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Abstract

Although bird eggs have been used in biomonitoring studies on perfluoroalkyl acids (PFAAs), effects of environmental concentrations on reproduction remain largely unknown in wild birds. In the present study we examined the associations between the concentrations of 4 perfluoroalkyl sulfonic acids (PFSAs) and 11 perfluoroalkyl carboxylic acids (PFCAs) in the eggs of great tits (*Parus major*), collected along a distance gradient from a pollution source, and multiple reproductive parameters (including the start of egg laying, clutch size, hatching success, fledging success and total breeding success) along with egg shell thickness and body condition of the nestlings.

The PFAA concentrations measured at the plant site were among the highest ever reported in wild bird eggs. PFAA concentrations decreased sharply with increasing distance (0 – 11 km) from the plant, but remained relatively elevated in the adjacent sites. PFAAs were grouped into principal components (PCs) to prevent collinearity. High concentrations of PFOS, PFDS, PFDoDA, PFTrDA and PFTeDA (grouped as PC1) were associated with a reduced hatching success of nests where at least one egg hatched, thinner egg shells and increased survival of the hatched chicks. High concentrations of PFDA (PC2) were associated with a reduced hatching success, especially in nests where no eggs hatched, an earlier start of egg laying and a reduction of total breeding success, mainly caused by the failure in hatching.

Although the major manufacturer of PFAAs phased out the production of perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA) and related products in 2002, concentrations appear to have increased since previous measurements. Surprisingly, despite the very high concentrations close to the fluorochemical plant, there was no clear evidence for reproductive impairment as the observed associations between PFAA concentrations and reproductive parameters were rather limited compared to previous studies in songbirds. These findings also suggest potential differences in sensitivity between species.

Keywords: Perfluoroalkyl acids; PFOS; Birds; Eggs; Belgium; Reproductive impairment

Capsule Despite the very high PFAA concentrations at the perfluorochemical hotspot, correlations with reproductive parameters were limited.

Introduction

Perfluoroalkyl acids (PFAAs) are chemicals with distinctive physicochemical properties, which result from the strong C-F binding and the hydrophobic and lipophobic character that make them highly persistent and bioaccumulative in the environment. They have been produced and used since 1950 for numerous applications, such as textile stain and soil repellents, food-contact paper and fire-fighting foams (Buck et al., 2011; Kissa, 2001). Consequently, PFAAs have been detected globally in the environment, wildlife and humans (Butt et al., 2010; D'Hollander et al., 2010; Giesy & Kannan, 2001, 2002; Groffen et al., 2017, 2018; Houde et al., 2006; Miller et al., 2015), which can all be polluted either directly or via environmental degradation of precursor compounds (Buck et al., 2011; Martin et al., 2010; Prevedouros et al., 2006). During the last decades, regulatory agencies and researchers have mainly focused on long chain perfluoroalkyl carboxylic acids (PFCAs) and perfluoroalkyl sulfonic acids (PFSAs), rather than their short-chained analogues, which have a lower bioaccumulative potential. Particularly perfluorooctanoic acid (PFOA, $C_7F_{15}COOH$) and perfluorooctane sulfonate (PFOS, $C_8F_{17}SO_3H$) have been studied often (Buck et al., 2011).

Based on their persistence, widespread distribution and potential health effects, the major manufacturer of PFAAs, 3M, phased-out the production of PFOS, PFOA and related compounds in 2002. Furthermore, PFOS was included in the Stockholm Convention on Persistent Organic Pollutants in 2009. These measures appear to have reduced environmental PFOS concentrations in many cases, whereas concentrations of other PFAAs are rising (Ahrens et al., 2011; Filipovic et al., 2015; Miller et al., 2015).

Although bird eggs have been used in numerous studies to monitor PFAA concentrations on a global scale (e.g., Gebbink & Letcher, 2012; Giesy & Kannan, 2001; Holmström et al., 2005; Miller et al., 2015; Yoo et al., 2008), only very few of these studies have focused on terrestrial birds (Ahrens et al., 2011; Custer et al., 2012; Groffen et al., 2017; Holmström et al., 2010; Lopez-Antia et al., 2017; Rüdel et al., 2011; Yoo et al., 2008).

Previous studies on PFAA concentrations in wildlife near a fluorochemical plant in Antwerp, Belgium, revealed the highest concentrations ever found in wildlife (Dauwe et al., 2007; D'Hollander et al., 2014; Groffen et al., 2017; Hoff et al., 2005; Lopez-Antia et al., 2017). PFOS concentrations in liver from great tits (*Parus major*) and blue tits (*Cyanistes caeruleus*) were higher in this area than those measured in top predators in other regions (Dauwe et al., 2007). In addition, PFOS concentrations in great tit eggs were among the highest ever reported in bird eggs worldwide (Groffen et al., 2017; Lopez-Antia et al., 2017). Furthermore, studies on the effects of PFAAs in the vicinity of this plant in Antwerp were restricted to PFOS and mainly reported biochemical effects in wood mice (Hoff et al., 2004) and great tits (Hoff et al., 2005; Lopez-Antia et al., 2017). Hoff et al. (2004, 2005) observed significantly positive associations with liver weight in both species and lipid peroxidation level in liver of mice. Plasmatic biochemical biomarkers in great tits were not affected by PFAA concentrations (Lopez-Antia et al., 2017). Biomonitoring of PFAA concentrations and their composition profile in the surroundings of the fluorochemical plant in Antwerp is therefore extremely important.

Reproductive effects of PFAAs have been studied in a wide variety of taxa, including nematodes (e.g. Chen et al., 2018), arthropods (e.g. Princz et al., 2018), fish (e.g. Lee et al., 2017; Xia & Niu, 2017) and humans (e.g. Foresta et al., 2018; Song et al., 2018). Despite the ubiquity of PFAAs, not much is known about their effects on the individual and population level in terrestrial bird species. To the best of our knowledge, only a few studies investigated the associations between PFAA concentrations and reproductive parameters in birds. Most of these studies were performed under laboratory conditions, where bird eggs were injected with PFAAs or where birds were exposed to PFAAs through their diet, whereas field studies remain scarce. In addition, the majority of these studies only focus on PFOS as their target analyte.

Two field studies have studied the relationship between PFOS concentrations and hatching success in tree swallows (*Tachycineta bicolor*; Custer et al. 2012, 2014). Custer et al. (2012) have reported negative associations between PFOS concentrations starting from 150 ng/g ww in eggs of tree swallows and the

hatching success of the remaining eggs in the nest. Furthermore, a 20% decrease in hatching success at PFOS concentrations of 283 ng/g in eggs has been observed (Custer et al., 2014).

In ovo exposure to PFOS, under laboratory conditions, did not affect hatching rate in white leghorn chickens (*Gallus gallus domesticus*), but did cause a reduced body and wing length (Peden-Adams et al., 2009). However, other laboratory studies have observed reproductive dysfunction after *in ovo* exposure to perfluorohexane sulfonic acid (PFHxS), PFOS and PFOA (Cassone et al., 2012; Molina et al., 2006; Yanai et al., 2008). A significant reduction in hatching success by 20% and 63% was observed after injection of 5000 ng/g PFOA and 38000 ng/g PFHxS, respectively (Cassone et al., 2012; Yanai et al., 2008). In addition, tarsus length and body weight were reduced at the same concentrations (Cassone et al., 2012). Treatment-related mortalities or effects on body weight and reproductive parameters were not observed in a study in which northern bobwhite quail (*Colinus virginianus*) were exposed to perfluorobutane sulfonic acid (PFBS) through diet (Newsted et al., 2008). Furthermore, no effects of PFOS on body weight and reproductive performance have been found in mallard ducks (*Anas platyrhynchos*; Newsted et al., 2007).

In the present study, we investigated possible relationships between multiple PFAA concentrations in great tit eggs and multiple reproductive parameters (including the start of egg laying, clutch size, hatching success, fledging success and total breeding success), egg shell thickness and body condition of the nestlings along a distance gradient, starting from a fluorochemical plant in Antwerp. This study can help to understand possible effects of these pollutants on wild birds.

Materials and method

Study species and sample collection

Great tits (*Parus major*) are insectivorous songbirds that feed mainly on caterpillars during the breeding season and berries and seeds during the winter (del Hoyo et al. 2007; Lopez-Antia et al. 2017). They are

considered to be a model species for ecotoxicological studies as they nest in man-made nestboxes, are abundant and can be attracted to polluted areas (Dauwe et al. 1999, 2004, 2005; Eens et al. 1999; Eeva & Lehikoinen 1995, 1996; Eeva et al. 1998; Van den Steen et al. 2006).

Nestboxes were placed during autumn of 2015 at five sampling sites (Figure 1), representing a gradient from a fluorochemical plant (3M) in Antwerp, Belgium. These sites were the 3M fluorochemical plant (28 nestboxes), Vlietbos (24 nestboxes; 1 km SE from 3M), Rot-Middenvijver (further called Rot; 20 nestboxes; 2.3 km ESE from 3M), Burchtse Weel (21 nestboxes; 3 km SE from the plant) and Fort 4 in Mortsel (58 nestboxes; 11 km SE from the plant).

From just before egg laying until incubation, nestboxes were checked every other day or daily to be able to determine the start of the egg laying period and clutch size. From each nest, the third egg was collected by hand before the incubation had started. From 10 days after incubation onwards, nests were daily checked for hatching to determine hatching success. Body condition, determined according to the scales mass index of Peig and Green (2009) of the nestlings was determined 14 days after hatching. Finally, nestboxes were checked after approximately 25 days to determine the number of fledglings.

Egg parameters

Prior to the chemical analysis eggs were weighed (± 0.1 g, Mettler Toledo, Zaventem, Belgium) and their length and width were measured using a digital caliper (± 0.01 mm, Mitutoyo Belgium NV, Kruibeke, Belgium). Shell thickness was measured using the methodology described by Lopez-Antia et al. (2013). Three small pieces of shell from the equatorial region were collected and dried. Hereafter, the thickness of these pieces was measured with a micrometer (± 0.01 mm, Mitutoyo Belgium NV, Kruibeke, Belgium).

Chemical analysis

All used abbreviations of PFAAs are according to Buck et al. (2011). Target PFAAs included 11 PFCAs (PFBA, PFPeA, PFHxA, PFHpA, PFNA, PFOA, PFDA, PFUnDA, PFDoDA, PFTrDA and PFTeDA) and 4 PFSAs (PFBS, PFHxS, PFOS and PFDS). Isotopically mass-labelled internal standards (ISTDs) were purchased by

Wellington Laboratories (Guelph, Canada) and comprised $^{13}C_4$-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, 1,2[$^{13}C_2$]PFDoDA, $^{18}O_2$-PFHxS and [1,2,3,4-$^{13}C_4$]PFOS. HPLC grade Acetonitrile (ACN) and water (VWR International, Leuven, Belgium) were used.

Sample extraction

Egg content was transferred into a polypropylene (PP) tube and homogenized by repeatedly sonicating and vortex-mixing. The extraction procedure was based on solid-phase-extraction. Approximately 0.4g of homogenized egg was used for the analysis. Samples were spiked with 10 ng of each ISTD (in 50:50 ACN/HPLC grade water). Hereafter, 10 mL ACN was added and the samples were sonicated (3 x 10 min, Branson 2510) and left overnight on a shaking plate (135 rpm, room temperature, GFL 3020, VWR International, Leuven, Belgium). After centrifugation (4°C, 10 min, 2400 rpm, Eppendorf centrifuge 5804R, rotor A-4-44), the supernatant was transferred into a 14 mL PP tube. Chromabond HR-XAW SPE cartridges (Application No 305200, SPE department, Macherey-Nagel, Germany, 2009) were conditioned with 5 mL ACN and equilibrated with 5 mL Milli-Q (MQ) water. After loading the samples, the columns were washed with 5 mL 25 mM ammonium acetate and 2 mL ACN. The elution was performed with 2 x 2 mL 2% ammonium hydroxide in ACN and the eluent was completely dried using a rotational-vacuum-concentrator at 30°C (Eppendorf concentrator 5301, Hamburg, Germany). The dried eluent was reconstituted with 200 μL 2% ammonium hydroxide in ACN and vortex-mixed during one minute. Samples were filtered through an Ion Chromatography Acrodisc 13 mm Syringe Filter with 0.2 μm Supor (PES) Membrane (VWR International, Leuven, Belgium) and collected into a PP auto-injector vial before analysis.

UPLC-TQD analysis

PFAAs were analyzed by UPLC coupled tandem ES(-) mass spectrometry (ACQUITY, TQD, Waters, Milford, MA, USA). An ACQUITY BEH C18 column (2.1 x 50 mm; 1.7 μm, Waters, USA) was used to separate the analytes. The mobile phase solvents were 0.1% formic acid in water (A) and 0.1% formic acid in ACN (B),

with a solvent gradient starting at 65% A to 0% A in 3.4 min to 65% A at 4.7 min and a flow rate of 450 µL/min and an injection volume of 10 µL. An ACQUITY BEH C18 pre-column (2.1 x 30 mm; 1.7 µm, Waters, USA) was inserted, between the solvent mixer and injector, to retain any PFAAs contamination originating from the system. PFAAs were identified and quantified based on multiple reaction monitoring (MRM) of the following diagnostic transitions: 213 → 169 (PFBA), 217 → 172 ($^{13}C_4$PFBA), 263 → 219 (PFPeA), 313 → 269 (PFHxA), 313 → 119 (PFHxA), 315 → 269 ($^{13}C_2$PFHxA), 315 → 119 ($^{13}C_2$PFHxA), 363 → 319 (PFHpA), 363 → 169 (PFHpA), 413 → 369 (PFOA), 413 → 169 (PFOA), 417 → 372 ($^{13}C_4$PFOA), 417 → 172 ($^{13}C_4$PFOA), 463 → 419 (PFNA), 463 → 169 (PFNA), 468 → 423 ($^{13}C_5$PFNA), 468 → 172 ($^{13}C_5$PFNA), 513 → 469 (PFDA), 513 → 219 (PFDA), 515 → 470 ($^{13}C_2$PFDA), 515 → 220 ($^{13}C_2$PFDA), 563 → 519 (PFUnDA), 563 → 169 (PFUnDA), 565 → 520 ($^{13}C_2$PFUnDA), 565 → 170 ($^{13}C_2$PFUnDA), 613 → 569 (PFDoDA), 613 → 319 (PFDoDA), 615 → 570 ($^{13}C_2$PFDoDA), 615 → 320 ($^{13}C_2$PFDoDA), 663 → 619 (PFTrDA), 663 → 319 (PFTrDA), 713 → 669 (PFTeDA), 713 → 169 (PFTeDA), 299 → 99 (PFBS), 299 → 80 (PFBS), 399 → 99 (PFHxS), 399 → 80 (PFHxS), 403 → 103 ($^{18}O_2$PFHxS), 403 → 84 ($^{18}O_2$PFHxS), 499 → 80 (PFOS), 499 → 99 (PFOS), 503 → 80 ($^{13}C_4$PFOS), 503 → 99 ($^{13}C_4$PFOS), 599 → 99 (PFDS) and 599 → 80 (PFDS).

Calibration

Calibration curves were prepared by adding a constant amount of internal standard to varying amounts of unlabeled standards. The dilutions of these standards were performed in ACN and water. The relationship between the ratio of concentrations of unlabeled and labeled PFASs and the area of unlabeled and labeled PFASs was described by a linear regression function with a highly significant linear fit for all target analytes (all $p < 0.001$; $R^2 > 0.98$). Individual PFAAs were quantified using the corresponding ISTD with exception of PFPeA, PFHpA, PFTrDA, PFTeDA, PFBS and PFDS which were quantified using the ISTD of the compound closest in terms of functional group and size, i.e. the ISTD of PFBA to quantify PFPeA, PFHxA to quantify PFHpA, PFDoDA to quantify both PFTrDA and PFTeDA, PFHxS to quantify PFBS and PFOS to quantify PFDS.

Quality assurance

Procedural blanks were regularly (one per batch of 10 samples) analyzed and contained contamination of PFBA (<0.25 ng/μL). Concentrations observed in blanks were subtracted from the concentrations found in samples in the same batch. The limit of quantification (LOQ) was calculated based on a signal-to-noise ratio of 10 and ranged from 0.045 ng/g to 0.59 ng/g for PFBA, PFOA, PFNA, PFDA, PFDoDA, PFTrDA and PFTeDA. LOQs were considerably higher for PFOS and PFDS and were 2.55 ng/g and 5.92 ng/g respectively. LOQs for PFPeA, PFHxA, PFHpA, PFUnDA, PFBS and PFHxS could not be determined as these PFAAs were not detected in any sample. Individual LOQs of the detected compounds are displayed in Table 1. Recoveries for each sample were determined based on the ISTD of the corresponding sample and an ISTD solution. Detection frequencies of the detected compounds varied between 25% and 100% and should be interpreted with caution due to high variation in LOQs.

Statistical analysis

Statistical analyses were performed using SPSS 23. To obtain a normal distribution, data were log transformed. The level of significance was set at $p \leq 0.05$. PFAA concentrations below the LOQ were given a value of LOQ/2 (Bervoets et al. 2004; Custer et al. 2000).

Differences in PFAA concentrations among sampling sites were evaluated by using a one way ANOVA, followed by Tukey's honest significant differences Post-hoc analysis. Correlations between individual compounds and between ∑PFSA and ∑PFCA were assessed in each study site using Spearman rank correlation analyses. PFAAs composition profiles were calculated as the proportions of individual compounds to the total PFAAs, PFSA and PFCA concentration in each egg. These percentages were averaged for all the eggs at a site.

Generalized Linear Models (GLMz) were used to test for correlations between PFAA concentrations and reproductive parameters. In order to reduce the number of covariates and to account for collinearity among them, we conducted a Principal Component Analysis (PCA) on the 9 detected PFAAs, i.e. PFBA,

PFOA, PFNA, PFDA, PFDoDA, PFTrDA, PFTeDA, PFOS and PFDS. To study the correlations between PFAAs and the different reproductive parameters, we used the following distributions: a Poisson distribution to study correlations with the clutch size, a normal distribution to study correlations with the egg laying date, egg parameters (length, width and shell thickness) and the mean condition of the chicks. For ratios, we used a binary logistic distribution; hatching success (number of hatched eggs divided by the number of incubated eggs), fledging success (number of fledglings divided by the number of hatched eggs), overall breeding success (number of fledglings divided by the number of incubated eggs). Finally we studied the total failure of hatching (those nests where any egg hatched) and the total failure of reproduction (nests where incubation did not occur or hatching or fledging success completely failed), both based on a negative binomial distribution type.

Results

PFAA concentrations

Table 1 gives an overview of median concentrations, ranges and detected frequencies of PFAAs in great tit eggs. PFOA, PFNA, PFDA, PFDoDA, PFTrDA, PFTeDA and PFOS were detected at all locations, whereas PFDS was only detected at 3M and PFBA was not detected at Burchtse Weel. With exception of PFBA, short-chained PFSAs (PFBS) and PFCAs (PFPeA, PFHxA, PFHpA) were not detected in any sample from any location. In addition, PFHxS and PFUnDA were not detected as well.

Significant differences among sampling sites were observed for PFBA, PFOA, PFNA, PFDA, PFDoDA, PFTrDA, PFTeDA, PFOS and PFDS (all $p < 0.007$; Figure 2). Eggs collected at the 3M site showed significantly higher concentrations of PFOA, PFNA, PFDA, PFDoDA, PFOS and PFDS compared to all other locations (all $p < 0.001$). In addition, PFBA concentrations were higher at 3M than at Burchtse Weel ($p = 0.002$) and Fort 4 ($p = 0.011$), PFTrDA concentrations were higher at 3M than at Vlietbos ($p = 0.009$), Burchtse Weel ($p = 0.003$) and Fort 4 ($p < 0.001$) and PFTeDA concentrations were higher at 3M than at Burchtse Weel ($p =$

0.015). PFOA and PFDoDA concentrations were higher at Rot compared to Vlietbos ($p = 0.046$ and $p = 0.003$, respectively). Furthermore, PFOS concentrations at Rot were significantly higher than those measured at the sites furthest away from the plant, i.e. Burchtse Weel ($p = 0.004$) and Fort 4 ($p < 0.001$). PFOS concentrations at Fort 4 were also lower than those at Vlietbos ($p < 0.001$). Eggs taken from Rot also contained higher PFTrDA concentrations compared to Fort 4 ($p = 0.019$) and PFTeDA concentrations compared to Burchtse Weel ($p = 0.048$). Finally, PFDA concentrations were higher at Fort 4 than at Vlietbos ($p = 0.005$) and Burchtse Weel ($p = 0.032$).

PFAA profile

PFOS was the dominant contributor to both the ∑PFSAs (Figure S1) and the ∑PFAAs at each sampling site. Its contribution to the ∑PFSAs was lowest at Vlietbos (82.9%) and highest at 3M (99.5%). The major contributor to the ∑PFCAs was PFOA at 3M (25.5%) and PFTrDA at all other locations (37.0% at Vlietbos, 41.4% at Rot, 28.5% at Burchtse Weel and 24.5% at Fort 4; Figure 3).

PFAAs correlations

Table S1 summarizes the correlations found among PFAAs at the different sampling locations. All significant correlations were positive. 3M had the highest number of significant correlations (20), followed by Vlietbos (18), Fort 4 (17), Rot (14) and Burchtse Weel (11). These results show a high degree of collinearity between PFAA compounds and thus confirm the use of PCs in further analysis.

Reproductive parameters

Reproductive parameters amongst the different sampling sites are reported in Table 2. The day of the first egg and the hatching success did not differ significantly among locations. The shell thickness of the eggs was significantly lower at 3M compared to all other locations (all $p < 0.02$). In addition, the thickness was also lower at Rot and Fort 4 compared to Burchtse Weel (both $p < 0.001$). Furthermore, the breeding success differed significantly among locations, which was caused by a significantly lower success at Fort 4 compared to Vlietbos and Burchtse Weel (both $p < 0.001$). Similarly, the condition of the chicks was

significantly higher at Vlietbos and Burchtse Weel compared to 3M and Rot (all $p < 0.001$) and the survival of the chicks was higher at Vlietbos and Burchtse Weel than at Fort 4 ($p = 0.050$ and $p = 0.016$, respectively). Finally, the clutch size was significantly lower at Fort 4 than at 3M ($p = 0.010$) and Burchtse Weel ($p = 0.036$).

Only a few reproductive parameters were correlated (Table S2). At all locations, breeding success was positively correlated with hatching success and chicks' survival. In addition, at Vlietbos a significant negative correlation was observed between clutch size and the day of the first egg and at Fort 4, we observed a significant negative correlation between clutch size and chicks' survival.

Two principal components were selected according to Kaiser criterion (eigenvalue higher than 1; Kaiser 1960). The first Principal Component (PC1) explained 61.61% of the variance and the second Principal Component (PC2) explained a further 14.38% (Table S3). PC1 was mainly influenced by PFOS, PFDS, PFDoDA, PFTrDA and PFTeDA and to minor extent by PFOA and PFNA; high concentrations of these compounds corresponded with high values of PC1. PC2 was mainly influenced by PFDA, therefore high values of PC2 mainly indicated high PFDA concentrations (Table S3).

Table 3 summarizes the results (Wald χ^2, p, beta and standard error of the beta) of the GLMz for the reproductive parameters. Both PC1 ($p = 0.003$) and PC2 ($p < 0.001$) were negatively associated with the hatching success. Further analysis demonstrated that high values of PC2 were significantly associated with the total failure of hatching ($p = 0.025$; nests where no egg hatched, Figure 4D) while PC1 was negatively associated with the hatching success in those nests where at least one egg hatched ($p < 0.001$; Figure 4A). Moreover, high values of PC1 (PFOS, PFDS, PFDoDA, PFTrDA and PFTeDA) were associated with thinner egg shells ($p < 0.001$; Figure 4B), but also with an increased survival of hatched chicks ($p = 0.027$). PC2 (influenced mainly by PFDA) was negatively associated with the day of the 1st egg ($p = 0.005$; Figure 4C).

Finally, high PC2 values were significantly associated with a reduction of the total breeding success ($p = 0.05$; figure 4D), which was mainly due to a failure in hatching success.

Discussion

PFAA concentrations

PFOS, PFOA and PFDS concentrations at the 3M fluorochemical plant were among the highest ever reported in eggs of free-living birds with median concentrations of 48056 ng/g ww, 18 ng/g ww and 315 ng/g ww, respectively.

To the best of our knowledge, only six papers on PFAAs in passerine bird eggs have been published. Table 4 shows some the PFAA concentrations detected in these studies. The highest PFCA concentrations have been observed by Yoo et al. (2008) in parrot bill (*Paradoxornis webbiana*) eggs collected around the shores of a lake that receives wastewater from an industrial complex in Korea. Yoo et al. (2008) observed median PFNA and PFDA concentrations of 40 ng/g and 114.2 ng/g, respectively. In the present study, PFNA (7.7 ng/g ww) and PFDA (13 ng/g ww) concentrations were much lower, suggesting different types of contamination between both places. Until now, the highest PFSA concentrations, have been detected by Groffen et al. (2017) in great tit eggs collected near the same fluorochemical plant as in the present study, with median concentrations of 10380 ng/g ww PFOS, 99.3 ng/g ww PFHxS and 47.7 ng/g ww PFDS. As a result of the phase-out of PFOS, PFOA and related products in 2002, it was expected that environmental concentrations of long-chained PFAAs would decrease, whereas those of short-chained alternatives would increase (Ahrens et al., 2011; Filipovic et al., 2015; Miller et al., 2015). Lopez-Antia et al. (2017) detected PFOS concentrations ranging from 19 ng/g ww to 5635 ng/g ww in great tit eggs collected at Vlietbos and Burchtse Weel in 2006. In the present study, PFOS concentrations at these locations were lower and ranged from <LOQ – 4035 ng/g ww at Vlietbos and 17.6 – 690 ng/g ww at Burchtse Weel. On the contrary, median PFOS concentrations in great tit eggs from the fluorochemical plant were approximately 3.5 times

higher in the present study compared to 2011 (Groffen et al., 2017). With exception of PFDS, concentrations of other PFAAs were comparable to concentrations detected in 2011 (Groffen et al., 2017). However, the PFOS concentrations in the study by Groffen et al. (2017) exceeded the linear range of the calibration curve and where therefore extrapolated. Therefore, these concentrations should be treated with caution, as real concentrations may have been higher.

Our earlier studies support the present one, reporting high PFOS concentrations in the liver of wood mice (*Apodemus sylvaticus;* D'Hollander et al., 2014; Hoff et al., 2004), livers of great tit and blue tit nestlings (Hoff et al., 2005) and great tit blood and plasma (Dauwe et al., 2007) near this fluorochemical hotspot.

Although there was a steep decrease for most PFAAs with increasing distance from the fluorochemical plant, differences between Vlietbos and Rot were less evident, as PFOA and PFDoDA concentrations were higher at Rot. In addition, PFDA concentrations were higher at Fort 4, furthest away from the fluorochemical plant, than at Vlietbos and Burchtse Weel. As was mentioned before, this could be explained by direct exposure closer to the fluorochemical plant and indirect exposure due to degradation of fluorotelomer alcohols (FTOHs) further away. Nevertheless, the decrease of PFAA concentrations with increasing distance from the fluorochemical plant was also observed in previous studies conducted in the area (Dauwe et al., 2007; D'Hollander et al., 2014; Groffen et al., 2017; Hoff et al., 2004, 2005; Lopez-Antia et al., 2017) and in chicken eggs, soil and water near a fluorochemical plant in China (Wang et al., 2010).

Variation in PFAA concentrations within a nest has been demonstrated before in the Audouin's gull (*Larus audouinii*), where PFOS concentrations decreased with laying order (Vicente et al., 2015). Therefore, it was expected that sampling a fixed egg from each nest would reduce the variation among nests at a site compared to randomized sampling. However, the variation in PFAA concentrations among nests remained as large as in our previous study (Groffen et al. ,2017), in which eggs were collected randomly. This

suggests that other factors such as the age or dispersal status of the bird, might cause this variation. Unfortunately, we do not have information about the origin of most female birds.

PFAA profile

A similar PFAA profile was observed in the present study compared to a study conducted in the same area in 2011 (Groffen et al., 2017), with PFOS being the major contributor to the total PFAAs and ∑PFSA concentrations. The dominance of PFOS was in agreement with literature on PFAAs in bird eggs (Ahrens et al., 2011; Custer et al., 2012; Nordén et al., 2013; Rüdel et al., 2011). At the plant site, PFOA was the dominant contributor to the ∑PFCAs in both the present study and the study performed by Groffen et al. (2017), whereas further away from the plant, PFTrDA and PFDoDA were more dominant in both studies. This profile can be explained by a direct deposition of PFOA close to the plant, whereas further away atmospheric and biological degradation of volatile polyfluorinated precursor compounds could explain the dominance of PFTrDA. In addition, the bioaccumulative potential of PFAAs increases with increasing chain length, indicating that long-chained PFAAs such as PFDoDA and PFTrDA are more bioaccumulative than the shorter-chained PFOA (Armitage et al., 2009; Conder et al., 2008; Ellis et al., 2004; Houde et al., 2006).

Are high PFAA concentrations associated with reproductive impairment?

In the present study, hatching success was negatively correlated with PC1 (influenced by PFOS, PFDS, PFDoDA, PFTrDA and PFTeDA and to minor extent by PFOA and PFNA) in nests where at least one egg hatched. Fledging success, on the other hand, was positively correlated with PC1, which was probably caused by a higher chance of survival of the chicks in nests with a lower number of hatched eggs. In addition, PC2 (PFDA) was negatively correlated with both hatching success, in nests where no egg hatched, and total breeding success. A possible explanation for the negative correlation between PFAAs and hatching success and hatching and fledging probability might be that parents have a reduced fertility or

that toxic effects on the development of the embryo occur (Molina et al., 2006; Yanai et al., 2008). Custer et al. (2014) observed an association between PFOS exposure and embryo death in tree swallows.

Studies on the associations of PFAAs on reproductive parameters of birds remain scarce. To the best of our knowledge, only two studies (Custer et al., 2012; Custer et al., 2014) suggest effects of environmental PFAA concentrations on reproduction. Similar outcomes were observed by Custer et al. (2012, 2014), who also observed negative associations between PFOS concentrations and hatching success in tree swallows. However, the effects they observed started from 150 ng/g ww, which is approximately 1000 times lower than the PFOS concentrations detected in the present study. In addition, the associations with reproductive parameters of great tits (present study) were less evident compared to the associations in tree swallows. This suggests that differences in sensitivity between species may exist. Karchner et al. (2006) have reported species-specific differences in sensitivity to other POPs between chickens and free-living wild birds. These species-specific differences in sensitivity to pollutants have also been observed for PFAAs. Norden et al. (2016) reported that the toxic effects of PFOS and PFOA, after *in ovo* injection, were higher in White Leghorn chicken (*Gallus gallus domesticus*) compared to herring gull (*Larus argentatus*) and great cormorant (*Phalacrocorax carbo sinensis*). Another explanation for the differences between the great tits and the tree-swallows, but highly speculative, is that great tits near the fluorochemical plant in Antwerp may have adapted to PFAAs pollution, as fast trait changes in response to changing environmental factors, including toxics, have been reported before (Marzluff, 2016).

A reduced hatching success was also observed in multiple laboratory studies (e.g., Cassone et al., 2012; Molina et al., 2006; O'Brien et al., 2009). *In ovo* PFHxS exposure resulted in a reduction of hatching success, tarsus length and embryo mass at concentrations of 38,000 ng/g in white leghorn chicken (Cassone et al., 2012). Hatchability of eggs of white leghorn chicken was reduced after *in ovo* exposure to PFOS concentrations of 0.1 μg/g and higher (Molina et al., 2006). However, no effects of PFOA, PFUnDA

and PFDS on hatching success were observed at concentrations up to 10 μg/g after *in ovo* exposure in white leghorn chicken (O'Brien et al., 2009). Median PFOS concentrations in the present study were higher at 3M than reported by Molina et al. (2006), indicating that hatching success at 3M might be influenced by high PFOS concentrations.

Surprisingly, higher values of PC2 (mainly influenced by PFDA) were correlated with the earlier start of the egg laying period, which is contradictory to studies on pesticides (Bustnes et al., 2007; Helberg et al., 2005; Lopez-Antia et al. 2015 a,b), where a delayed start of the egg laying period was observed at higher concentrations. However, for metals, often no effects are observed (Dauwe et al., 2005; Eeva & Lehikoinen 1995; Janssens et al., 2003). Early breeding is typically associated with a higher reproductive output and quality. The positive association with early egg laying may therefore indicate that PFAAs stimulate self-maintenance mechanisms in birds. This hypothesis has also been suggested by Blévin et al. (2017) who reported a positive relationship between PFAAs contamination and telomere dynamics, which is also a measure of quality. A decline in reproductive output, could be the result of direct or indirect effects of differences in the quality of breeders (Kwon et al., 2018; Stenseth & Mysterud, 2002), but also other factors such as seasonal timing (Kwon et al., 2018). This could possibly explain the negative correlation between the start of the egg laying period and the clutch size at Vlietbos as higher quality breeding will start laying earlier and will thus have larger clutch sizes. At Fort 4, the quality of the breeders is possible lower, which might explain the negative correlation between clutch size and chicks' survival there. To the best of our knowledge, this is the first study that reports the correlation between PFAA concentrations and the timing of egg laying.

The reduced shell thickness of the eggs may have also resulted in a reduced hatching, especially for nests where at least one egg hatched, since both parameters were negatively correlated with PC1, and overall breeding success. Contaminant-induced eggshell thinning is a major threat to populations of avian species, as it reduces the survival of the embryos and the hatchability (Miljeteig et al., 2012). Studies that relate

eggshell thickness with PFAA concentrations are scarce. Miljeteig et al. (2012) observed no significant association between PFAAs and eggshell thickness in ivory gulls. Similarly, Vicente et al. (2015) observed no associations between PFAA concentrations and egg dimensions in yellow legged gulls. However, concentrations in both these studies were much lower compared to the present study. Eggshell formation is a complex process and disruption of this process, or any of its steps, may lead to alterations in eggshell thickness. Some of the steps in eggshell formation are under hormonal control, indicating that compounds influencing estrogens, androgens and thyroid hormones may alter eggshell thickness (Miljeteig et al., 2012). Nost et al. (2012) reported positive associations between total thyroxin and PFAA concentrations in black-legged kittiwake and northern fulmar chicks, which indicates that PFAAs may affect the hormones that control eggshell formation.

Finally, our results show that reproductive output of great tits were not necessarily related to the distance from the pollution source, as reproduction, in terms of the studied reproductive parameters, tended to be better at Vlietbos and Burchtse Weel compared to the other locations. To the best of our knowledge, only three studies, which all focused on metals, have been performed that associate environmental pollutant concentrations with reproduction in great tits. Similar results were observed by Eeva & Lehikoinen, who did not observe a variation in clutch size, hatching success and egg shell thickness at different distances from a copper smelter in Finland. However, at the same study site, Eeva & Lehikoinen (1996) reported an increased breeding success with increasing distance from the factory complex. Finally, the hatching and breeding success were lower in great tits closer to a large nonferrous smelter in Belgium compared to lesser-polluted sites (Janssens et al., 2003).

Conclusions

Although PFAA concentrations in the area surrounding the fluorochemical plant in Antwerp were among the highest ever reported in wildlife, there were no severe declines in reproductive output of great tits.

Only a few, weak, associations between PFAA concentrations and reproductive parameters, such as hatching success and total breeding success, were observed. Stronger associations have been reported for other species at lower PFAA concentrations, suggesting either a lower sensitivity to PFAAs of great tits compared to other species, or an adaptation to PFAAs contamination. The association with a reduced hatching success, shows the possibility of PFAAs to affect populations of great tits and implies the need for future monitoring of PFAA concentrations in the environment, as well as monitoring of the population dynamics of this species. Nevertheless, other environmental factors and/or other pollutants, which have not been studied in the present study, could also play an important role in explaining the differences in reproduction among sites. Therefore, future research should try to include other important factors that could affect the reproduction of free-living bird species.

The outcome of this study can be used in other monitoring studies that use both minimally invasive sampling (eggs; Furness and Greenwood, 1993) and a species that has demonstrated to be a useful sentinel species for local contamination of Persistent Organic Pollutants (Dauwe et al. 2003, 2007; Van den Steen et al. 2006, 2009). But as the great tit does not seem to be very sensitive to PFAAs (which ideally should be confirmed in additional studies, given that the current study only covers one breeding season), other bird species may be preferable. More research on PFAAs pollution in the vicinity of the fluorochemical plant is necessary to understand 1) the environmental distribution of PFAAs in multiple matrices along the terrestrial food chain, 2) the possible effects at different levels of biological organization both in a wild population, as well as under controlled laboratory conditions.

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Figures and tables

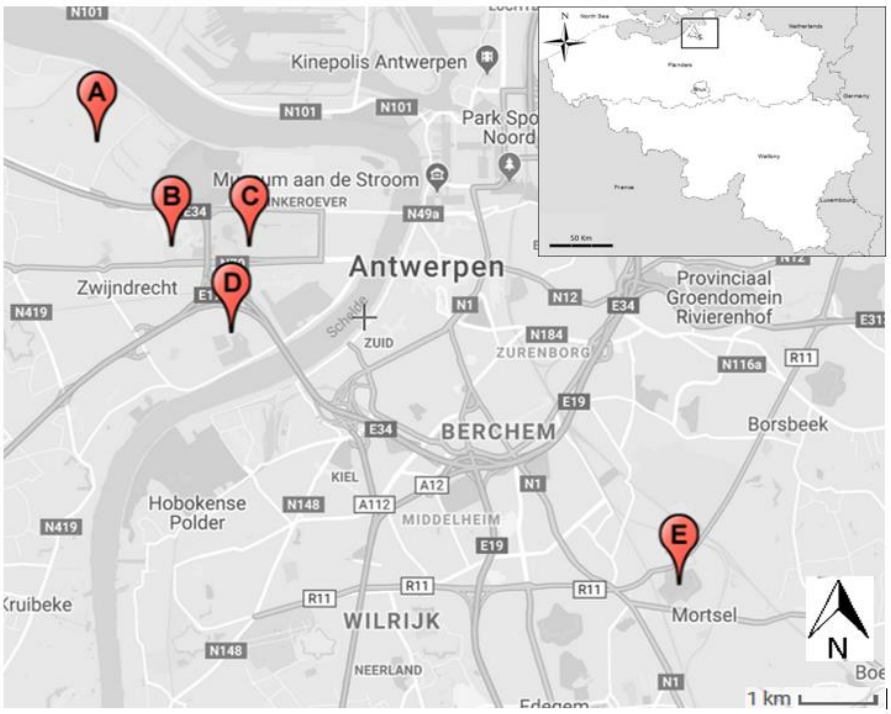
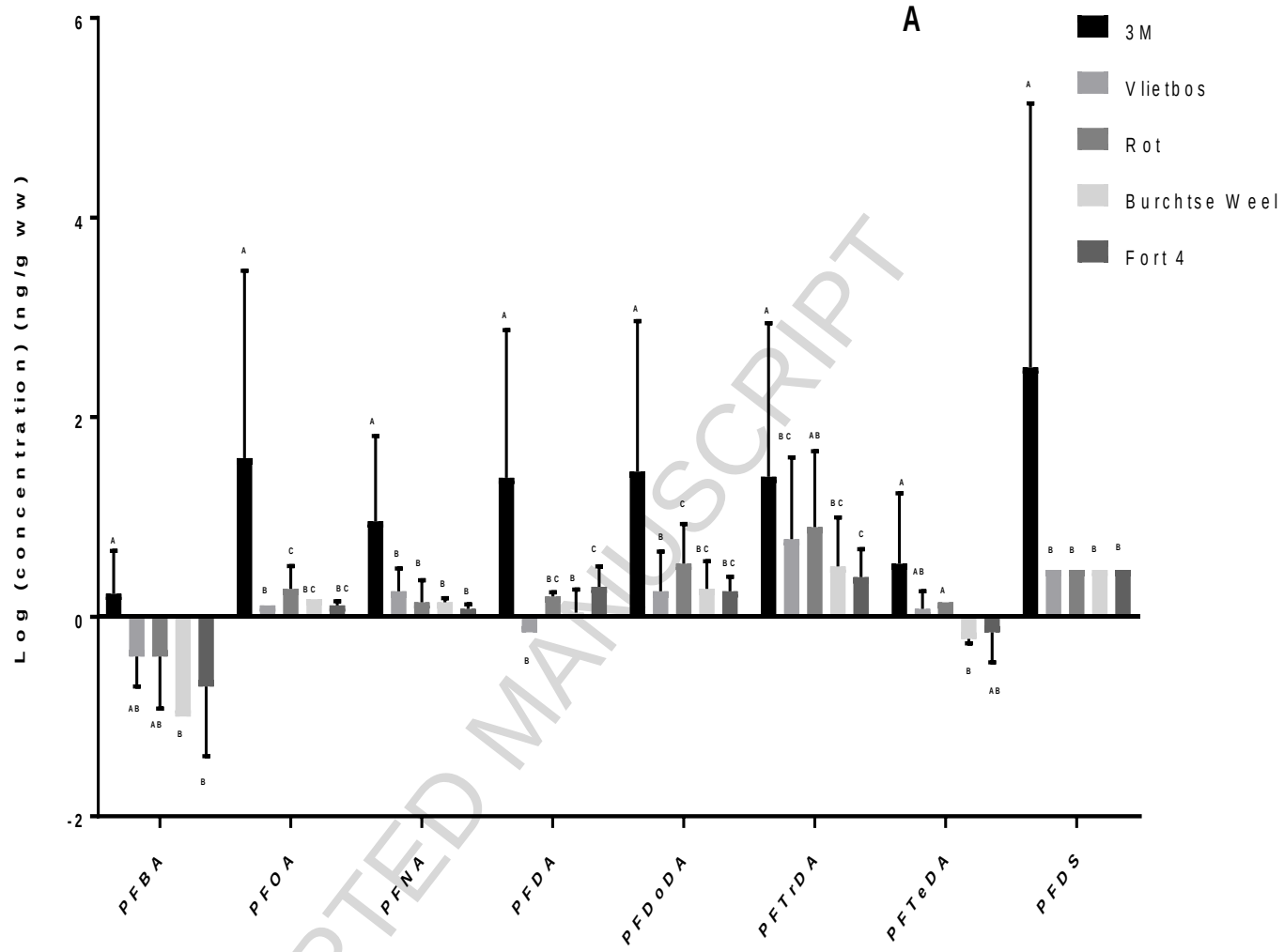


Figure 1. Overview of the study area in Antwerp, Belgium. Sampling locations are indicated as letters: A. Fluorochemical plant 3M, B. Vlietbos, C. Middenvijver-Rot, D. Burchtse Weel, E. Fort 4



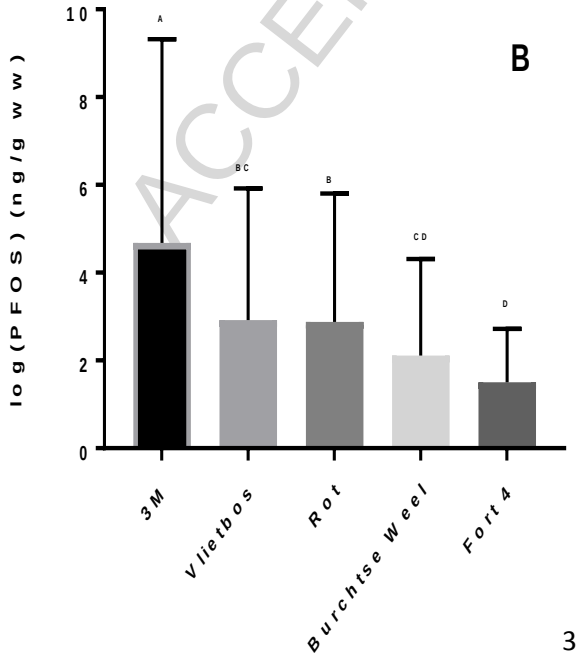


Figure 2. Concentrations of multiple PFAAs at each study site. A) Mean concentrations (logarithmic) of PFBA, PFOA, PFNA, PFDA, PFDoDA, PFTrDA, PFTeDA and PFDS (+ Standard deviation). B) Mean concentrations (logarithmic + standard deviation) of PFOS at each site. Different letters indicate significant differences in PFAA concentrations between the different locations.

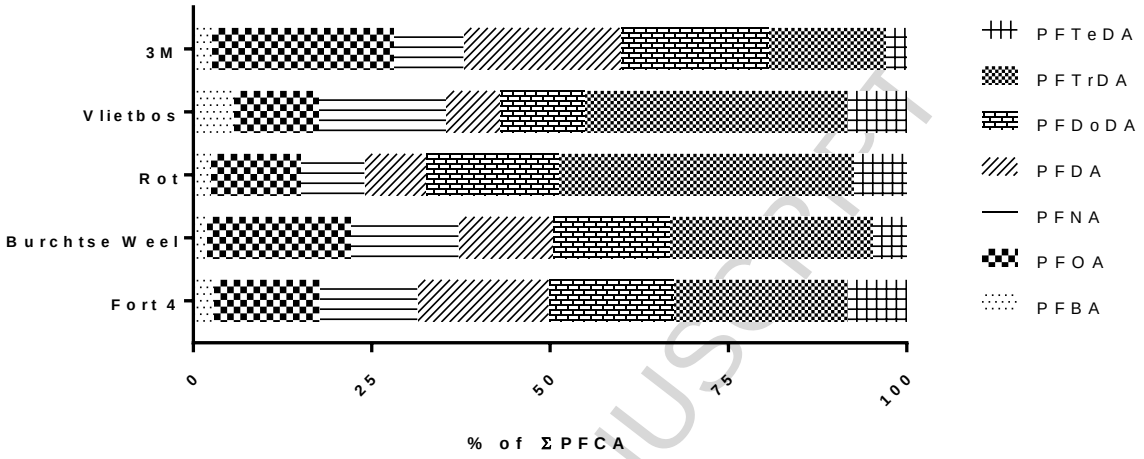


Figure 3. Composition profile of PFCAs in eggs of great tit at the five sampling sites, with increasing distance from the fluorochemical plant of 3M: Vlietbos (1 km), Rot (2.3 km), Burchtse Weel (3 km) and Fort 4 (11 km).

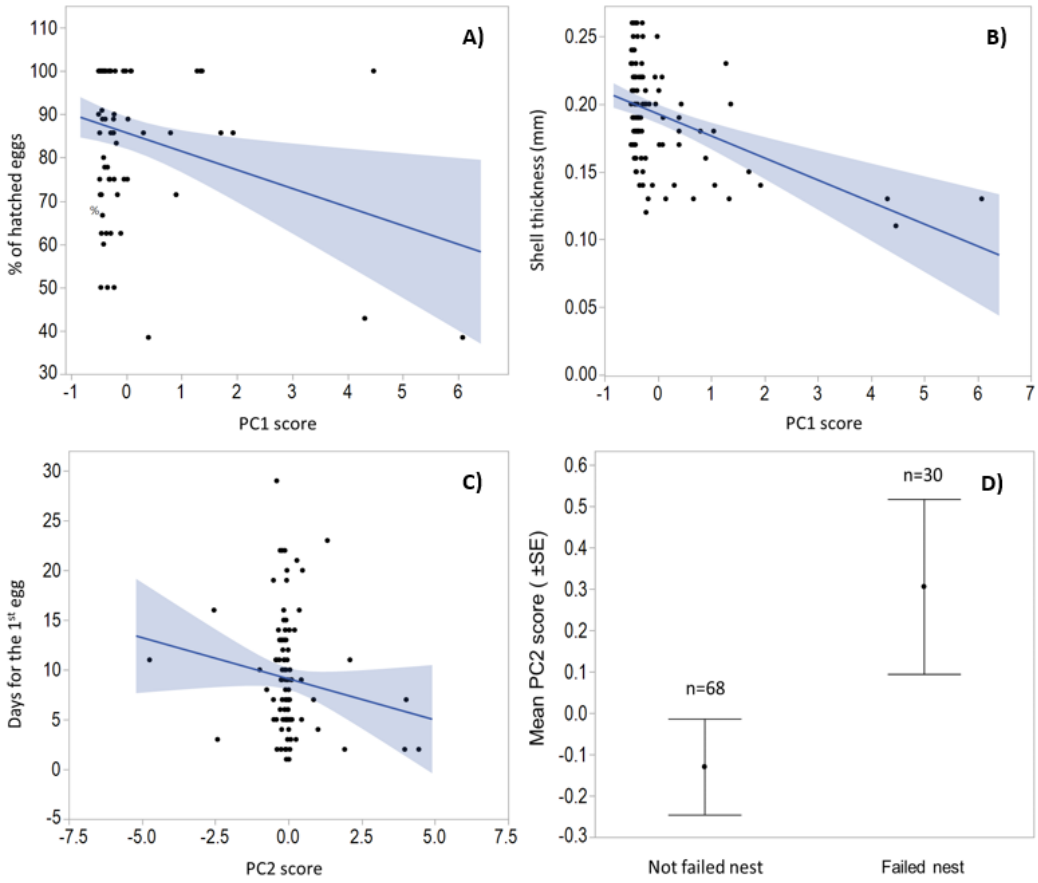


Figure 4. Correlations between the Principal Components (PCs) and reproductive parameters. A) Negative correlation between PC1 factor scores and the percentage of hatched eggs in a nest. B) Negative correlation between PC1 factor scores and shell thickness (mm). C) Negative association between PC2 factor scores and the egg laying date. D) Effects of factor scores of PC2 on reproduction total failure. The blue band represents the 95% confidence intervals of the correlation coefficients.

Table 1. Individual limits of quantification (LOQ: ng/g determined as 10 times the signal to noise ratio), median and mean concentrations (ng/g ww), range (ng/g ww) and detection frequencies (Freq; %) of PFAAs in eggs of great tit at the five sampling sites with increasing distance from the fluorochemical plant of 3M: Vlietbos (1 km), Rot (2.3 km), Burchtse Weel (3 km) and Fort 4 (11 km). Compounds that are not detected (i.e. PFPeA, PFHxA, PFHpA, PFUnDA, PFBS and PFHxS) are excluded from the table.

		PFCAs							PFSAs	
		PFBA	**PFOA**	**PFNA**	**PFDA**	**PFDoDA**	**PFTrDA**	**PFTeDA**	**PFOS**	**PFDS**
LOQ		0.261	0.045	0.586	0.425	0.444	0.256	0.355	2.55	5.92
3M (n = 23)	**Median**	<LOQ	18	7.7	13	18	14	1.3	34251	82
	Mean	1.7	39	9.1	25	29	25	3.4	48056	315
	Range	<LOQ – 11	3.4 – 359	2.1 – 28	1.6 – 102	1.1 – 133	<LOQ – 156	<LOQ – 22	5111– 187032	9.4 – 1489
	Freq	39	100	100	100	100	91	61	100	100
Vlietbos (n = 21)	**Median**	<LOQ	1.3	1.4	<LOQ	<LOQ	4.1	<LOQ	416	<LOQ
	Mean	<LOQ	1.8	1.8	0.7	1.8	6.0	1.2	830	<LOQ
	Range	<LOQ – 1.7	<LOQ – 3.5	<LOQ – 5.7	<LOQ – 4.1	<LOQ – 7.8	<LOQ – 22	<LOQ – 4.1	<LOQ – 4035	<LOQ
	Freq	29	71	71	29	38	76	38	81	0
Rot (n = 18)	**Median**	<LOQ	1.4	1.4	1.4	2.9	6.6	1.2	454	<LOQ
	Mean	0.4	1.4	1.4	1.6	3.4	7.9	1.4	764	<LOQ
	Range	<LOQ – 1.0	0.6 – 8.3	<LOQ – 2.3	<LOQ – 4.0	<LOQ – 12	1.7 – 26	<LOQ – 4.2	207 – 3806	<LOQ
	Freq	39	100	94	78	94	100	83	100	0
Burchtse Weel (n = 16)	**Median**	<LOQ	1.5	1.2	<LOQ	1.6	2.5	<LOQ	87	<LOQ
	Mean	<LOQ	1.4	1.4	1.1	1.9	3.2	0.6	130	<LOQ
	Range	<LOQ	<LOQ – 3.3	<LOQ – 3.7	<LOQ – 5.5	<LOQ – 6.9	<LOQ – 12	<LOQ – 3.6	18 – 690	<LOQ
	Freq	0	81	69	25	63	81	31	100	0
Fort 4 (n = 33)	**Median**	<LOQ	1.2	1.3	1.9	1.6	2.5	0.7	30	<LOQ
	Mean	<LOQ	1.2	1.2	2.0	1.8	2.5	0.7	32	<LOQ
	Range	<LOQ – 0.9	<LOQ – 6.9	<LOQ – 4.5	<LOQ – 5.7	<LOQ – 6.7	<LOQ – 8.3	<LOQ – 2.0	<LOQ – 73	<LOQ
	Freq	27 76	97		73	88	91	67	97	0

Table 2. Mean values and standard deviations for the different reproductive parameters at each location. Different letters indicate significant differences in this parameter between the locations.

Reproductive parameter	**Location**				
	3M	Vlietbos	Rot	Burchtse Weel	Fort 4
Average day 1st egg	11 ± 8A	8 ± 4A	12 ± 6A	8 ± 4A	8 ± 5A
Shell thickness (mm)	0.16 ± 0.03A	0.22 ± 0.03BC	0.19 ± 0.05C	0.23 ± 0.03B	0.19 ± 0.02C
Hatching success[a]	0.80 ± 0.21A	0.92 ± 0.13A	0.78 ± 0.15A	0.89 ± 0.13A	0.87 ± 0.17A
Failure of total hatching (%)[b]	20	5	25	7	45
Breeding success[c]	0.54 ± 0.42AB	0.81 ± 0.31B	0.51 ± 0.42AB	0.83 ± 0.26B	0.30 ± 0.40A
Total failure of reproduction (%)[d]	39	18	41	13	55
Mean chick condition	15 ± 2A	17 ± 2B	15 ± 2A	16 ± 2B	16 ± 2AB
Chicks' survival[e]	0.84 ± 0.34AB	0.93 ± 0.23A	0.83 ± 0.39AB	1.0 A	0.63 ± 0.43AB
Clutch size	9 ± 3 A	9 ± 2 AB	8 ± 2 AB	9 ± 3 A	7 ± 2 B

[a] Number of hatched eggs divided by the number of incubated eggs (we considered only those nests with at least one hatched egg). 3M (N = 16), Vlietbos (N = 19), Rot (N = 12), Burchtse Weel (N = 14), Fort 4 (N = 18).

[b] Percentage of nests where no egg hatched.

[c] Number of fledglings divided by the number of incubated eggs.

[d] Percentage of nests where incubation, hatching or chicks survival failed.

[e] Number of fledglings divided by the number of hatched eggs. Only nests where at least one egg hatched were included.

Table 3. Overview of the results of the GLMz (Wald χ^2, p, beta and standard error of the beta) for the significant PCs at each reproductive parameter assessed.

Parameter	Sig. PC	Wald χ2	p	beta	SE
Days for the 1st egg	PC2	4.660	0.031	-0.063	0.0290
Shell thickness	PC1	22.19	0.00	-0.016	0.0035
Hatching success[a]	PC1	14.58	0.00	-0.270	0.0707
Total failure of hatching[b]	PC2	5.046	0.025	0.595	0.2649
Chicks survival	PC1	4.868	0.027	0.603	0.273
Breeding success[c]	PC2	7.955	0.005	-0.198	0.07
Total failure of reproduction[d]	PC2	3.181	0.074	-0.438	0.246
Egg weight	Not significant				
Egg length					
Egg width					
Chicks' survival[e]					
Mean chick condition					
Clutch size					

[a] Number of hatched eggs divided by the number of incubated eggs (we considered only those nests with at least one hatched egg).

[b] Binomial variable, nests where any egg hatched vs. nests where at least one egg hatched.

[c] Number of fledglings divided by the number of incubated eggs.

[d] Binomial variable, nests where incubation, hatching or chicks survival failed vs nests with at least one fledgling.

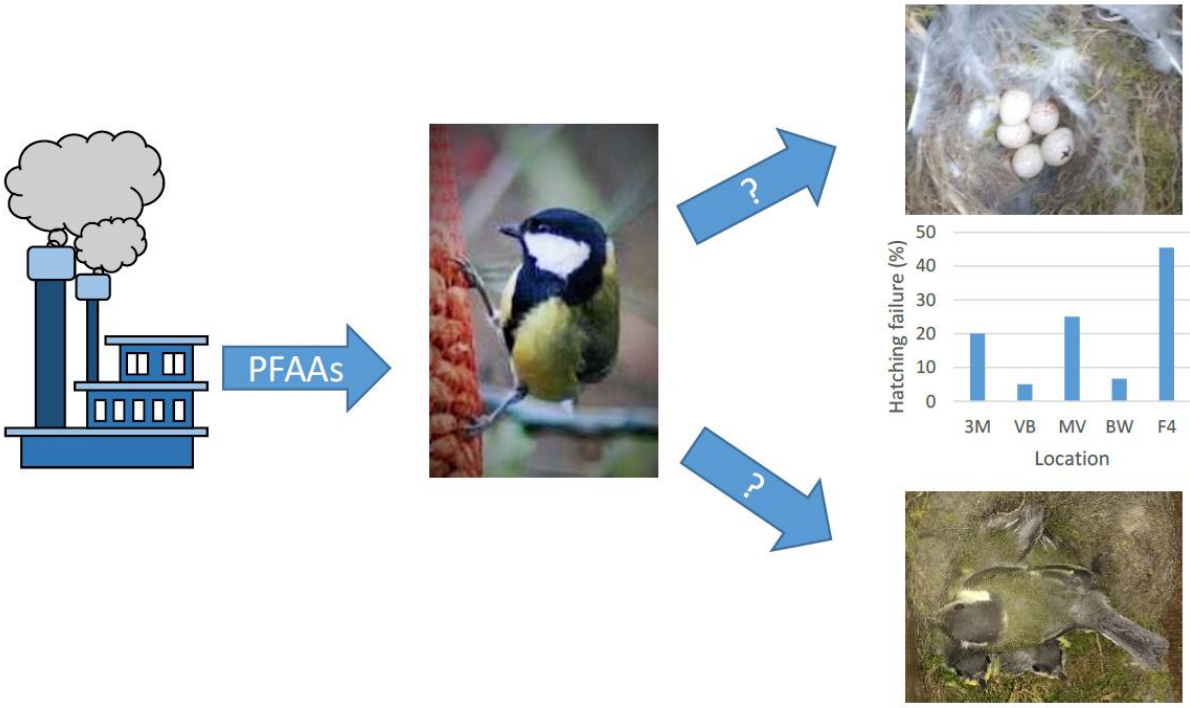
[e] Number of fledglings divided by the number of hatched eggs.

*Table 4. Median PFAA concentrations in eggs (ng/g ww) of passerine birds. * range; NP = concentrations were analyzed but not reported; NA = not assessed. # = concentrations detected at a fluorochemical plant.*

Species	Country	Year	PFHxS	PFOS	PFDS	PFOA	PFNA	PFDA	PFDoDA	PFTrDA	Publication
Corvus frugilegus	Germany	2009	<LOQ	5.3	NA	0.5	2.1	0.8	NA	NA	Rüdel et al., 2011
Paradoxornis webbiana	Korea	2006	1.3	314.1	1.1	0.8	40	114.2	25.6	NA	Yoo et al., 2008
Tachycineta bicolor	USA	2008 – 2009	NP	141	NA	<LOD	NP	5.51	NP	NA	Custer et al., 2012
Tachycineta bicolor	USA	2007 – 2011	0.95	270	NA	18.7	3.10	5.47	1.96	NA	Custer et al., 2014
Parus major[*]	Belgium	2006	NA	19 – 5635	NA	NA	NA	NA	NA	NA	Lopez Antia et al., 2017
Parus major[#]	Belgium	2011	99.3	10380[a]	47.7	19.88	<LOQ	12.0	13.7	5.6	Groffen et al., 2017

[a] Extrapolated concentration.

Graphical Abstract



Highlights

- Data on associations between PFAA concentrations and reproduction remain scarce in wildlife
- Eggs of great tit were sampled along a distance gradient from a fluorochemical plant
- Reproduction of great tits was monitored along the same distance gradient
- PFAA concentrations in the eggs were among the highest ever reported in wild birds
- Despite the high concentrations, there was limited evidence of reproductive impairment