from Lasters et al (2019).

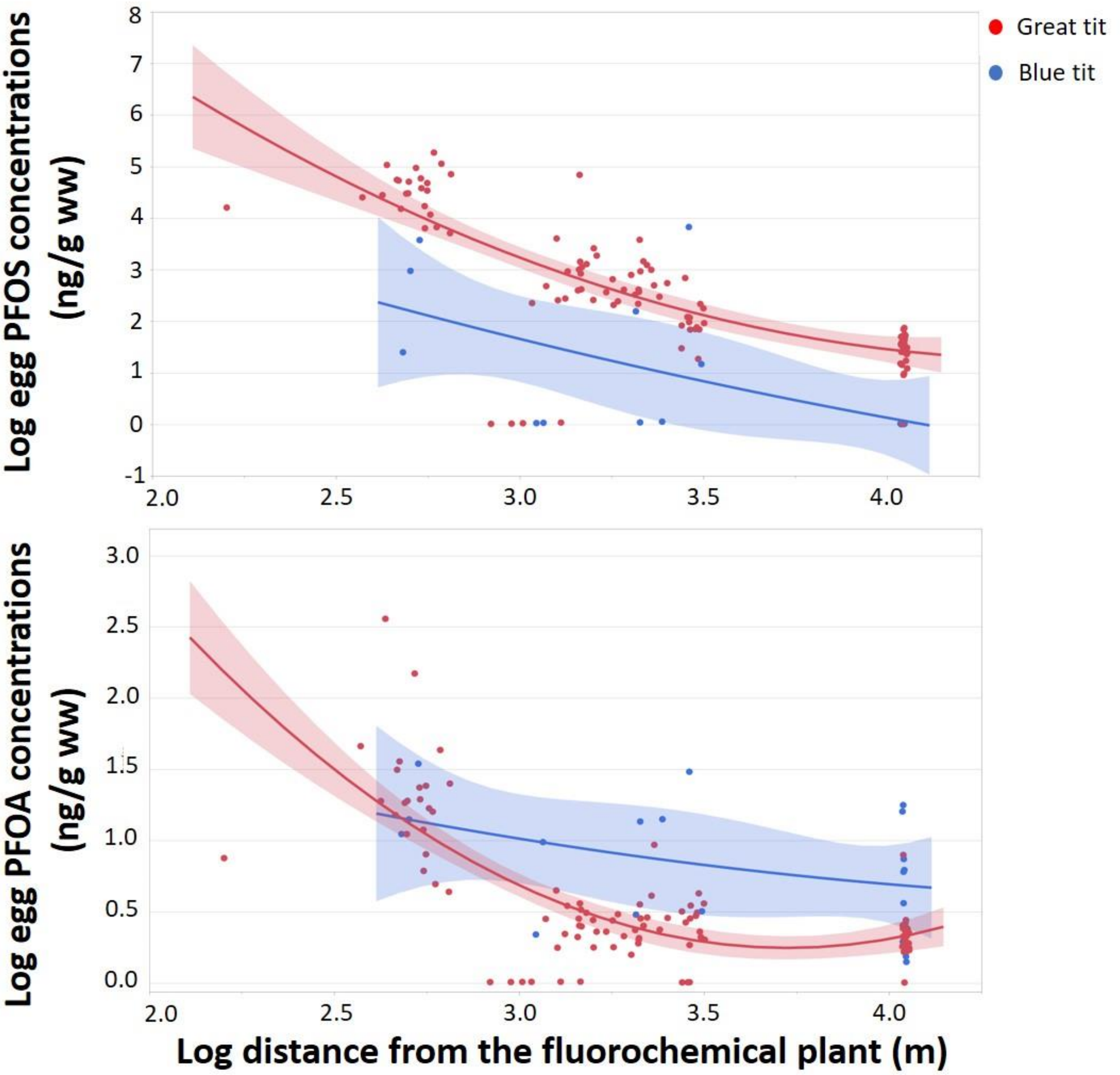

Fig. 5: Comparison of log egg PFOS (top graph) and PFOA (lower graph) concentrations in third eggs (ng/g wet weight) along the PFAA pollution gradient (3M; Vlietbos; Rot; Burchtse Weel; Fort 4) between blue tits (open dots; dashed regression curve; *N* = 17) and great tits (filled dots; solid regression curve; *N* = 111). The gray band denotes the 95% confidence interval for the regression estimates of each slope. PFOS blue tit curve adjusted $R^2 = 0.32$, $P < 0.05$; PFOS great tit curve adjusted $R^2 = 0.66$, $P < 0.001$. PFOA: blue tit curve: adjusted $R^2 = 0.15$, $P < 0.01$ and PFOA great tit curve adjusted $R^2 = 0.60$, $P < 0.001$. Great tit third egg data were adopted from Groffen et al. (2019a).

Supplementary information

Tables

Table S1: Schematic overview of the number of collected eggs and clutches of each tit species from all the study areas.[1] Third egg data adopted from Groffen et al. (2019a); [2] Complete clutch data were adopted from Lasters et al. (2019). Grey filled area = no data.

	Number third eggs		**Number complete clutches**	
Study area	**Blue tit**	**Great tit[1]**	**Blue tit**	**Great tit[2]**
3M	4	23		
Vlietbos	1	21		
Rot	2	18		
Burchtse Weel	2	16		
Fort 4	8	33	8	8

Table S2: MRM transitions, mass-labelled internal standards (ISTDs), cone voltages (V) and collision energy (eV) for the target perfluoroalkyl substances and their internal standard (Table was adopted from Groffen et al. (2019c)).

Compound	**Precursor ion (m/z)**	**Product ion (m/z)**		**Cone Voltage (V)**	**Collision energy (eV) for diagnostic transition1**	**Collision energy (eV) for diagnostic transition 2**	**Internal standard (ISTD) used for quantification**
		Diagnostic product Ion 1	**Diagnostic product Ion 2**				
PFBA	213	169	169	19	19	50	$^{13}C_4$-PFBA
PFPeA	263	219	219	15	10	45	$^{13}C_4$-PFBA
PFHxA	313	269	119	19	21	65	[1,2-$^{13}C_2$]PFHxA
PFHpA	363	319	169	24	40	30	[1,2-$^{13}C_2$]PFHxA
PFOA	413	369	169	22	13	60	[1,2,3,4-$^{13}C_4$]PFOA
PFNA	463	419	169	28	17	20	[1,2,3,4,5-$^{13}C_5$]PFNA
PFDA	513	469	219	25	29	29	[1,2-$^{13}C_2$]PFDA
PFUnDA	563	519	169	18	30	35	[1,2-$^{13}C_2$]PFUnDA
PFDoDA	613	569	319	22	21	30	[1,2-$^{13}C_2$]PFDoDA
PFTrDA	663	619	319	26	21	30	[1,2-$^{13}C_2$]PFDoDA
PFTeDA	713	669	169	28	21	21	[1,2-$^{13}C_2$]PFDoDA
PFBS	299	80	99	40	65	45	$^{18}O_2$-PFHxS
PFHxS	399	80	99	22	30	60	$^{18}O_2$-PFHxS
PFOS	499	80	99	60	58	58	[1,2,3,4-$^{13}C_4$]PFOS
PFDS	599	80	99	29	63	63	[1,2,3,4-$^{13}C_4$]PFOS
$^{13}C_4$-PFBA	217	172	172	19	19	50	
[1,2-$^{13}C_2$]PFHxA	315	269	119	19	21	65	
[1,2,3,4-$^{13}C_4$]PFOA	417	372	172	22	13	60	
[1,2,3,4,5-$^{13}C_5$]PFNA	468	423	172	28	17	20	
[1,2-$^{13}C_2$]PFDA	515	470	220	25	29	29	
[1,2-$^{13}C_2$]PFUnDA	565	520	170	18	32	35	
[1,2-$^{13}C_2$]PFDoDA	615	570	320	22	21	30	
$^{18}O_2$-PFHxS	403	84	103	22	30	60	
[1,2,3,4-$^{13}C_4$]PFOS	503	80	99	60	58	58	

Table S3: Minimum and maximum extraction recovery range, expressed in %, for the egg samples of the great tit and blue tit. Great tit data adopted from Lasters et al. (2019).

PFAA compound	Great tit (% min-max)	Blue tit (% min-max)
PFBA	35-114	32-107
PFPeA	35-114	32-107
PFHxA	28-170	10-162
PFHpA	28-170	10-162
PFOA	27-107	3.7-98
PFNA	22-108	1.9-88
PFDA	17-114	1.9-105
PFUnDA	11-91	4.7-117
PFDoDA	8.6-71	2.8-63
PFTrDA	8.6-71	2.8-63
PFTeDA	8.6-71	2.8-63
PFBS	6-112	2.9-24
PFHxS	6-112	2.9-24
PFOS	5.5-81	6.2-14
PFDS	5.5-81	6.2-14

Table S4: Mean values (± SE) of the breeding and egg parameters for blue tits and great tits from Fort 4 (Antwerp) in 2016, controlling for the egg laying order. Significant differences between both species are denoted with * (*P* < 0.05), ** (*P* < 0.01) or *** (*P* < 0.001). Great tit data adopted from Lasters et al. (2019).). [A] Laying date 1st egg = the number of days after which the first egg was laid in Fort 4.

Breeding and egg parameters	**Blue tit (*N* = 81)**	**Great tit (*N* = 47)**
Egg content weight (g)	**1.1 (0.05)**	**1.5 (0.08) *****
Volume (mm^3)	**1065 (47)**	**1323 (67) *****
Shell thickness (mm)	0.21 (0.01)	0.21 (0.02)
Laying date 1st egg (day) [A]	11 (1.1)	11 (1.6)
Clutch size (*N*)	**10.7**	**6.8 *****

Table S5: min-max concentration ranges (ng/g ww) of the target PFAA analytes among the individual clutches of blue tits ang great tits from Fort 4 near Antwerp (Belgium) in 2016. [1] Great tit data were adopted from Lasters et al. (2019). <LOQ = below the limit of quantification.

	Min - max concentration range (ng/g ww)								
Blue tit Clutch ID	PFBA	PFHxA	PFOA	PFNA	PFDA	PFUnDA	PFDoDA	PFTrDA	PFTeDA
1	<LOQ-81	<LOQ-0.15	0.40-11	<LOQ	<LOQ	<LOQ-1.4	<LOQ	<LOQ	<LOQ
2	<LOQ-2.6	<LOQ-0.79	3.7-58	<LOQ	<LOQ-7.2	<LOQ-1.7	<LOQ	<LOQ	<LOQ
3	<LOQ-85	<LOQ	<LOQ-11	<LOQ	<LOQ-1.1	<LOQ-11	<LOQ	<LOQ	<LOQ
4	<LOQ-11	<LOQ	<LOQ-18	<LOQ	<LOQ	<LOQ-4.2	<LOQ	<LOQ	<LOQ
5	0.23-22	<LOQ	<LOQ-32	<LOQ	<LOQ	<LOQ-4.3	<LOQ	<LOQ	<LOQ
6	0.29-1.6	<LOQ-1.2	4.1-15	<LOQ	<LOQ-2.0	<LOQ-3.8	<LOQ	<LOQ	<LOQ
7	0.06-47	<LOQ	0.20-6.4	<LOQ	<LOQ	<LOQ-2.1	<LOQ	<LOQ	<LOQ
8	0.46-0.38	<LOQ-0.35	3.1-5.6	<LOQ	<LOQ	<LOQ-1.1	<LOQ	<LOQ	<LOQ
Great tit Clutch ID	PFBA	PFHxA	PFOA	PFNA	PFDA	PFUnDA	PFDoDA	PFTrDA	PFTeDA
1	<LOQ	<LOQ	1.1-3.2	<LOQ-1.9	1.2-3.5	<LOQ	1.2-2.2	<LOQ-2.4	<LOQ-0.44
2	<LOQ	<LOQ	0.93-2.2	<LOQ-1.1	0.48-1.5	<LOQ	1.3-2.7	0.66-1.5	<LOQ-0.18
3	<LOQ	<LOQ	0.79-2.3	<LOQ-1.6	0.51-2.4	<LOQ	0.90-2.8	<LOQ-3.1	<LOQ-0.91
4	<LOQ	<LOQ	1.1-2.8	<LOQ-1.9	<LOQ-2.2	<LOQ	1.7-4.8	0.47-5.7	<LOQ-0.96
5	<LOQ	<LOQ	1.2-2.9	<LOQ-1.2	1.0-2.2	<LOQ	1.5-2.9	0.41-3.2	<LOQ-0.71
6	<LOQ	<LOQ	1.1-3.2	<LOQ-1.4	1.1-2.1	<LOQ	1.7-3.5	0.52-2.3	<LOQ-0.65

7	<LOQ	<LOQ	0.72-3.0	0.79-2.4	<LOQ-2.3	<LOQ	1.3-2.9	0.40-2.5	<LOQ-0.69
8	<LOQ	<LOQ	0.73-3.7	0.77-2.0	1.1-3.0	<LOQ	2.0-3.6	0.68-2.6	<LOQ-1.1

Figures

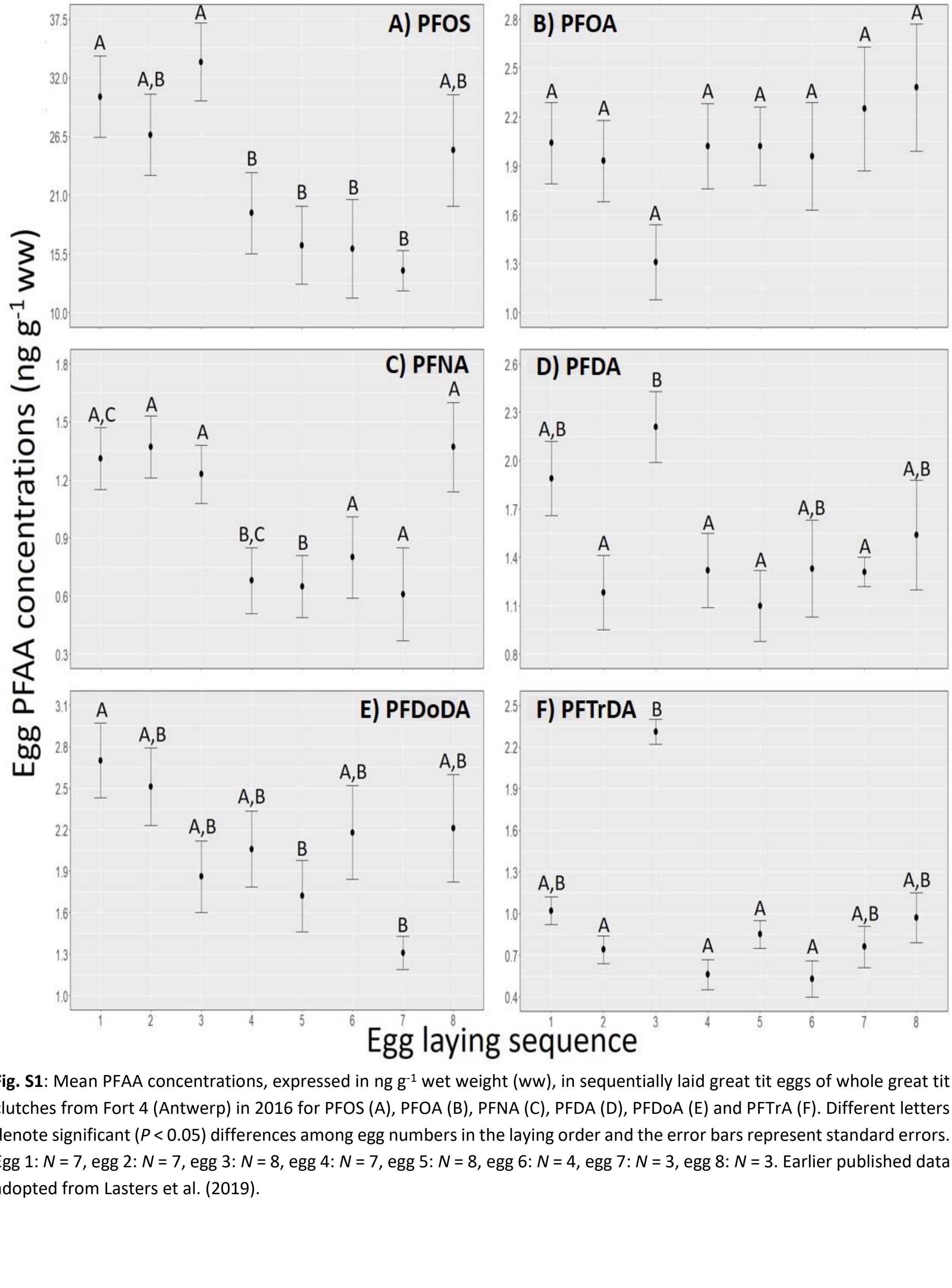

Fig. S1: Mean PFAA concentrations, expressed in ng g^{-1} wet weight (ww), in sequentially laid great tit eggs of whole great tit clutches from Fort 4 (Antwerp) in 2016 for PFOS (A), PFOA (B), PFNA (C), PFDA (D), PFDoA (E) and PFTrA (F). Different letters denote significant ($P < 0.05$) differences among egg numbers in the laying order and the error bars represent standard errors. Egg 1: $N = 7$, egg 2: $N = 7$, egg 3: $N = 8$, egg 4: $N = 7$, egg 5: $N = 8$, egg 6: $N = 4$, egg 7: $N = 3$, egg 8: $N = 3$. Earlier published data adopted from Lasters et al. (2019).