Perfluoroalkyl acids (PFAAs) concentrations and oxidative status in two generations of great tits inhabiting a contamination hotspot

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Abstract

The ubiquity of perfluoroalkyl acids (PFAAs) contrasts with the limited information about their effects. We report here PFAA plasma concentrations in wild populations of great tits (*Parus major*) settled at and in the vicinity of a fluorochemical plant in Antwerp (Belgium). Using two generations we obtained novel results on some poorly known issues such as differences between sexes, maternal transfer of the compounds and potential associations with the oxidative status. For five out of the 13 detected PFAAs, the concentrations were the highest ever reported in birds' plasma, which confirms that Antwerp is one of the main hotspots for PFAAs pollution. Contrary to other studies conducted in birds, we found that females presented higher mean concentrations

and detection frequencies for two compounds (perfluorooctane sulfonic acid (PFOS) and perfluoroundecanoic acid (PFUnDA)) than males. Maternal transfer and the dietary intake appear to be the main route of exposure for nestlings to PFOS but not to other compounds. Finally, PFAA concentrations tended to correlate positively with protein damage in adult birds while in nestlings they positively correlated with higher activity of antioxidant enzymes (glutathione peroxidase and catalase). Experimental work is needed to confirm oxidative stress as a pathway for the pernicious effects of PFAAs.

Keywords: Perfluorinated compounds, sex differences, maternal transfer, oxidative stress, Antwerp.

Introduction

Perfluoroalkyl acids (PFAAs) are highly persistent substances produced and extensively used for more than six decades. Historically, long chain (LC) perfluoroalkyl carboxylic acids (PFCAs; with ≥7 perfluorinated carbons) and perfluoroalkyl sulfonic acids (PFSAs; with ≥6 perfluorinated carbons) have been the most used ones, concretely perfluorooctanoic acid (PFOA, $C_7F_{15}COOH$) and perfluorooctane sulfonic acid (PFOS, $C_8F_{17}SO_3H$). The widespread use of these PFAAs, together with their persistence and bioaccumulation potential, has resulted in a global contamination of the environment, wildlife and humans[1-4].

Since 2000, the widespread distribution and potential health effects of LC-PFAAs (reviewed by OECD[5]), led the industry and regulators to take action by reducing the use and the release of these compounds. In 2002, the 3M company voluntarily phased out the production of PFOS and in 2009 PFOS and related substances were listed under Annex B (restriction of production and use) of the Stockholm Convention on Persistent Organic Pollutants. Other LC-PFCAs and PFSAs have been recently included in the Candidate List of Substances of Very High Concern for Authorization under the European Chemicals Regulation (REACH[6]). Due to these actions, a transition is taking place in the industry to replace LC-PFAAs with short chain (SC) PFAAs and polyfluorinated substitute compounds[7,8]. However, but for many of these alternatives, information on actual releases and exposures is missing. Moreover, their risks and potential toxicity to various biota remains largely unexplored[7-10].

Previous studies on the bioaccumulation and effects in birds[11-16] have been conducted near the 3M fluorochemical plant in Antwerp using great tits and blue tits (*Cyanistes caeruleus*), lapwing (*Vanellus vanellus*) and the Mediterranean gull (*Larus melanocephalus)*. These studies have revealed the highest PFOS concentrations ever found in wildlife (e.g. mean concentration of 48056

ng/g found in the eggs of great tits breeding at the fluorochemical plant[15]). Furthermore, concentrations of other PFSA such as perfluorodecane sulfonic acid (PFDS) and perflurohexane sulfonic acid (PFHxS) and concentrations of PFOA were also the highest reported in bird eggs[13,15].

Previous studies performed in birds described negative effects of PFAAs on reproduction[15,17], chick survival[17] and the immune system[18,19]. The oxidative status of individuals could be used as an indicator of the pernicious effects of PFAAs[20]. Immune system cells or sperm cells are vulnerable targets to the oxidative damage produced by many pollutants[21]. Furthermore, organisms might need to use dietary antioxidants to deal with oxidative stress (OS), which causes an imbalance of the trade-off in the allocation of these substances among physiological functions (e.g. reproduction, sexual signalling[21,22]). Therefore, studying the oxidative status is a key element in toxicological studies. Nevertheless, not much is known about PFAAs effects on birds' antioxidant system. The study of the transcriptional response of chicken hepatocytes exposed to PFOS pointed to OS as a cause of gene alteration[23]. Similarly, wild common cormorants (*Phalacrocorax carbo*) livers, naturally exposed to PFAAs, presented an altered transcriptional response of genes involved in the antioxidant system[24]. Despite this, a study performed in white-tailed eagle (*Haliaeetus albicilla*) nestlings, did not find any relationship between PFAA concentrations and the activity of the antioxidant enzyme superoxide dismutase (SOD) in plasma[25].

In the present study we examined plasma concentrations and the composition profile of fifteen PFAAs (11 PFCAs and 4 PFSAs) in wild populations of great tits (*Parus major*) settled along a distance gradient of 11 km from an active fluorochemical plant in Antwerp (Belgium). We studied differences in PFAA concentrations and composition profile along the gradient. We also examined the association between the measured PFAAs concentrations and the body condition and the OS status of the birds. Moreover, we sampled adult birds, their eggs and their nestlings, which enabled

us to explore the maternal transfer of PFAAs to the offspring[17,26]. The outcome of this study will reveal the current exposure status of wildlife to PFAAs in one of the main hotspots in the world. It will also improve our understanding of OS as a potential underlying mechanism for pernicious effects of PFAAs and predicting the exposure consequences for wild bird populations.

2. Material and methods

2.1. Sample collection

Nestboxes were placed during autumn of 2015 at five sampling sites, representing a distance gradient from a fluorochemical plant in Antwerp (Figure S1). These sites were the fluorochemical plant (25 nestboxes), Vlietbos (22 nestboxes; 1 km SE from the plant), Rot-Middenvijver (shortly Rot; 18 nestboxes; 2.3 km ESE from the plant), Burchtse Weel (19 nestboxes; 3 km SE from the plant) and Fort 4 in Mortsel (31 nestboxes; 11 km SE from the plant).

The first blood sampling period was performed before the start of the breeding season between the 8th of February and the 9th of March of 2016. During this period, all nestboxes were visited after sunset and roosting birds were captured. Captured birds were ringed (if not already ringed), tarsus length and body mass were measured and age (yearlings versus older) and sex were determined following Svensson[27]. Body condition was calculated according to the scaled mass index[28]. We also took a blood sample (maximum 150 μL) from the brachial vein using microhaematocrit heparinized capillary tubes (Microvette®). These samples were kept refrigerated and centrifuged at 10,000 × *g* for 10 min at 4°C to separate plasma from the red blood cells (RBC), which were stored separately at -80°C for later analysis. The number of sampled birds was 79 (between 13 and 18 per location).

From just before egg laying until incubation, nestboxes were checked every other day or daily to determine the start of the egg-laying period. From each nest, the third egg was collected before the incubation started. Later, in the nestling period, the second blood sampling was performed, in May and June 2016. When nestlings were 10 days old, parents (mostly the female) were captured inside the nestbox, using a trap door in the entrance hole, and we proceeded as was explained above. In this way we sampled 60 birds (45 females and 15 males). Finally, when nestlings were 14 days old, all nestlings in each nest were ringed, measured (tarsus length and body mass) and a blood sample was taken. A total of 441 nestlings from 79 nests were sampled, from which 179 samples were selected for PFAAs and OS parameters analyses: 1) we selected 2 nestlings per nest (the lightest and the heaviest); 2) we selected one complete brood per site. A small portion of nestlings' RBC (≈1μL) was used to determine the sex genetically following the method described by Griffiths et al.[29] with minor modifications[30].

Due to great tits being highly resident with birds staying close to or in their breeding area during the winter, we have repeated measurements from 18 individuals (birds sampled both in winter and in the nestling period).

2.2. PFAAs analysis in plasma

The used abbreviations for PFAA compounds are according to Buck et al.[31](Table S1). Eleven PFCAs (PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFDoDA, PFDTrDA and PFTeDA) and 4 PFSAs (PFBS, PFHxS, PFOS and PFDS) were selected as target analytes. A mixture of isotopically mass-labelled internal standards (ISTDs) were used (supplementary material).

Sample extraction

Samples were extracted by using a solid-phase extraction technique, which is based on the chemical principle of weak-anion exchange described by Groffen et al.[15] (supplementary material). Briefly, 80 µL of isotopically mass-labelled internal standard mixture and 10 µL of acetonitrile was added to each sample (10 µL of plasma / ~0.4g of homogenized egg). After sonication, samples were left overnight on a shaking plate. After centrifugation the supernatant was transferred into a 14 mL tube and loaded on HR-XAW columns.

UPLC-TQD analysis

UPLC coupled tandem ES(-) mass spectrometry (ACQUITY, TQD, Waters, Milford, MA, USA) was used to analyse PFAAs.

Individual LOQs of the detected compounds are shown in Table S2 (Table S3 those compounds with detection frequency < 20%). Based on the ratio of the mean ISTD area of the sample over the area of a blank ISTD solution, recoveries were determined. PFBS and PFHxS recoveries were too low and therefore they were excluded from further analysis. Further details about the analysis conditions, the calibration method and the quality assurance can be found in the supplementary material and in Groffen et al.[15].

2.3. Antioxidant and oxidative stress parameters measurement in red blood cells

Total antioxidant capacity (TAC) was estimated by ferric ion reducing antioxidant power (FRAP) assay[32]. Reduced and total glutathione (GSH) was detected using a reversed-phase HPLC of Shimadzu (Shimadzu, 's Hertogenbosch, The Netherlands[33]). The ratio between GSH and the oxidized form (GSSG) was used as an index of redox state[34]. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) were determined by homogenizing haemolysates of red blood cells of 0.01 M PBS buffer (pH 7.4) contained 1.15% KCl and 0.02 M

149 EDTA[35]. SOD, CAT and GPX activities were determined by measuring the decrease in nitroblue
150 tetrazolium (NBT) reduction[36], H_2O_2[37] and NADPH contents[38], respectively. Protein carbonyls as
151 oxidative damage markers was measured using Protein Carbonyl Colorimetric Assay Kit by
152 Cayman Chemical's (Ann Arbor, MI, USA; see also Levine et al.[39]). All above-mentioned
153 parameters have previously been successfully quantified in the great tit[40]. Detailed protocols can
154 be found in the supplementary material.

155 *2.4. Statistical analysis*

156 To perform statistical analyses we used JMP Pro 14. In each location, we only considered those
157 compounds with detection frequency ≥ 50% , values below LOQ were replaced by LOQ/2[17,41]. All
158 PFAA concentrations were log transformed to obtain a normal distribution. Temporal data from
159 adult birds were pooled together (adults from both the late winter and the spring) and analysed
160 separately from nestlings' data. To study the maternal transfer we used a database that included
161 data from mothers captured in the nestling period (spring) and data from their eggs and their
162 offspring.
163 We performed linear mixed models to compare the concentrations of the different PFAAs and
164 levels of different oxidative stress parameters among locations. For adult birds, we included the
165 location, sex and age of the bird, the sampling period and the interactions between them as factors
166 and we followed a backward elimination. We included bird identity (determined by ring number)
167 as a random effect. To calculate the mean, median, range and detection frequency values of PFAA
168 compounds in adults in each sampling site (Table S2), each bird was only considered once (the
169 winter measurement in case the bird was captured both in winter and in spring). For nestlings, we
170 included nest identity (determined by nestbox number) as a random effect and we included clutch
171 size as a factor in the models. To compare the detection frequency of the different PFAAs among

locations we performed a Generalized Linear Model (GLMz) with binomial distribution and we proceeded as for the concentrations but only including each bird once. When significant results were found ($p \leq 0.05$) post-hoc analyses (Tuckey test) were conducted for pairwise comparison. To compare the distribution of single PFAA compounds in the mothers, their eggs and the offspring, data were treated using methods of survival analyses for left-censored data, i.e. reverse Kaplan-Meier method[42,43]. To compare ∑PFAA and ∑PFCA concentrations and to test for effects of the body condition on PFAA concentrations we used an ANOVA analysis including the type of sample as a factor and the body condition as covariate. Relationship between compound concentrations in each location and relationships between mothers', eggs' and nestlings' concentrations were investigated using Spearman's correlation test.

To study the relationship between PFAAs and OS parameters or body condition, firstly, in order to reduce the number of covariates and to account for collinearity among them, we conducted Principal Component Analysis (PCA). In this analysis we included all those compounds with a detection frequency ≥ 20% (7 compounds for adults and 4 compounds for nestlings; Table S4), for these compounds values below LOQ were replaced by LOQ/2. The number of significant principal components was selected according to the Kaiser criterion (i.e. eigenvalue higher than 1[44]). Two Principal Components (PCs) were selected for adults (hereafter adults-PC1 and adults-PC2) and one for nestlings (hereafter nestlings-PC1). Each compound loading and variance explained by each PC are shown in Table S4. Adults-PC1 explained 62% of the variance and adults-PC2 explained a further 19%. Nestlings-PC1 explained 73% of the variance.

We performed linear mixed models for each OS parameter and for the body condition of the birds. OS parameters were log transformed to obtain a normal distribution when necessary. For adults, the age and sex of the bird, the season, the PCs and the interactions between them were included

as explanatory variables and followed a backward elimination, while including the ring number as a random effect. For nestlings we proceeded as for adults but including the nestbox number as random effect.

3. Results

3.1. *PFAA concentrations, detection frequencies, correlations and profiles in adult birds*

PFOS concentration in the plasma of adult birds decreased significantly with the distance from the plant (all $p<0.001$;Figure 1, Table S2). PFDoDA concentration at the plant site was significantly higher than at the other sites ($p<0.001$). For PFOA there is a season-dependent site effect (site*season interaction $p=0.05$), with significantly higher concentrations at the plant ($p<0.0001$) but only in winter. There was not significant difference between sites for PFUnDA ($p>0.13$; Figure 1).

For PFOS ($p=0.01$) and PFUnDA ($p=0.02$), females had significantly higher concentrations than males (Figure S2). This sex effect was independent of the sampling site (site*sex interactions $p>0.09$) but it was more apparent at 3M where mean concentrations (± SE) were (females / males): PFOS 94153 ± 33531 / 46337±17596 pg/uL, PFUnDA 21.3 ± 6.2 / 12.3 ± 4.1 pg/uL. The age of the birds did not affect the concentrations of PFAAs.

Differences between periods were found for PFOA and PFDoDA (all $p<0.01$). Concentrations (mean ± SE) were higher in winter for PFOA (winter=60.2±4.2 pg/uL, spring=42.2±5.2 pg/uL; these differences were significant in Vlietbos and Fort 4 (both $p\leq0.003$)) whereas for PFDoDA higher concentrations were found in spring (winter=6.1±1.2 pg/uL, spring=9.6±2.9 pg/uL) regardless of the sampling site (site*season interactions $p=0.54$).

Regarding the detection frequencies of the different PFAA compounds (Table S2), PFOA was found above it LOQ at 99% of the samples. PFOS was detected above its LOQ at 72% of the

samples. Significant differences existed in the detection frequency of PFOS among locations ($p<0.0001$) appearing less frequently at Burchtse Weel (60%) than at the plant and Vlietbos (100%), and at Fort 4 (25%) than at all the other locations. PFOS detection frequency was higher in females (80.0%) than in males (62.3%; $p=0.03$) at all sampling sites (site*sex interaction $p=0.85$). PFUnDA overall detection frequency was 48% and significant differences existed among locations ($p\leq0.01$) with lower detection frequency at Burchtse Weel (32%) and Fort 4 (26%) than at Vlietbos (62%) and Rot (70%) and similar than at the plant (50%). PFUnDA appeared more often in female (61%) than in male (32%) birds ($p=0.001$) regardless of the sampling site (site*sex interaction $p=0.38$). PFDoDA overall detection frequency was 63% with no significant differences among locations. PFDoDA was detectable more often ($p=0.001$) in spring (80%) than in winter (52%). Detection frequencies of PFNA (overall 27%), PFDA (overall 24%) and PFDTrDA (overall 33%) were only at the plant site ≥50% (70, 50 and 55% respectively).

An overview of the correlations found among compounds (with a detection frequency of ≥ 50%) at the different locations is given in Table S5. When a compound was < LOQ it was substituted by LOQ/2. Almost all the compounds were correlated with each other at the plant site except for the following pairs: PFOA/PFUdA, PFOA/PFDTrDA, PFNA/PFDTrDA and PFDA/PFTrA. By contrast, no correlations were found for Vlietbos. At Rot PFDoDA was significantly correlated with PFUnDA and PFOS and at Burchtse Weel PFOA and PFOS were significantly correlated.

The PFAAs profile was clearly dominated by PFOS at the plant (93% of the PFAAs) but this percentage decreased with the distance from the plant to only 30% at Fort 4. On the other hand, the contribution of PFOA to the total of PFAAs increased from 1% at the plant to 41% at Fort 4. The PFCAs profile was dominated by PFOA at all the locations (from 46 to 58%) followed by PFDA at the plant site and by PFUnDA at the other locations (Table S2).

241 For PFHpA and PFDS, concentrations in all samples were below the LOQ (7.4 and 5.1 pg/µL
242 respectively). Moreover, values above the LOQ were only detected in four samples for PFHxA
243 (range 8.5 – 9.7 pg/µL), five samples for PFBA (9.4 – 133.2 pg/µL), seven samples for PFTeDA
244 (1.4 – 2.4 pg/µL) and 15 samples for PFPeA (52 – 202 pg/µL). LOQ and detection frequencies of
245 these compounds in each sampling site are shown in Table S3.
246
247 3.2. *Spatial PFAAs contamination in nestlings*

248 Mean concentrations of PFAAs found in nestlings are shown in Figure 1 (median concentrations,
249 LOQs, ranges and detection frequencies shown in Table S6).
250 Significantly different concentrations were found among locations for all compounds (all $p<0.000$;
251 Figure 1) and post hoc analysis revealed that differences occurred between the plant and all the
252 other locations. No significant differences in concentrations of PFAAs between sexes were found
253 (all $p>0.185$). Clutch size did not have a significant effect on nestling concentrations (all $p>0.08$).
254 For PFOS, among nests, concentrations varied up to 58-fold at the plant, 143-fold at Vlietbos, 9-
255 fold at Rot, 11-fold at Burchtse Weel and 6 fold at Fort 4. For ∑PFCAs, concentrations varied up
256 to 15 fold at the plant and around 3-fold at the other locations. The maximum variation of PFOS
257 concentrations within nests was similar at the plant, Vlietbos and Burchtse Weel (around 4-fold)
258 and slightly higher at Rot (7-fold) and at Fort 4 (6 fold). For ∑PFCAs, the maximum variation
259 within nest was around 3-fold at the plant site and Vlietbos and around 2-fold at the other locations.
260 PFOA (LOQ: 2.9 pg/ml) was detected in all samples whereas PFOS (LOQ: 46.6 pg/ml), PFDoDA
261 (LOQ: 1.8 pg/ml) and PFBA (LOQ: 6.5 pg/ml) were detected in 61%, 34% and 20% of the
262 samples, respectively. Differences exist in the detection frequencies of these compounds among
263 locations (all $p<0.001$;Table S6). The detection frequency of PFOS decreased with the distance

264 from the plant because many samples fell below the LOQ. Like in adults, PFOS appeared more
265 often in females than in males (p=0.014), mainly due to the differences found at Rot (p=0.005).
266 All the studied compounds in nestlings from the plant were correlated with each other (Table S7).
267 PFOA was correlated with PFOS at Vlietbos but not at Rot.
268 PFOS dominated the PFAAs profile at the plant (98%) and at Vlietbos (70%) but at Rot it
269 represented only 47% of the ∑PFAAs, exactly the same as PFOA. PFOS ratio decreased in farther
270 locations where PFOA was the dominant compound. Regarding the PFCAs profile, PFOA was the
271 dominant compound at all the locations (ratio range from 81 to 91%).

272 PFPeA, PFHxA, PFHpA, and PFDS concentrations were below their respective LOQ in all the
273 samples. For PFNA we found 16 samples with concentrations above the LOQ (range 4.70 - 18.4
274 pg/μL), with 15 of these samples belonging to nestlings sampled at the plant. Moreover, these
275 nestlings belonged only to 8 nestboxes (out of 14 with nestlings at the plant). Similarly, for
276 PFDTrDA we found 11 samples above the LOQ (2.92 - 12.1 pg/μL), most of them at the plant
277 site. We found 10 samples above the LOQ for PFDA (6.9 - 14.7 pg/μL) and PFUnDA (8.6 - 24.7
278 pg/μL). Finally, we found three samples with concentrations above the LOQ of PFTeDA (1.7 - 6.7
279 pg/μL): LOQ and detection frequencies of these compounds in each sampling site are shown in
280 Table S8.
281

282 3.3. *Relationship between mothers, eggs and offsprings concentrations*

283 Concentrations in females plasma in spring (mothers), and in the plasma of offspring (the heaviest
284 and the lightest nestlings in the nest) were compared (Table S9, Figure S3). We only compared
285 those compounds with a detection frequency ≥ 50% in at least one of the sampling sites (i.e. PFOA,
286 PFDoDA and PFOS). PFOA concentrations were significantly higher in the nestlings than in the

287 mothers ($p<0.001$). PFOS concentrations were higher in the mothers than in the nestlings ($p\leq0.04$).
288 No differences between sample types were found for PFDoDA. ∑PFAA and ∑PFCA
289 concentrations were higher in the mothers than in the nestlings ($p<0.01$ and $p<0.0001$
290 respectively). No significant differences were found in the concentration of any compound
291 between siblings. Body condition did not explain the differences between mothers and offspring
292 for any of the compounds (all $p>0.14$).
293 Correlations were studied between mother, the third egg and offspring concentrations. We found
294 significant correlations in ∑PFAA concentrations between mothers and eggs ($p<0.001$; $r=0.52$),
295 mothers and offspring (all $p<0.0001$; $r\geq0.70$) and between siblings (the heaviest and the lightest;
296 $p<0.0001$; $r=0.71$), but not between eggs and nestlings (all $p>0.11$; $r<0.26$). Very similar
297 correlations were found in PFOS concentrations with slightly higher correlations between eggs
298 and nestlings (both $p=0.06$, $r=0.31$; Figure 2). PFOA concentrations in mothers were correlated
299 with the concentration in the eggs ($p<0.01$; $r= 0.41$) but no correlations were found between
300 mothers/eggs and offspring, nor between siblings. For PFDoDA and ∑PFCAs no correlations were
301 found.
302
303 3.4. *Correlation of PFAA concentrations with body condition and the oxidative status*
304 Body condition and OS parameters' results at the five sampling sites for adults and nestlings are
305 shown in Table S10 and S11 respectively (differences between sites on these parameters are also
306 indicated).
307 Adults-PC1 was mainly influenced by PFOS, PFDA, PFNA, PFOA, PFUnDA and PFDoDA; high
308 concentrations of these compounds corresponded with high values of adults-PC1. Adults-PC2 was

mainly influenced by PFDTrDA, with high values of aduts-PC2 mainly indicating high concentrations of PFDTrDA (Table S4).

A trend existed in adult birds to present higher levels of protein carbonyls with higher values of Adults-PC1 ($p=0.08$) and Adults-PC2 ($p=0.07$; Figure S4). This means that birds with higher concentrations of PFAAs tended to have also higher oxidative protein damage. There was also a significant effect of the sampling season in protein carbonyls' concentrations ($p<0.0001$) with higher concentrations in winter, although the interaction between Adults-PCs and the season was not significant ($p\geq0.26$). We did not find significant correlations (all $p>0.14$) between Adults-PCs and the body condition or between Adults-PCs and the other measured stress parameters (GSH and GSSG concentrations or the ratio between them, SOD, CAT and GPX activity or the measurement of the TAC) in adult birds.

Nestlings-PC1 was influenced by PFOS, PFOA, PFDoDA and PFBA: therefore high concentrations of these compounds corresponded with high values of nestlings-PC1.

Nestlings' GPX activity was positively correlated with Nestlings-PC1 ($p=0.006$; Figure 3) and the body condition of the chick ($p=0.007$). There was also a marginally significant result in the interaction between Nestlings-PC1 and the sex ($p=0.06$). When we performed a separate analysis for males and females, the relationship between Nestlings-PC1 and GPX activity was only significant in females ($p=0.002$; Figure 3) with increased enzyme activity detected in higher exposed females.

Nestlings' CAT activity was positively correlated with Nestlings-PC1 ($p=0.05$; Figure 3) , body condition ($p=0.012$) and marginally affected by the sex of the chick ($p=0.06$), with higher activity in females. We did not find significant correlations between PFAA concentrations and the other stress parameters or the body condition in nestlings (all $p>0.23$).

4. Discussion

4.1. *PFAA concentrations in adults and nestlings*

Concentrations found in this study are, like in previous studies performed in the area[11-15,45], among the highest ever reported in wildlife. According to previous studies[11-15], a pollution gradient was detected for PFOS but this decrease was not so evident for other PFAA compounds[13,15]. Considering the literature on plasmatic PFAAs concentrations in birds (Table S12) it is evident that the entire study area is influenced by the presence of the fluorochemical plant[11,13,15].

For five of the detected compounds (PFBA, PFOA, PFDA, PFDoDA and PFOS) concentrations found in the present study (in adults and in most cases also in nestlings) were the highest ever reported in birds' plasma. Concentrations of other four compounds (PFNA, PFUnDA, PFDTrDA and PFTeDA) were only surpassed by concentrations found in bald eagle (*Haliaeetus leucocephalus*) nestlings sampled in the upper mid-west of the USA[46], a region with several sources of PFAAs, including a 3M fluorochemical plant. PFDA concentrations in nestlings of the present study were also surpassed by those found in bald eagle nestlings[46]. PFAA compounds are highly bio-accumulative (especially LC ones) and, at similar exposure condition, higher concentrations would be expected in a top predator (bald eagle) compared to a small passerine (great tit).

We only found two studies that measured PFAA concentrations in blood of passerine species. In a study by Custer et al.[26] tree swallow (*Tachycineta bicolor*) nestlings that hatched very close to a US 3M fluorochemical plant were analysed. Compared to that study, concentrations in the nestlings hatched at the Antwerp 3M plant were higher for all the compounds measured in both studies (PFOA, PFNA, PFDA, PFUnDA, PFDoDA and PFOS). Moreover, even when comparing concentrations in tree swallows with the ones we found in the nestlings from Fort 4 (10 km away

355 from the plant), they were higher for all the compounds but PFOS in the present study. Remarkable
356 is the difference in PFOA concentrations, which were around 50 times higher in our study.
357 The second study that measured PFAAs in blood of passerine birds was by Dauwe et al.[12] who
358 measured PFOS concentrations in adult great tits in 2005. The study area was the same as in the
359 current study but birds were only sampled in Vlietbos, Rot and Burchtse Weel (concentrations
360 ranges were 173-1625, 154-234 and 24.3-123 pg/μL respectively). Whole blood was used as the
361 matrix. When comparing both studies we found that the mean concentrations of PFOS were higher
362 in the present study: 3-, 1.2- and 1.7-fold higher at Vlietbos, Rot and Burchtse Weel respectively.
363 These differences are very probably due to the different matrix used, whole blood vs plasma. It
364 has been calculated that concentrations measured in whole blood are 2 to 5-fold lower than in
365 plasma[47]. Taking this into account, the concentrations we found are very similar to the ones found
366 by Dauwe et al.[12]. This, or even a decrease in the concentrations, was expected as 3M plant has
367 phased out PFOS production since 2002. Other studies performed in the same area have detected
368 a decrease in PFOS concentrations measured in great tit eggs[13] from 2006 to 2011 and also in wood
369 mice liver from 2002 to 2006[45]. In other places in Europe and USA the same decrease has been
370 detected since 2000 in several bird species[46,48-50].
371 PFAA concentrations and profiles found in the present study appear to correspond with both a high
372 historical contamination, with high concentration of PFOS and LC-PFCA, and a recent
373 contamination, with the presence of SC-PFCAs such as PFBA and PFHxA, all related to current
374 fluorinated compounds production (as final compounds, degradation products or impurities[7]). In
375 the future, the analytical method should be improved to increase the recoveries, and thus the
376 detection possibilities, of SC-PFSAs in blood, including PFBS and PFHxS. Also other currently
377 used per- and polyfluoroalkyl substances such as 3H-perfluoro-3-[(3-methoxy-propoxy)propanoic

acid], ammonium salt (ADONA) or dodecafluoro-2-methylpentan-3-one (3M™ Novec™ 1230) should be included in future analyses.

A remarkable result is the higher concentrations and detection frequencies of PFUnDA and PFOS found in adult females compared with males (Figure S2), although most pronounced at the plant site, these differences were consistent at all sites and in both sampling periods. Most of the studies on PFAA concentrations in birds did not observe differences between sexes (reviewed in Sturm and Ahrens[51]), and the ones that did always have reported higher concentrations in males[52-55]. Moreover, in two studies performed previously in great tits' blood[12] and liver[11] in the same area, no differences in PFOS concentrations between sexes were found. In general for PFAA compounds, as for other contaminants, females could present lower concentrations due to the excretion of these compounds through the eggs[56]. We know that female great tits actually excreted PFOS through the eggs, as very high concentrations of PFOS were detected in the eggs analysed in this study. On the other hand, no PFUnDA concentrations were found in those eggs which can sometimes be due to low exposure and modest detection limits

In mammals, sex differences in the elimination half-life of some PFAA compounds have been observed, and the elimination is not always faster in females[57]. The reason for the differences in elimination is not well understood but some studies pointed to a hormonal regulation of the elimination[57]. These sex differences could also be explained by behavioural reasons such as differences in foraging strategies[58], or ecological reasons such a greater longevity and thus higher accumulation in females. Further research is therefore necessary to better understand the sex differences and their consequences.

4.2. *Relationship between mothers, eggs and offspring concentrations*

401 The distribution of PFOS in mothers, their eggs and nestlings, and the fact that concentrations in
402 mothers and nestlings (and to a lesser extent in eggs and nestlings) correlated with each other, are
403 suggesting that the main exposure of nestlings to this compound is through maternal transfer and/or
404 the diet (provided by the parents). The transfer of PFOS from females to the eggs was previously
405 described in birds[16,17,54,59] but as far as we know this is the first study that correlates plasmatic
406 concentrations in the mother with plasmatic concentrations in the offspring. On the other hand, for
407 ∑PFCAs, the lack of correlation between mothers, eggs and nestlings, and even between siblings,
408 could be indicating that maternal transfer or the diet is not the main route of exposure for these
409 compounds. Moreover, higher concentrations of PFOA (~1.6 times) and PFBA were found in the
410 offspring while ∑PFCAs were higher in the mothers (Table S9, Figure S3). These differences in
411 PFCAs profile could be explained in two non-exclusive ways: 1) Mothers and offspring were
412 exposed differently during the nestling period. 2) Birds were exposed to precursor substances (e.g.
413 fluorotelomer alcohols) and these compounds follow different biotransformation pathways in
414 adults and nestlings[60]. This second hypothesis is supported by correlations found between PFNA
415 concentrations in the mothers and PFOA concentrations in the offspring and between LC-PFCAs
416 (PFOA, PFDA and PFDTrDA) in the mothers and PFBA in the offspring. Future studies to be
417 performed in this hot-spot should include the study of "precursors" together with the study of
418 PFAAs.
419
420 4.3. *Associations between PFAA concentrations and the oxidative status*
421 In adult great tits, a trend existed for more exposed birds to have higher levels of protein damage
422 (measured as protein carbonyls). This could mean that the antioxidant defences failed in
423 neutralizing the extra reactive oxygen species (ROS) generated because of the pollutants, and thus

oxidative damage occurred. Similarly, a recent study performed in Artic black-legged kittiwakes (*Rissa tridactyla*)[61] found that high blood levels of protein damage were associated with high plasma concentrations of certain LC-PFAA compounds (i.e. PFDoDA, PFTriA and PFTeDA). Additionally, they found negative associations between the non-enzymatic antioxidant capacity (i.e. vitamins, carotenoids, glutathione) of these birds and high plasma concentrations of other LC-PFAAs such as PFUnA, PFTeDA or PFOS. .

In nestlings, we detected a positive correlation between PFAAs load and antioxidant defences. More exposed nestlings presented higher activity of GPX and CAT enzymes, both part of the first line of defence against ROS. Their increased activity seemed to efficiently neutralized ROS, as no changes in other endogenous antioxidants (glutathione) or oxidative damage were detected.

Due to the presumable long duration of the physiological stress[62] and the susceptibility of early stages of life to oxidative damage[63], detrimental effects could occur in these birds. We also must consider that, due to limitations in sample volume, we were not able to measure exogenous antioxidant concentrations (i.e. vitamins and carotenoids) or other oxidative damage parameters. Therefore, consequences for the birds are difficult to predict.

Finally, it is also important to note that the tissue we used (RBC) is not the main target of PFAAs[57], and therefore, we hypothesize that the effects of oxidative damage produced by PFAAs would be more evident in other tissues (e.g. liver or adipose tissue) but this remains to be tested. A previous study performed in tree shallows nestlings from the Great Lakes did not find any association between oxidative stress parameters measured in the liver and PFAAs concentrations in the plasma[20].On the other hand, a previous study performed in wood mice living in the vicinity of the fluorochemical plant in Antwerp, found positive associations between PFOS concentrations and the level of lipid peroxidation in the liver of these mice[64]

Our study provides evidence that OS is a possible pathway for the pernicious effects of PFAAs, however the causal relationship has to be proven. In humans, PFOA has been recently classified by the Agency for Research on Cancer as "possibly carcinogenic"; DNA damage secondary to oxidative stress has been pointed out as the cause of this carcinogenic effect[65].

The obtained data represent an important step towards the understanding of the consequences of exposure to these compounds for wild birds at the individual and the population level. Continuous monitoring of exposure and effects in these populations will give us longitudinal and multigenerational data, which are essential for PFAAs risk assessment.

ASSOCIATED CONTENT

Supporting Information: Detailed description of the methods (including PFAAs analysis in plasma and antioxidant and oxidative stress parameters measurement in red blood cells). Map of the study area (Figure S1). Tables showing target PFAA compounds and their acronyms (based in Buck et al. 2011; Table S1), limits of quantification, mean and median concentrations, range and detection frequencies of PFAAs in plasma of adult great tits (Table S2), limits of quantification and detection frequencies of PFAA compounds with detection frequency < 20% in plasma of adult great tits (Table S3), results of the Principal Component Analysis conducted on the PFAA compounds measured in adults and nestlings (Table S4), coefficient and probability of the correlations found between different PFAAs at the five sampling sites in the plasma samples of the adults (Table S5), limits of quantification, mean and median concentrations, range and detection frequencies of PFAAs in plasma of great tits' nestlings (Table S6), coefficient and probability of the correlations found between different PFAAs at the five sampling sites in the plasma samples of the nestlings (Table S7), limits of quantification and detection frequencies of PFAA compounds with detection

frequency < 20% in plasma of great tits nestlings (Table S8), ∑PFAAs, ∑PFCAs, PFOA, PFDoDA and PFOS mean concentrations in mothers, eggs and both nestlings (the lightest and the heaviest in the nest) at the five sampling sites (Table S9), mean values of body condition and oxidative stress biomarkers in red blood cells of adult great tits at the five sampling sites (Table S10), mean values of body condition and oxidative stress biomarkers in red blood cells of nestlings at the five sampling sites (Table S11), PFAA concentration (range) measured in plasma of different bird species around the world (Table S12). Additional graphic representation of the concentrations of PFUnDA and PFOS found in adult birds' plasma, at the five sampling sites, separated by sex (Figure S2), mean concentrations of PFOA, PFOS and ∑PFCAs found in the mother, egg and offspring (the lightest and the heaviest nestlings in the nest; Figure S3), relationship between Adults-PC1, Adults-PC2 and protein carbonyl content in blood of adult birds sampled in winter and spring (Figure S4).

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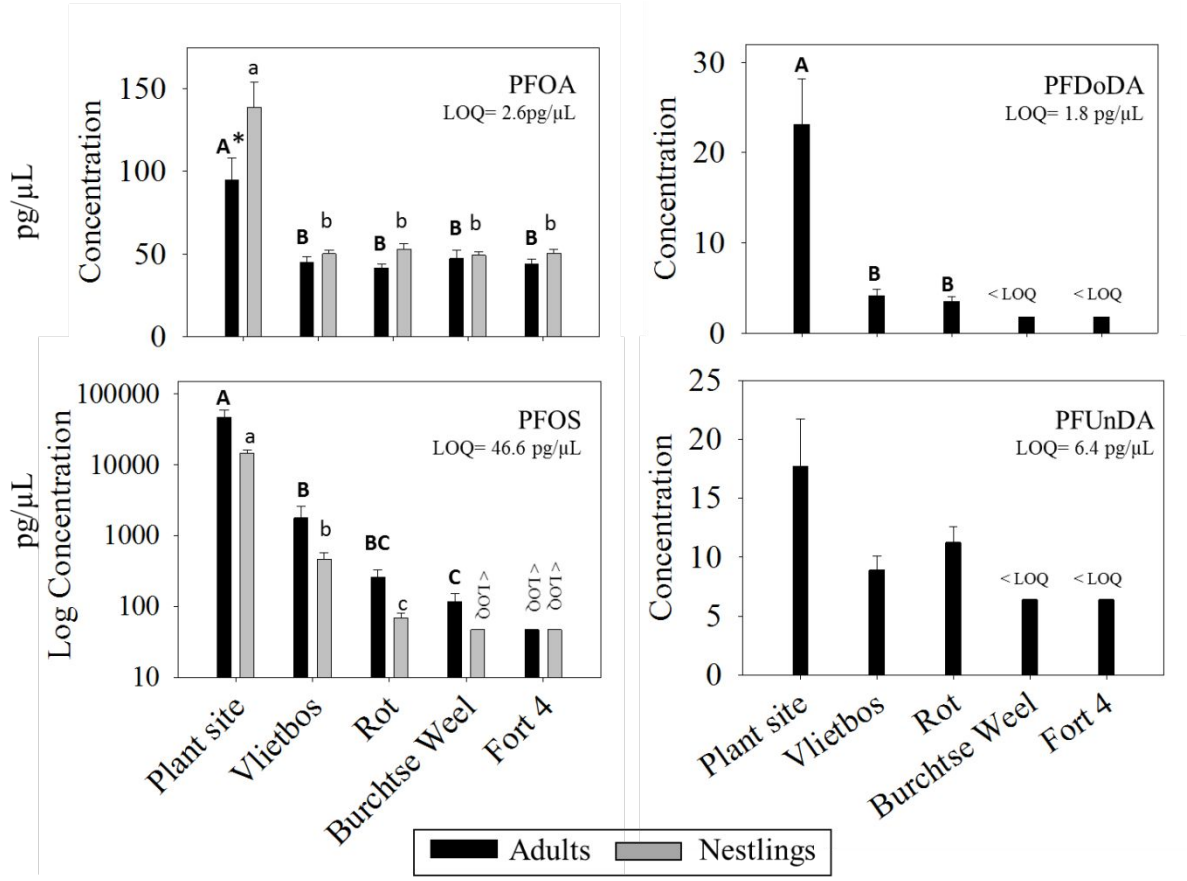

Figure 1.Mean concentrations (±SE) of different PFAAs found in adult birds'(black) and nestlings'(grey) plasma at the five sampling sites in 2015: a fluorochemical plant in Antwerp and four sites with an increasing distance from the plant site (i.e. 1 km Vlietbos, 2.3 km Rot, 3 Km Burchtse Weel and 11 km Fort 4). Different upper case letters and lower case letters indicate different concentrations among sampling sites in adults birds and nestlings respectively. Additionally, mean concentrations (±SE) could be calculated (detection frequency ≥ 50%) at the plant site in adults for PFNA (21.8±5.3) and PFDTrDA (8.5±2.3) and in nestlings for PFBA (24.1±4.6) and PFDoDA (12.2±1.9).
Temporal data from adult birds were pooled together (adults from both the late winter and the spring).
*For PFOA, concentration at the plant site was only significantly higher in winter (no differences in spring).
<LOQ; detection frequency was below 50% and mean values were not calculated (the value given is the LOQ).

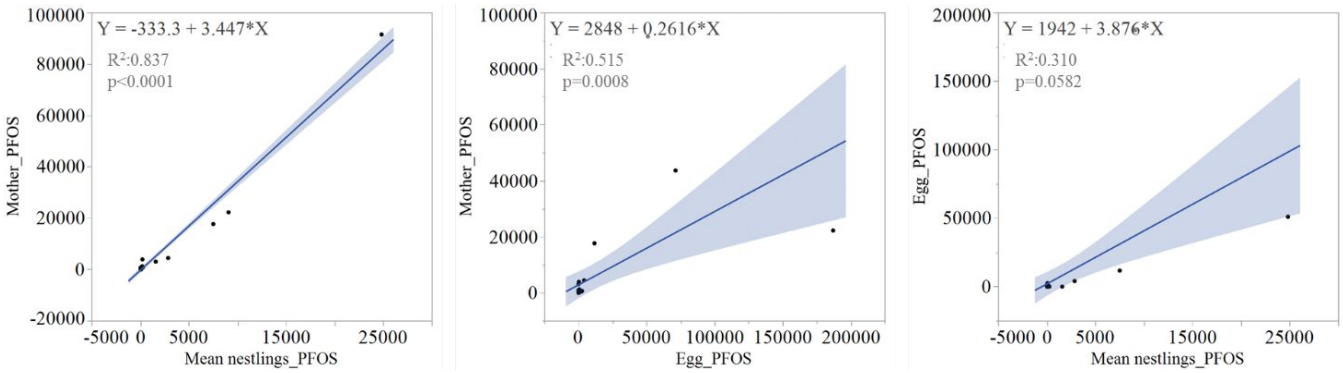

Figure 2. Correlations between mother, offspring (pg/µL;mean values were calculated for the lightest and the heaviest nestlings in the nest) and egg (ng/g) PFOS concentrations. Spearman correlation values, ps and the regression equations are given. Regression lines are shown with 95% confidence bands shaded.

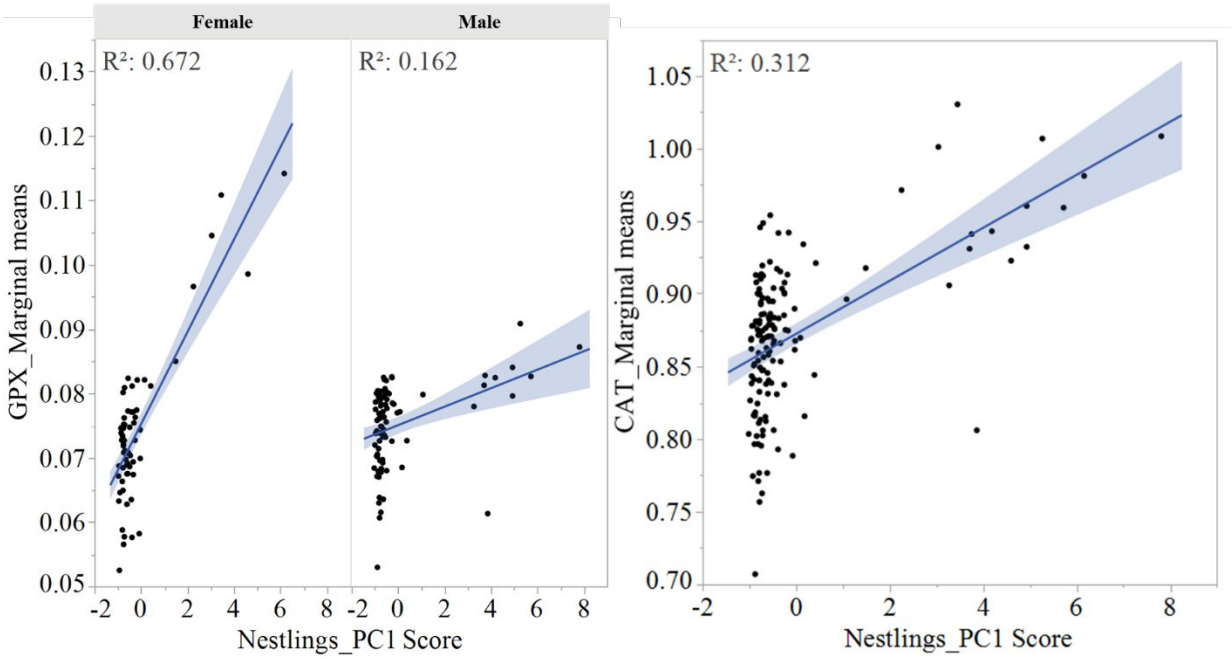

Figure 3. Relationship between nestlings-PC1 and the glutathione peroxidase (separated by sex) and catalase activity (marginal means as obtained in the mix models when considering body condition as a covariate and nestbox as random effect). in 2015 at 5 sites in the vicinity of Antwerp, Belgium. Nestlings-PC1 was influenced by PFOS, PFOA, PFDoDA and PFBA: therefore high concentrations of these compounds corresponded with high values of nestlings-PC1. Regression lines are shown with 95% confidence bands shaded.

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