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

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

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Abstract

Over the past decades, there has been growing scientific attention and public concern towards perfluoroalkyl acids (PFAAs), due to their widespread presence in the environment and associations with adverse effects on various organisms. Bird eggs have often been used as less-invasive biomonitoring tools for toxicological risk assessments of persistent organic pollutants, including some PFAAs. Hereby, it is typically assumed that one random egg is representative for the PFAA concentrations of the whole clutch. However, variation of PFAA concentrations within clutches due to laying sequence influences can have important implications for the egg collection strategy and may impede interpretations of the quantified concentrations. Therefore, the main objective of this paper was to study variation patterns and possible laying sequence associations with PFAA concentrations in eggs of the great tit (*Parus major*). Eight whole clutches (4 - 8 eggs) were collected at a location in the Antwerp region, situated about 11 km from a known PFAA point source. The ∑ PFAA concentrations ranged from 8.9 - 75.1 ng g^{-1} ww. PFOS concentrations ranged from 6.7 - 55.1 ng g^{-1} ww and this compound was the dominant contributor to the total PFAA profile (74%), followed by PFDoA (7%), PFOA (7%), PFDA (5%), PFTrA (4%) and PFNA (3%). The within-clutch variation (70.7%) of the ∑ PFAA concentrations was much larger than the among-clutch variation (29.3%) and concentrations decreased significantly for some PFAA compounds throughout the laying sequence. Nevertheless, PFAA concentrations were positively and significantly correlated between some egg pairs within the same clutch, especially between egg 1 and egg 3. For future PFAA biomonitoring studies, we recommend to consistently collect the same egg along the laying sequence, preferably the first or third egg if maximizing egg exposure metrics is the main objective.

Keywords

Perfluorinated compounds; PFAAs; laying order; clutch variation; terrestrial passerine; great tit

Capsule

Due to large within-clutch variation in PFAA concentrations, we recommend to consistently use the same egg, preferably the first or third, when maximizing egg exposure metrics is the main objective.

1. Introduction

Since industrialization took place in the 18th century, human-induced environmental change has led to the concept of "the Anthropocene" (Corlett, 2015; Rose, 2015). Particularly, it also refers to the dramatically increased emission of persistent organic pollutants (POPs) into the environment (Zalasiewicz *et al.*, 2015). After detection of their global presence in nature, these pollutants have received worldwide research attention (Fernández and Grimalt, 2003; Jaspers *et al.*, 2014). Therefore, well-known POPs such as pesticides, polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) have been studied extensively for toxic effects among various wildlife and humans (Li *et al.*, 2006; Ross and Birnbaum, 2010; Jaspers *et al.*, 2014; Ashraf, 2017). However, much less is known about the environmental impact of the more recently detectable perfluoroalkyl acids (PFAAs) (Domingo and Nadal, 2017; Mudumbi *et al.*, 2017).

PFAAs are a diverse family of synthetic, organic compounds that consist of a perfluoroalkyl chain with strong C-F bonds and a functional acid group (Buck *et al.*, 2011). These physicochemical properties make them extremely resistant to different types of degradation (Beach *et al.*, 2006; Surma *et al.*, 2017). As a result of these physicochemical properties, combined with both their lipophobic and hydrophobic character, they have been produced at large-scale for more than 60 years and used for diverse applications. These include surface coating for textiles, soil repellents, food contact paper, cleaning products, fire-fighting foams and pesticides (Buck *et al.*, 2011; Zhou *et al.*, 2013; Ulrich *et al.*, 2016). Consequently, PFAAs can enter the environment directly via industrial production and usage but also via degradation of precursor compounds as an indirect pathway (Martin *et al.*, 2010; Butt *et al.*, 2014).

Biomonitoring of PFAAs has shown that they bioaccumulate and can biomagnify through the trophic chain (Conder *et al.*, 2008; Fang *et al.*, 2014; Groffen *et al.*, 2018). Hence, PFAAs have been globally

reported from 2000 onwards in the environment, in various organisms (Giesy and Kannan, 2001, 2002; Butt *et al.*, 2010; Rodriguez-Jorquera *et al.*, 2016) and in humans (Hansen *et al.*, 2001; Roosens *et al.*, 2010; Olesen *et al.*, 2016). Over the past decades, there has been growing scientific attention and public concern towards long-chain perfluoroalkyl sulfonic acids (PFSAs) and perfluoroalkyl carboxylic acids (PFCAs), due to their higher bioaccumulation potential and toxicity to various organisms (Conder *et al.*, 2008). As a consequence, the production of these long-chain PFAAs has been regulated in Europe and North-America (Gebbink *et al.*, 2015; Kim and Oh, 2017). Despite these regulatory measures, global PFAA concentrations are still high and even increasing in several countries (Ahrens *et al.*, 2011; Miller *et al.*, 2015; Groffen *et al.*, 2017), highlighting that it remains crucial to continuously monitor these PFAAs.

Although PFAAs exposure in terrestrial animals occurs generally via inhalation of dust and air and ingestion of contaminated food and water (Gebbink *et al.*, 2015), the latter two are thought to be the most important intake routes for biota (D'Hollander *et al.*, 2015). Unlike the majority of POPs, which generally bind to fatty tissues, PFAAs show high affinity toward protein-rich organs such as blood serum, liver and kidney (Jones *et al.*, 2003; Lau *et al.*, 2007). Therefore, these matrices have often been used as biomonitoring method for PFAAs (Dauwe *et al.*, 2007; D'Hollander *et al.*, 2014; Jaspers *et al.*, 2014). Nevertheless, because of ethical and practical reasons, the application of non-destructive biomonitoring methods is highly recommended.

Bird eggs have already been successfully used in many studies as a less-invasive biomonitoring method for PFAAs in various regions (Gebbink and Letcher, 2012; Nordén *et al.*, 2013; Custer *et al.*, 2014; Lopez-Antia *et al.*, 2017; Sedlak *et al.*, 2017), although very few of these studies have focused on terrestrial birds (Ahrens *et al.*, 2011; Groffen *et al.*, 2017; Gewurtz *et al.*, 2018). Most of the previously mentioned studies determined PFAAs in one random egg per nest. It is well known that pollutant concentrations are deposited in eggs, but still very little is known on the overall variation of PFAA release across entire clutches and which factors (eg. diet, age, PFAA chemical properties) are contributing to this process.

100 Surprisingly, there exist only very few studies to date that investigated within-clutch variation of PFAA
101 concentrations in birds (Custer *et al.*, 2012; Vicente *et al.*, 2015). Nevertheless, this information can be
102 very useful in further biomonitoring studies, e.g. to know whether a single egg represents the PFAA
103 contamination of an entire clutch and if so, to minimize the impact of biomonitoring on the population.
104 Furthermore, Vicente *et al.* (2015) demonstrated that PFOS concentrations decreased with laying
105 sequence in Audouin's gull (*Larus audouini*) eggs. It should be noted that females in this species have
106 relatively small clutch sizes of maximum three eggs per nest which may make it more difficult to detect
107 influences of laying sequence on PFAA concentrations.

108 In comparison, passerines may be more appropriate candidate birds to study PFAA clutch variation and
109 laying sequence influences. They generally have large clutch sizes which makes them more suitable to
110 study pollutant variation and associations with laying sequence (Van den Steen *et al.*, 2006). The great
111 tit, *Parus major*, can be considered as a promising model species to study the accumulation and
112 possible effects of PFAAs. Great tits are terrestrial, insectivorous passerine birds which are common
113 and abundant in nearly every urban or wooded area throughout Europe (Van den Steen *et al.*, 2009a).
114 They are very useful as biomonitors of local contamination, because of their small home ranges (Eens
115 *et al.*, 1999). They are cavity-nesting birds and make readily use of nest boxes (Dauwe *et al.*, 2007),
116 which makes it easy to collect samples such as eggs.

117 Great tits have relatively large clutch sizes of 6 - 12 eggs (Van den Steen *et al.*, 2009a), making them
118 suitable to study PFAA accumulation patterns and egg laying sequence influences. Great tits lay eggs
119 daily and need replenishment of endogenous resources with exogenous resources for the eggs.
120 Maternal resources are used for the first eggs and energy from the daily diet for the later eggs, which
121 might contain lower PFAA concentrations than maternal resources, due to less accumulated time
122 within the mother (Van den Steen *et al.*, 2009a). Therefore, a relatively large within-clutch variation
123 and decrease in PFAA concentrations along the laying sequence can be expected.

To the best of our knowledge, there are no studies which have examined possible egg laying sequence influences on PFAAs in a terrestrial bird species. Nevertheless, this information is crucial with respect to future biomonitoring studies, especially for a bird species which has been frequently used in this context. Recent measurements close to a fluorochemical plant near Antwerp reported PFOS concentrations up to 69 218 ng g^{-1} in great tit eggs (Groffen *et al.*, 2017), despite the phase-out of this compound among others in 2002 by 3M (3M Company, 2000). Therefore, biomonitoring of PFAAs in proximity of the fluorochemical plant in Antwerp is extremely important.

The main objective of this study was to assess the variation of different PFAAs, i.e. PFCAs and PFSAs, both within and among clutches of the great tit. Furthermore, possible laying sequence associations between egg parameters (egg weight, egg volume and shell thickness) and PFAA concentrations were studied. In addition, possible relationships of PFAAs among eggs from the same clutch were investigated to infer potential implications for future biomonitoring studies.

2. Materials and method

2.1. Study site

The data collection was conducted at Fort IV (51°10'24"N, 4°27'37"E), which is a park characterized by loam soil and groves dominated with deciduous trees (Hoff *et al.*, 2005). This study site is situated about 11 km from a known PFAAs pollution source in Antwerp (Fig. 1). One of the largest perfluorochemical plants (3M) is located in Antwerp near the river Scheldt and has been a primary production site for PFAAs since 1971. Its importance as a major point source of perfluoroalkyl substances has recently become clear, as a range of monitoring studies have reported among the highest PFAA concentrations in wildlife from 2003 onwards (Hoff *et al.*, 2005; Dauwe *et al.*, 2007; D'Hollander *et al.*, 2014; Groffen *et al.*, 2017; Lopez-Antia *et al.*, 2017).

2.2. Data sampling

147 In total 56 nestboxes were installed at least six months prior to the breeding season and at similar
148 distances from each other to minimalize local differences in great tit densities. Whole clutches were
149 collected from eight nests during March - May 2016 at Fort IV (clutch size: 4 - 8 eggs ± 1.3 (min - max
150 ± SD); *n* = 47). The nest-building phases of each nest were followed-up and advanced nests were
151 checked daily in sequence to determine the egg laying date and identify the eggs individually. Every
152 egg was then cautiously numbered with a non-toxic marker according to the laying sequence. After
153 an entire clutch was completed, all the present eggs with known egg laying sequence were collected
154 before incubation started and stored in 50 mL polypropylene (PP) tubes in a freezer (-20 °C) for later
155 chemical analysis. The collection of the eggs was approved by the Ethical Committee for Animal Testing
156 of the University of Antwerp (2014 - 90).

157 *2.3. Egg parameters*

158 Prior to chemical analysis, egg length (EL) and width (EW) were measured to the nearest 0.01 mm with
159 digital callipers (Mitutoyo Belgium NV, Kruibeke, Belgium). The egg volume (EV) was then estimated
160 using the equation (1) described in Ojanen *et al.* (1978), which can be applied as a specific measure for
161 determining volume of great tit eggs:

162 $$EV = 0.042 + 0.4673 \times EL \times EW^2 \quad (1)$$

163 Afterwards, the content of every egg sample was weighed on a precision balance to the nearest 0.01
164 mg (Mettler Toledo, Zaventem, Belgium). Shell thickness was measured following the methodology
165 described in Lopez-Antia *et al.* (2013). Briefly, three small shell pieces (approximately 0.5 cm^2 each)
166 were obtained from the equatorial region and were dried. Then, the thickness of these pieces was
167 measured with a micrometer (Mitutoyo Belgium NV, Kruibeke, Belgium) to the nearest 0.01 mm and
168 egg shell thickness was then calculated as the average value of all pieces.

169 *2.4. Chemical analysis*

Samples were analyzed for a range of PFAAs and the mentioned abbreviations are conforming to Buck *et al.* (2011). In total four target PFSAs (PFBS, PFHxS, PFOS and PFDS) and 11 PFCAs (PFBA, PFPeA, PFHxA, PFHpA, PFNA, PFOA, PFDA, PFUdA, PFDoA, PFTrA and PFTeA) were examined (Table S1). Analyses were conducted by using isotopically mass-labelled internal standards (ISTDs) including $^{18}O_2$-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-$^{13}C_2$]PFUdA and 1,2[$^{13}C_2$]PFDoA which were purchased from Wellington Laboratories (Guelph, Canada). The stock ISTD mixture (1.2 mL solution containing 2000 pg μL^{-1} of each previously mentioned mass-labelled internal standard with chemical purities of > 98%) was diluted in a 50:50 mixture of HPLC grade acetonitrile (ACN) and water (VWR International, Leuven, Belgium) at a concentration of 125 pg μL^{-1} to spike the samples.

2.5. Sample extraction

The extraction was performed using solid-phase extraction based on the principle of weak-anion exchange. Whole egg content was transferred into a new PP tube and homogenized by alternatively vortex-mixing and sonicating. About 0.3 g of homogenized sample was weighed with a precision balance (± 0.01 mg, Mettler Toledo, Zaventem, Belgium) and used for analysis. Briefly, the homogenates were spiked with 80 µL of 125 pg μL^{-1} ISTD mixture. Subsequently, 10 mL of ACN was added and the samples were sonicated (three times 10 min) with vortex-mixing in between periods. Then, the samples were left overnight on a shaking plate (± 135 rpm, 20 °C, GFL 3020, VWR International, Leuven, Belgium) for approximately 16 hours. Afterwards, the samples were centrifuged for 10 min in a type 5804R centrifuge (2400 rpm, 4 °C, Eppendorf centrifuge, rotor A-4-44) to precipitate and remove insoluble particles. The supernatant was transferred into a 14 mL PP tube.

PFAA extraction was performed by solid phase extraction (SPE) using Chromabond HR-XAW columns (Application-No 305200, SPE department, Macherey-Nagel, Germany, 2009). The column cartridges were conditioned with 5 mL ACN and 5 mL Milli-Q (MQ) water. After the samples were loaded, the column cartridges were washed with 5 mL 25 mM ammonium acetate and 2 mL ACN. Then, the

columns eluted with 2 x 1 mL 2% ammonium hydroxide and the purified extract was completely evaporated with an Eppendorf concentrator (30 °C, type 5301, Hamburg, Germany). The dried extract was dissolved in 200 µL 2% ammonium hydroxide in ACN and filtered through a 13 mm Acrodisc Ion Chromatography Syringe Filter with 0.2 µm Supor (PES) membrane (VWR International, Leuven, Belgium). Finally, the extract was transferred into a PP injector vial before instrumental analysis.

2.6. Instrumental analysis

PFAA measurements were conducted by UPLC coupled tandem mass spectrometry (ACQUITY, TQD, Waters, Milford, MA, USA) using negative electrospray ionization. Separation of the different PFAA target compounds was performed on an ACQUITY UPLC BEH C18 VanGuard Pre-column (2.1 x 5 mm; 1.7 µm, Waters, USA). The mobile phase consisted of HPLC grade water and ACN, both solvents dissolved in 0.1% HPLC grade formic acid. Then, the mobile phase was set up in a concentration gradient, initially consisting of 65% MQ and 35% ACN in 3.5 min and then changed to 10 % MQ and 90% in nearly 1.5 min. This was followed by a return to the initial conditions for 2 min up to the end and the flow rate was set at 450 µL min^{-1} throughout the whole sample run time. An ACQUITY BEH C18 pre-column (2.1 x 30 mm, 1.7 µm, Waters, USA) was inserted between the injector and the solvent mixer. In this way, any PFAA contamination from the system could be retained.

The mass spectrometer operated in multiple reaction monitoring (MRM) mode, which enables detection and quantification of the selected target PFAA analytes, based on their corresponding diagnostic transitions (Table S1).

2.7. Quality control

Two types of blanks were used to assure proper analysis and extraction method. One spiked blank of 10 mL ACN was used as procedural blank after each batch of ten samples to detect any contamination. The same extraction and filtration procedure as described earlier was applied to these blanks. A second

type of blank, consisting of 300 µL ACN, was immediately transferred into the injector vial every ten samples to prevent cross-over contamination between samples during detection in the UPLC-MS/MS.

A linear calibration curve was made by adding the same concentration of ISTD (125pg μL^{-1}) to different concentrations of an unlabeled PFAAs mixture of each PFAA compound in ACN. This calibration curve consisted of 15 calibration points. The relationship between the ratio of concentrations of unlabeled and labeled PFAAs was described by a linear regression function with a highly significant linear fit (R^2 > 0.99; $P < 0.001$) for all the target PFAA analytes.

2.8. Statistical analysis

All statistical analyses were conducted in R (version 3.2.3) and graphs were created with the package "ggplot2". Validity of the models' assumptions was examined with Shapiro-Wilks test and data were log-transformed when needed to fulfil normality assumptions. The level of significance for all statistical tests was set at $P \leq 0.05$. Unless stated otherwise, reported means are expressed as least square means ± standard errors (SEs). Statistical differences among variable levels were denoted with different letters. Limits of quantification (LOQs) were determined on a signal to noise ratio of 10. PFAA concentrations that were below the LOQ were given a concentration of LOQ/2 (Bervoets *et al.*, 2004; Loppi *et al.*, 2015; Groffen *et al.*, 2017). Whenever quantified concentrations of a given PFAA compound were below the LOQ in more than 50% of the samples, the compound was excluded from analyses.

Possible relationships of the laying sequence with the egg parameters and PFAA concentrations were tested with linear mixed-effect models using the package "lmerTest". The egg number (egg 1 - 8) according to the laying sequence in the clutch was considered as a fixed factor and the clutch identity as a random factor. These statistical models nested the individual eggs within their respective clutch and thus consider the dependency of the data. When significant differences were obtained, corrected Tukey's post-hoc tests were used to compare mean PFAA concentrations and mean egg content weight among different egg numbers. The laying date was converted into a continuous variable by considering

243 the first registered laying date (April 1) as day 1. Subsequently, linear regressions were used to identify
244 the relationship between the egg parameters and PFAA concentrations considering the laying date of
245 the 1ste egg as continuous covariate. PFAA variation within and among clutches was studied by
246 estimating variance components using the restricted maximum likelihood estimation method.
247 Correlations in PFAA concentrations among eggs from the same clutch were determined by Pearson's
248 correlation coefficient.

249 ## 3. Results

250 ### 3.1. General accumulation profile PFAAs in eggs

251 An overview of the mean egg concentrations, range, detection frequency and limit of quantification
252 for each detected PFAA compound is given in Table 1. PFOS was the only detected PFSA and showed
253 the highest PFAA concentrations ranging from 6.7 ng g^{-1} to 55.1 ng g^{-1}. Hence, this compound
254 accounted for a dominant contribution of 74% to the ∑ PFAAs (Fig. S1). Regarding the PFCAs, PFOA and
255 PFDoA were detected in all samples with concentrations ranging from 0.72 ng g^{-1} to 3.7 ng g^{-1} and 0.90
256 ng g^{-1} to 4.78 ng g^{-1}, respectively. Both PFCAs contributed 7% of the ∑ PFAAs, followed by PFDA, PFTrA
257 and PFNA (Table 1; Fig. S1). None of the following target PFAAs were detected in any sample and were
258 therefore omitted from further analyses: all the short-chain PFAAs (PFBS, PFBA, PFPeA, PFHxA and
259 PFHpA), some long-chain PFSAs (PFHxS and PFDS) and long-chain PFCAs (PFUnA and PFTeA).

260 ### 3.2. Within -and among-clutch variation in PFAA concentrations

261 Based on the estimated variance components, most of the variation in individual PFAA concentrations
262 in the eggs could be explained by variation within clutches (Fig. 2). The within-clutch variation (WCV)
263 of the ∑ PFAA concentrations was higher than the among-clutch variation (ACV), contributing for
264 respectively 70.7% and 29.3% of the total variation. Likewise, the WCV component of the ∑ PFCAs was
265 higher than the ACV component, accounting for 82.4% and 17.6% of the total variation, respectively.
266 For PFOS, the WCV and ACV accounted for 67.2% and 32.8% of the total variation in PFOS

concentrations, respectively (Fig. 2). Most of the variation in PFOA concentrations could be explained by the WCV component, which contributed for 96.3% of the total variation, whereas the ACV component only accounted for 3.7%. For PFNA, PFDA, PFDOA and PFTrA, the WCV accounted respectively for 89.4%, 92.3%, 82.7% and 99.9% of the total variation in PFAA concentrations (Fig. 2).

3.3. Relationships of egg laying sequence with PFAA concentrations

The large variation within clutches was reflected in significant changes of PFAA concentrations throughout the egg-laying sequence, both for PFOS and ∑ PFCAs (Fig. 3). Marked egg-laying sequence differences were observed for the detected PFAA compounds. For PFOS, egg 1 and egg 3 showed significantly higher concentrations compared to, respectively, egg 4, egg 5, egg 6 and egg 7 (all $P < 0.01$, $F_{7,38} = 5.7$; Fig. 4A). However, egg 8 was not different from egg 1, egg 2 or egg 3. Based on the total amount of PFOS transferred in each clutch, the mean percentage in each egg is presented in Table S2.

Approximately half of the clutches had similar PFOS concentrations in egg 8 as in egg 1 and three of the seven clutches showed a steady PFOS concentration decline throughout the egg laying period. The ∑ PFCAs showed an equally variable pattern with four of the seven clutches containing lower concentrations in later laid eggs, but three of the seven clutches containing higher or equal concentrations of ∑ PFCAs (Fig. 3). PFOA concentrations were not significantly associated with laying sequence ($P = 0.47$, $F_{7,38} = 1.3$; Fig. 4B). PFNA concentrations were significantly lower in egg 5 than in, respectively, egg 1 and egg 2 while egg 4 contained lower PFNA concentrations compared to egg 2 ($P = 0.001$, $F_{7,38} = 4.5$; Fig. 4C), but overall no clear egg order differences were observed. For PFDA, concentrations were significantly higher in egg 3 compared to egg 2, egg 4, egg 5 and egg 7 ($P < 0.01$, $F_{7,38} = 3.5$; Fig. 4D), but no clear trend throughout the laying order could be observed. Moreover, significantly higher PFDoA concentrations were found in egg 1 compared to egg 5 and egg 7 ($P < 0.05$, $F_{7,38} = 2.8$; Fig. 4E), but no egg order trend was found. Lastly, PFTrA concentrations were significantly higher in egg 3 than in egg 2, egg 4 and egg 5 ($P < 0.001$, $F_{7,38} = 5.6$; Fig. 4F).

3.4. Correlations PFAA concentrations among egg numbers

Correlations between PFAA concentrations in different egg numbers are shown in the correlation matrix (Table 2) and significantly positive correlations were observed between different egg pairs within the same clutch. PFOS, PFDA, PFTrA and ∑ PFCA concentrations were positively correlated between egg 1 and egg 3 (PFOS: $R = 0.66$, $P = 0.05$; PFDA: $R = 0.67$, $P < 0.05$; PFTrA: $R = 0.74$, $P < 0.05$; ∑ PFCAs: $R = 0.83$, $P < 0.05$, Table 2) whereas positive correlations could be found for ∑ PFAAs and PFNA, although marginally significant ($R \geq 0.60$, all $0.05 > P < 0.1$). PFDA concentrations were positively correlated between egg 2 and egg 4 ($R = 0.73$, $P < 0.05$, Table 2).

3.5. Relationships between PFAA concentrations and egg parameters throughout the laying order

The mean egg weight ranged from 1.38 g (mean max.) to 0.57 g (mean min.), with egg 1 weighing significantly heavier than egg 2, egg 4, egg 5 and egg 8 ($P < 0.05$, $F_{7,38} = 6.9$; Fig. 5). Egg 8 weighed significantly lighter compared to all the other recorded eggs in the laying sequence ($P < 0.05$; Fig. 5). There was a significant interaction between PFOS and the laying sequence ($P < 0.05$, $F_{7,25} = 3.1$), while the interaction term was not significant between ∑ PFCAs and the laying sequence ($P = 0.89$, $F_{7,25} = 0.37$). Specifically, there was a positive association between PFOS concentrations and the weight of egg 1 ($P = 0.01$, $t_{25} = 2.3$), egg 3 ($P = 0.03$, $t_{27} = 2.6$) and egg 8 ($P = 0.002$, $t_{25} = 3.4$). The egg volume and egg shell thickness (0.208 ± 0.004 mm) did not change significantly throughout the egg laying sequence (egg volume: $P = 0.88$, $F_{7,32} = 0.41$; egg shell thickness: $P = 0.56$, $F_{7,39} = 0.84$).

3.6. Correlations between egg-laying date of the 1st egg and PFAA concentrations

The laying date of the 1st egg was negatively and positively correlated with, respectively, the mean PFOS concentrations and mean egg weight of the clutches (Fig. 6). Clutches that were initiated later in the breeding season contained on average significantly higher PFOS concentrations ($R^2 = 0.58$, $P < 0.05$, Fig. 6), whereas a positive relationship was found between egg laying date and egg content weight ($R^2 = 0.49$, $P < 0.05$, Fig. 6). There was no significant association of 1st egg laying date with other PFAA concentrations and egg parameters (all $P > 0.05$).

4. Discussion

4.1. General accumulation profile PFAAs in eggs

The measured PFAA concentrations in the present study are relatively low in comparison with those found in previous monitoring studies conducted on bird eggs near Antwerp (Groffen *et al.*, 2017; Lopez-Antia *et al.*, 2017). The closest known PFAA point source (plant site, Fig. 1) is located about 11 km from Fort IV and previous monitoring studies in bird eggs at Antwerp indicated that PFAA concentrations follow a clear pollution gradient, with concentrations decreasing steeply from the plant site (D'Hollander *et al.*, 2014; Groffen *et al.*, 2017). However, mean PFOS and PFOA concentrations (resp. 12.0 ng g^{-1} and 0.45 ng g^{-1}) detected in free-range chicken eggs (*Gallus gallus*) only 1 km away from a fluorochemical plant in China (Wang *et al.*, 2010) were lower than those found in the present study (resp. 22.7 ng g^{-1} and 2.0 ng g^{-1}). Multiple factors might explain the differences between the present study including indirect pathways, such as environmental and biological degradation (Liu and Avendaño, 2013, Gebbink *et al.*, 2015; Brendel *et al.*, 2018), which probably become more important drivers of PFAA concentrations compared to direct pathways at distant sites from the point source. Furthermore, given the ubiquitous presence of PFAAs in numerous consumer products (Buck *et al.*, 2011; Zhou *et al.*, 2013; Ulrich *et al.*, 2016), the possible influence of local, unknown PFAA sources on the exposure to birds and their eggs cannot be completely excluded. Great tits are free-living birds that live in much more variable conditions than domestic chickens and could therefore be more likely exposed to these unknown PFAA sources (e.g. dietary intake). This could ultimately lead to higher PFOS and PFOA concentrations in great tits compared to domestic chickens.

The contribution profile was dominated by PFOS (Fig. S1), which is in accordance with other studies conducted on PFAAs in eggs of terrestrial birds (Ahrens *et al.*, 2011; Custer *et al.*, 2014; Groffen *et al.*, 2017), aquatic birds (Norden *et al.*, 2013) and other wildlife (Butt *et al.*, 2010; Fang *et al.*, 2014; Groffen *et al.*, 2018). PFOS tends to bioaccumulate in the liver due to the high amount of protein-rich tissue in this organ (Lau *et al.*, 2007; Gebbink *et al.*, 2012). These proteins are synthesized in the liver of the

342 mother and then transferred via the blood to the ovary and the eggs (Bertolero *et al.*, 2015), which
343 explains the dominant pattern of PFOS in eggs. Besides, the prevalent spatial presence of PFOS can
344 generally be explained due to its high bioaccumulation potential as PFOS is a terminal degradation
345 product of many perfluorinated compounds (Conder *et al.*, 2008; Buck *et al.*, 2011; Mudumbi *et al.*,
346 2017).

347 None of the target short-chain PFAAs were detected, while the majority of target long-chain PFCAs
348 (PFOA, PFNA, PFDA, PFDoA and PFTrA) could be detected in ≥ 80% of the egg samples. The dominance
349 of PFOA and PFDoA to the ∑ PFCAs is in accordance with some PFAA biomonitoring studies in bird eggs
350 (Haukas *et al.*, 2007; Groffen *et al.*, 2017), whereas other studies have not confirmed this dominant ∑
351 PFCA contribution of PFOA and PFDoA (Ahrens *et al.*, 2011; Custer *et al.*, 2012; Norden *et al.*, 2013).

352 Generally, long-chain PFAAs have greater bioaccumulation potential than their short-chain
353 homologues (Conder *et al.*, 2008; Olesen *et al.*, 2016) and thus are more likely to be transferred from
354 the mother to her respective eggs. Moreover, the frequent detection of long-chain PFAAs in this study
355 is in contrast with other reports in eggs from whole clutches of gulls (*Larus* sp.), in which long-chain
356 PFAAs were not or only sporadically detected (Vicente *et al.*, 2012; 2015). On the other hand, Custer
357 *et al.* (2012) also detected long-chain PFAAs in the eggs of tree swallows (*Tachycineta bicolor*).
358 Compared to gulls, great tits and tree swallows invest much larger amounts of resources in eggs
359 relative to their body weight and due to their large clutch size (Van den Steen *et al.*, 2009b).
360 Consequently, it could be that the persistent and bioaccumulative long-chain PFAAs are more prone
361 to transfer into the eggs of small passerines compared to gulls.

362 4.2. Within -and among-clutch variation in PFAAs

363 A remarkable variation in PFOS and in the sum of PFAAs concentrations within clutches was observed
364 (Fig. 2). Despite that the absolute PFOS concentrations were relatively low, WCV for all PFAAs was
365 consistently higher than the ACV. For the ∑ PFAAs, WCV of was still greater than the ACV. These results

366 support those of the few other studies in which PFAA clutch variation was assessed (Custer *et al.*, 2012;
367 Vicente *et al.*, 2015).

368 However, Van den Steen *et al.* (2006; 2009a; 2009b) found higher ACV than WCV for other classes of
369 organic pollutants (PCBs and PBDEs) in great and blue tits at Fort IV, although egg laying sequence
370 influences were present (Table S3). Different chemical properties (PCBs and PBDEs: lipophilic versus
371 PFAAs: both lipo- and hydrophilic) and hence different environmental transport mechanisms could
372 explain these diverging results. It could be that the PCBs and PBDEs are spatially distributed in a more
373 heterogeneous way than PFAAs. In contrast with PCBs and PBDEs, PFAAs have high water solubility
374 and are more volatile (Siddiqi *et al.*, 2003; Mudumbi *et al.*, 2017). As wind and water are mainly
375 responsible for a relatively homogeneous distribution pattern of most contaminants in general
376 (Fernández and Grimalt, 2003), PCBs and PBDEs might be restricted to only some places within a
377 location due to their more limited transport via these media. Lastly, natal dispersal distances of
378 females in tit species sometimes exceed more than 3 km (Greenwood *et al.*, 1979). Females that
379 dispersed from varying PCB and PBDE polluted sites to Fort IV could also contribute in explaining the
380 relatively large ACV contaminated sites in the former studies.

381 The very large WCV found in the present study is most likely related to the large clutch size of great
382 tits and the fact that they are 'income' breeders (Ward and Bryant, 2006; Van den Steen *et al.*, 2009a).
383 Because of their large clutch size, great tits invest relatively large amounts of resources (e.g. proteins
384 and lipids) in their eggs and most likely use resources from current, rather than stored nutrients.
385 Consequently, large variations in PFAA concentrations, which are associated with these nutrients,
386 could be expected. In addition, tits lay eggs on a daily basis and therefore rely on daily replenishment
387 of maternal resources with resources of their diet (Van den Steen *et al.*, 2009b). Therefore, the large
388 WCV is probably also a consequence of the large PFAAs variation in prey items or variation in the types
389 of prey being consumed.

390 During the breeding season, the diet of great tits mainly consists of caterpillars (Lepidoptera) (Dauwe
391 *et al.*, 2007). Variation in prey preferences for females throughout the days and local shifts in
392 availability of insects throughout the breeding season can also be contributing mechanisms to increase
393 WCV in great tits (Longcore *et al.*, 2007; Custer *et al.*, 2010). In order to understand better how
394 variation in prey items is translated in variation of PFAA egg concentrations, stable isotope analysis of
395 nitrogen and carbon could be a promising tool in future field studies investigating PFAA clutch
396 variation.

397 4.3. Relationships of egg laying sequence with PFAA concentrations

398 The results of the present study show marked egg laying sequence variation of PFAAs throughout the
399 clutch and an overall significant decrease on an average basis could be observed in the sum of PFAA
400 concentrations (Fig. 3, Fig. 4). While this was true especially for PFOS, it was not the case for all PFAA
401 congeners. For example, PFOA, PFNA, PFDA, PFDoA and PFTrA showed basically no difference across
402 the egg laying sequence (Fig. 4). On an individual clutch basis (Fig. 3), only three of the seven clutches
403 declined in these PFAA concentrations while two clutches increased and then started to decline in
404 pollutant concentrations. Three clutches alternately declined and increased in PFAA concentrations
405 throughout the laying order and had similar concentrations in egg 8 as in egg 1.

406 Vicente et al. (2015) demonstrated that PFOS decreased with the laying sequence of the eggs in gulls
407 (*Larus sp.*) and these results are also in line with studies assessing other organic pollutants in tits (Van
408 den Steen *et al.*, 2006; 2009a; 2009b). This observed pattern is likely due to decreasing concentrations
409 of PFAAs in the mother during the laying period. Tits rely on daily replenishment of endogenous
410 maternal resources with exogenous resources of their diet (Van den Steen *et al.*, 2009b). Throughout
411 the egg-laying period, these maternal resources are thus used for production of the first eggs, while
412 dietary lipids and proteins for the later eggs probably contain lower PFAA concentrations (Van den
413 Steen *et al.*, 2009a). Likewise, maternal tissues from the liver probably store higher amounts of
414 proteins to which PFAAs strongly bind and may therefore be more present in the endogenous maternal

415 resources than in the exogenous dietary resources. Hence, a decreasing pattern in PFAA
416 concentrations can be observed throughout the clutch.

417 Remarkably, the results of this study also show a significant increase in PFAA concentrations in egg 3
418 for PFDA and PFTrA. Although speculative, this result is best explained by a shortage in food availability
419 and could possibly reflect mother birds which laid their third under lower food conditions. Instead,
420 endogenous maternal reserves are exploited which may contain higher PFAA concentrations (Braune
421 and Norstrom, 1989). The precise reason or proximate mechanism why this result was only expressed
422 for these three mentioned compounds, remains to be elucidated. Notice that the sample sizes for the
423 last eggs were relatively low. Ideally, more data should be collected to reveal whether this result is a
424 spurious statistical relationship or there is a real biological mechanism behind this result.

425 4.4. Correlations PFAA concentrations among egg numbers

426 Despite the substantial within clutch variation, strong positive correlations were found in PFAA
427 concentrations between eggs from the same clutch, particularly between egg 1 and egg 3. In other
428 words, those clutches which contained high PFOS, PFDA and PFTrA concentrations in egg 1 also had
429 high concentrations of these compounds in egg 3 (Table 2, Fig. 3). Apart from PFTrA, concentrations in
430 egg 3 did not significantly differ from those found in egg 1. Therefore, taking into account that WCV
431 was large and marked egg-laying sequence associations with the PFAA concentrations were present,
432 we recommend two alternative sampling strategies depending on the main research goal.

433 For great tits (and other species provided that our results can be generalized for other species), the
434 first or third egg for future biomonitoring studies should be collected. Indeed, when maximizing egg
435 exposure metrics is the goal of biomonitoring, collection of the first or third egg is recommended.
436 Alternatively, two or three random eggs could be collected from a clutch to even out the large variation
437 in PFAA concentrations and hence obtaining a more representative sample. Importantly, this should
438 not interfere with collecting other data in another context, for instance studying associations between
439 pollutant concentrations and reproductive parameters (see Groffen et al., 2019). From a practical point

of view, random egg collection also prevents the need to visit the nest daily to identify and mark specific eggs in the laying sequence which may be practically beneficial.

Despite the present study being one of the very first in which PFAA variation along the egg-laying sequence is investigated, the previous statements concerning the sampling strategy are further supported by findings in other studies. In tree swallows (*Tachycineta bicolor*), up to 4-fold differences in PFOS concentrations within two clutches were found (Custer *et al.*, 2012). Moreover, Vicente *et al.* (2015) demonstrated considerable PFAA variation in eggs of Audouin gulls (*Larus audouini*). In contrast to passerines, gulls lay small clutches of mostly three eggs and also differ in terms of trophic position and feeding habits. However, both species are income breeders and therefore depend on exogenous resources for egg formation (Hobson, 1995; Meijer and Drent, 1999). Interestingly, Vicente *et al.* (2015) reported decreasing concentrations of PFAAs along the laying sequence and PFOS concentrations between egg 1 and egg 3 were also strongly and positively correlated. This finding not only enhances the general importance of considering the sampling strategy when monitoring PFAAs in bird eggs, but also suggests that the way of resource assimilation is a major proximate mechanism in explaining egg laying sequence associations with PFAAs, regardless of other life-history traits.

4.5. Relationships between PFAA concentrations and egg parameters throughout the laying order

The mean egg weight declined throughout the laying sequence (Fig. 5) and the significant interaction term between egg laying sequence and PFOS showed that concentrations of this compound were positively associated with the weight of egg 1, egg 3 and egg 8. Interestingly, these eggs also contained among the highest absolute PFOS concentrations. This result suggests that PFAA exposure may alter the egg composition which may change on its turn the egg weight, although it could equally well be that the egg composition changes the PFAA concentrations. For PCBs, higher concentrations in eggs of American kestrels (*Falco sparverius*) and blue tits (*Cyanistes caeruleus*) were also associated with heavier eggs (Fernie *et al.* 2000; Van den Steen *et al.*, 2009b).

464 Recent studies demonstrated that PFOS shows high affinity towards very low-density lipoproteins, for
465 instance phosvitin and lipovitellin, which are mainly present in egg yolk (Norden *et al.*, 2013; Bertolero
466 *et al.*, 2015). These egg nutrients are all synthesized in the liver of the mother bird before they get
467 transferred to the egg yolk via the ovaries (Bertolero *et al.*, 2015). Following this reasoning, heavier
468 eggs presumably have higher lipoprotein concentrations and therefore might result in higher PFOS
469 concentrations. Future studies on laying sequence associations with PFOS concentrations should
470 assess nutrient concentrations in eggs to further examine the plausibility of this hypothesis.

471 In addition, the significant decrease in PFOS concentrations throughout the laying sequence has direct
472 relevance to the potential toxicity of PFOS to the embryos. In many altricial bird species including great
473 tits, hatching asynchrony is a ubiquitous life-history treat which results in higher survival rates of chicks
474 from earlier laid eggs compared to those hatched from later laid eggs (Pijanowski, 1992; de Heij *et al.*,
475 2006). However, if the first laid eggs also contain higher PFOS concentrations, this general life-history
476 pattern may be disrupted in heavily polluted habitats as PFAAs have been associated with reduced
477 hatching success and growth rate of chicks (Molina *et al.*, 2006; Yanai *et al.*, 2008; Cassone *et al.*, 2012;
478 Custer *et al.*, 2012; 2014).

479 4.6. Correlations between egg-laying date first egg and PFAA concentrations

480 Interestingly, the egg-laying date of the first egg was significantly and negatively correlated with egg
481 PFOS concentrations, while heavier eggs were associated with late-breeding females (Fig. 6). This
482 result is in contrast with studies conducted on other POPs. For pesticides, higher concentrations were
483 associated with later breeding (Bustnes *et al.*, 2007; Lopez-Antia *et al.*, 2015a; 2015b) while no effect
484 was observed for PCBs (Van den Steen *et al.*, 2009b). To the best of our knowledge, this is the first
485 study reporting associations between laying date and PFAA concentrations.

486 It could be possible that these associations reflect an age effect, given that early-breeding females are
487 generally older than late-breeding females (Sydeman *et al.*, 1991; de Forest and Gaston, 1996; Tartu
488 *et al.*, 2014). Given the high bioaccumulation potential of PFOS, older birds would experience a higher

lifetime exposure to PFOS compared to younger birds and this may be reflected in the transfer of higher concentrations to their eggs. On the other hand, Blévin *et al.* (2017) found in black-legged kittiwakes (*Rissa tridactyla*) that higher PFOS concentrations were correlated with longer telomere lengths, which is considered to be a measure of quality. Therefore, the authors proposed that PFAAs may stimulate self-maintenance mechanisms in birds bearing the highest PFAA concentrations. If so, older birds which presumably accumulate higher PFOS concentrations may invest more energy in these self-sustaining mechanisms in favour of their own individual fitness. This could be at the expense of energy investment in the eggs and the fitness of their offspring, which could explain the found association between lighter eggs and early breeding (Fig. 6).

Although the above explanation is plausible, it should also be emphasized that the sample size for testing this hypothesis was rather low and that the age of the birds was unfortunately not known in the current study. Furthermore, to the best of our knowledge, the possible causal link of age with breeding date and egg weight, taking into account the PFAA pollution context, has never been examined. Future field studies on PFAAs in birds should include these variables.

5. Conclusions

In our study on great tits, an important model species in environmental research, the within-clutch variation was much higher for all PFAA compounds compared to the among-clutch variation, which is probably related with traits on (i) the species level (clutch size, variability in PFAA concentrations between exogenous and endogenous resources), (ii) individual level (e.g. age and variation in prey items) and (iii) the pollutant level (heterogeneous distribution of PFAAs). Significant and negative laying sequence relationships with both egg weight and PFAA concentrations were detected. These laying sequence associations may have important toxicological implications for developing embryos with potential disruption of general life-history patterns in bird species.

Regarding PFAA biomonitoring implications, one of the key findings is that in biomonitoring studies using bird eggs the sampling strategy chosen should depend on the main research objective. When

maximizing egg exposure metrics is the main goal, one should sample consistently the same egg of each nest. In the case of great tits, the sampling of the first or third egg is recommended. Finally, it should be noted that research focusing on possible egg-laying sequence influences on PFAAs remains very scarce and sample sizes are often rather small. Therefore, further biomonitoring studies on the same species and other species with similar large clutch sizes should be conducted to validate the reported results in this study.

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772 **8. Figures and tables**

773 ***Figures***

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Fig. 1*: Map of the study area (Fort IV) showing the distribution of the great tit nestboxes (black dots) from which whole clutches of great tit eggs were collected (n = 46) near Antwerp, Belgium in 2016. The right bottom map depicts the distance of the study area (rectangle) from the fluorochemical plant (star) in Antwerp.*

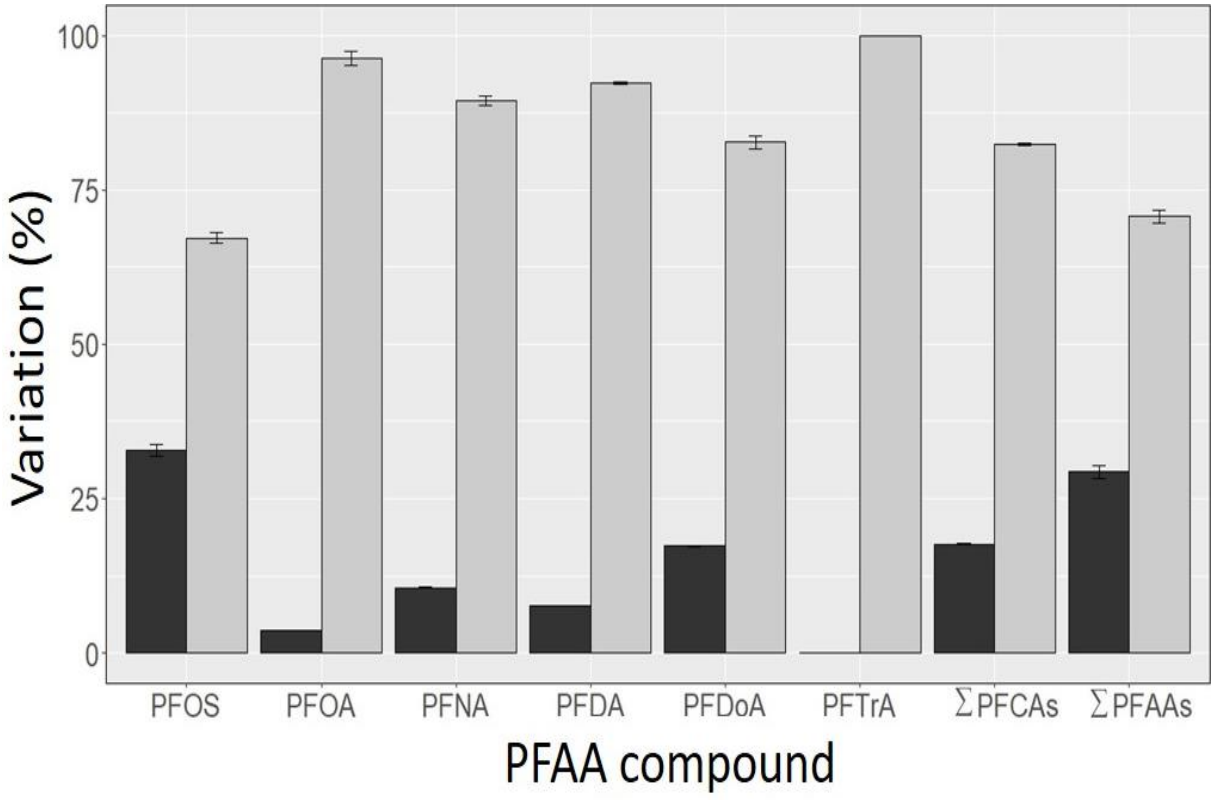

Fig. 2*: The estimated variance components, expressed in %, of the within-clutch variation (WCV: grey bar) and between-clutch variation (BCV: black bar) in eggs of whole clutches from great tit, nesting near Antwerp, Belgium in 2016. Error bars represent standard errors.*

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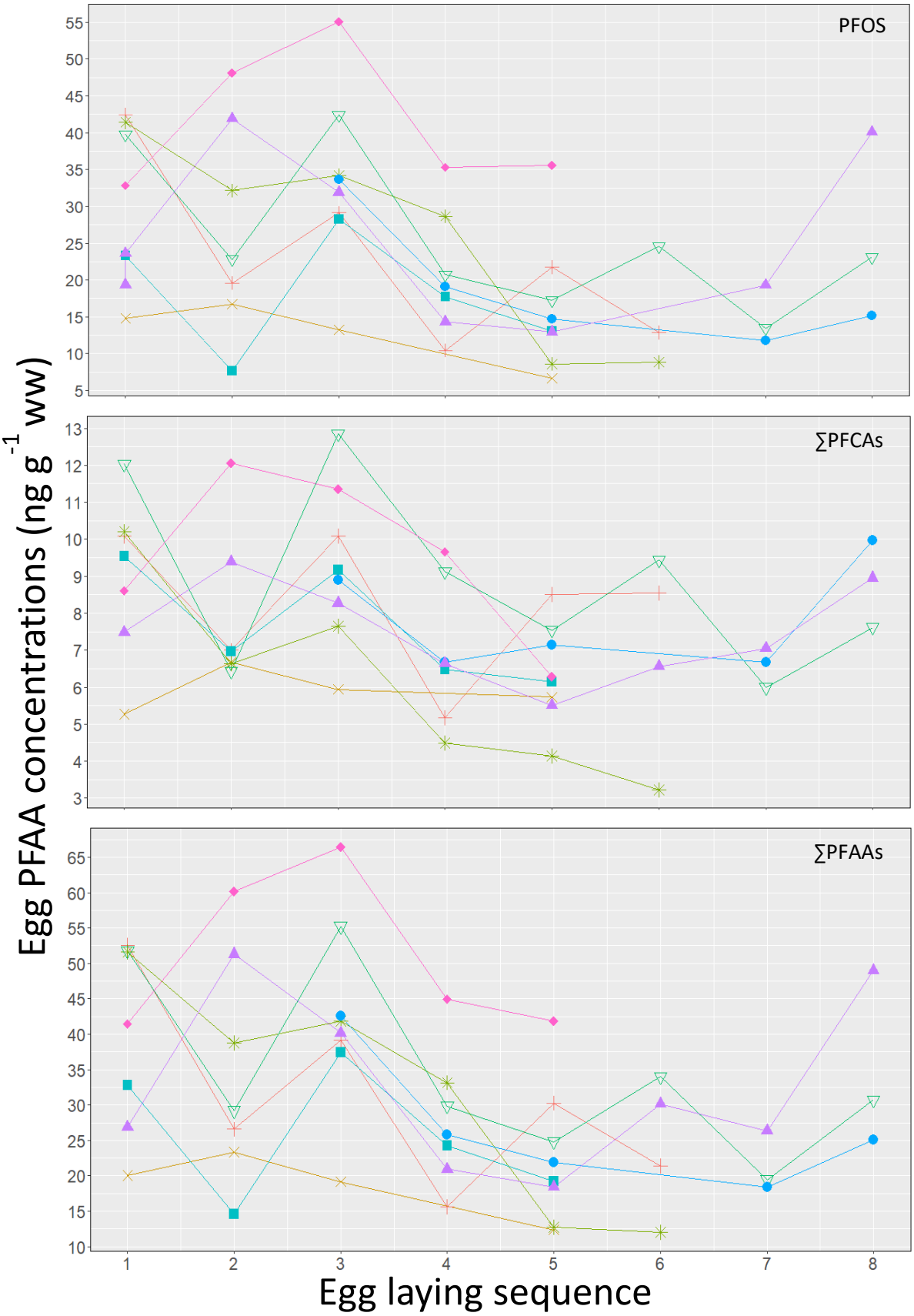

***Fig. 3**: The change in absolute PFAA concentrations, expressed in ng g^{-1} wet weight (ww), in sequentially laid great tit eggs within the same nestbox for PFOS (upper graph), ∑ PFCAs (middle graph) and the ∑ PFAAs (lower graph) near Antwerp, Belgium in 2016. Note that the PFSAs only includes PFOS as other target PFSAs were not detected. Colors represent different nestbox identities. 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.*

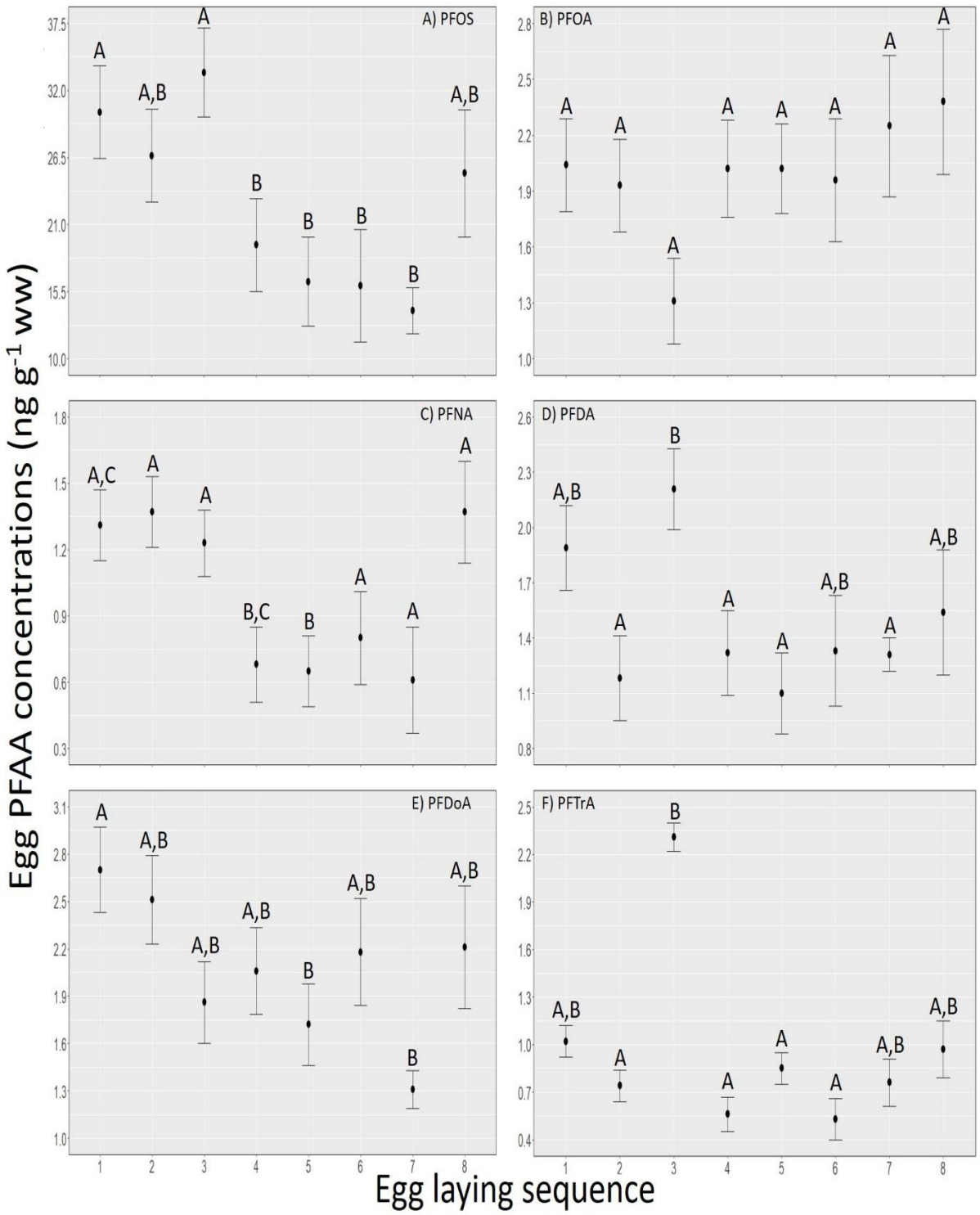

***Fig. 4**: Mean PFAA concentrations, expressed in ng g^{-1} wet weight (ww), in sequentially laid great tit eggs from whole clutches near Antwerp, Belgium in 2016 for PFOS (A), PFOA (B), PFNA (C), PFDA (D), PFDoA (E) and PFTrA (F). Different letters denote significant ($P < 0.05$) differences among 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.*

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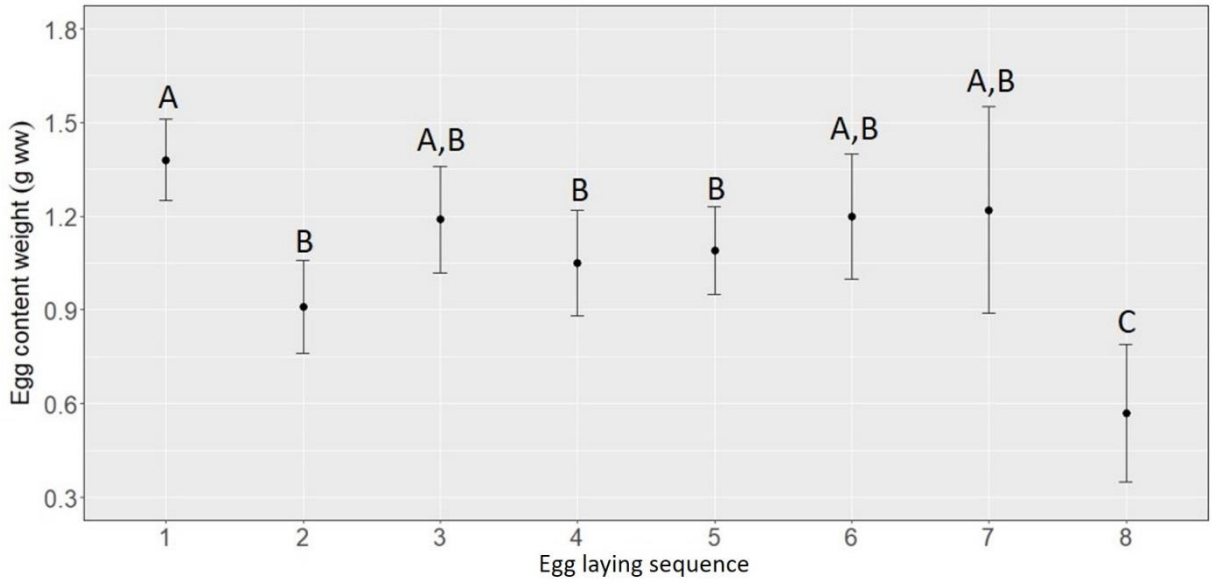

***Fig. 5**: Mean egg content weight, expressed in g wet weight (ww), in sequentially laid great tit eggs from whole clutches near Antwerp, Belgium in 2016. Different letters denote significant (P < 0.05) differences among egg numbers in the laying order and the error bars represent standard errors. Egg 1: n = 7, egg 2: n = 7, egg 3: n = 8, egg 4: n = 7, egg 5: n = 8, egg 6: n = 4,*

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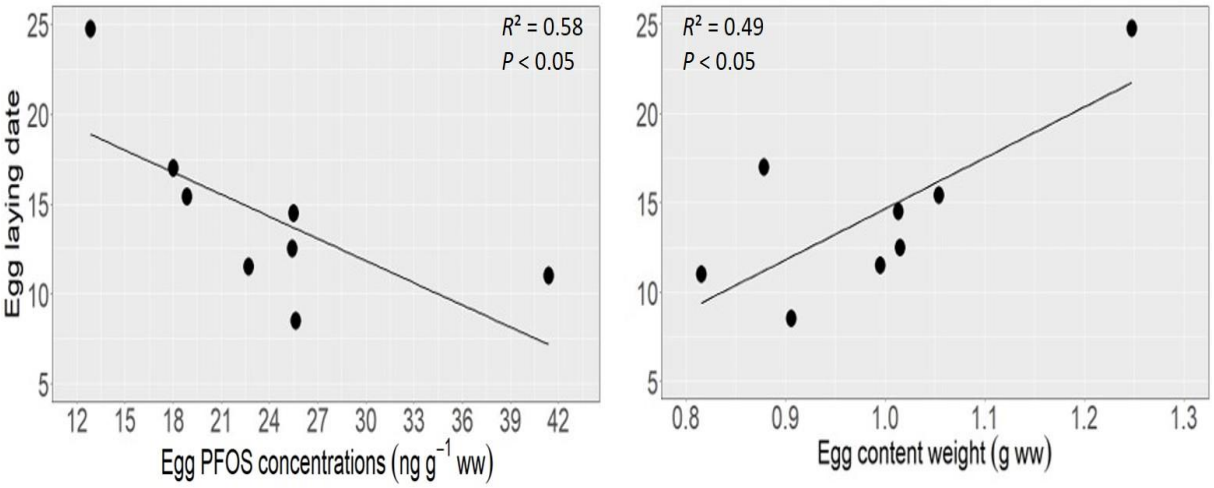

***Fig. 6**: Linear regression plots showing the significantly (P < 0.05, R^2 = 0.58) negative relationship between egg laying date and egg PFOS concentrations, in ng g wet weight (left graph) and the positive (P < 0.05, R^2 = 0.49) relationship between egg laying date and egg content weight, in g wet weight (right graph) of great tit clutches near Antwerp, Belgium in 2016. The data points represent the mean egg PFOS concentrations and egg laying date for each clutch (n = 8).*

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790 ***Tables***

Table 1: *Mean ± standard error (SE) concentrations with minimum-maximum ranges and limit of quantification (LOQ) in pooled great tit eggs from eight clutches near Antwerp, Belgium in 2016, expressed in ng g^{-1} wet weight, along with the detection frequency, expressed in %, for each PFAA compound. Note that the ∑ PFSAs only includes PFOS as other PFSAs were not detected.*

Compound	Mean ± SE	Min.	Max.	Det. freq.	LOQ
PFOA	2.0 ± 0.2	0.72	3.7	100	0.05
PFNA	1.0 ± 0.1	0.29	2.4	80.9	0.59
PFDA	1.5 ± 0.2	0.21	3.5	95.7	0.43
PFDoA	2.1 ± 0.3	0.9	4.8	100	0.44
PFTrA	0.97 ± 0.22	0.13	5.7	93.6	0.26
∑ PFCAs	7.8 ± 0.2	3.2	12.8		
PFOS	22.7 ± 3.8	6.7	55.1	100	2.6
∑ PFAAs	30.2 ± 0.8	8.9	75.1		

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Table 2: *Overview of the correlations in PFAA concentrations for all detected PFAA compounds between egg pairs of the same clutch near Antwerp, Belgium in 2016. Values represent Pearson correlation coefficients and bold values denote significant associations (significance levels: * = 0.05 > P < 0.10; ** = P < 0.05). Note that the ∑ PFSAs only includes PFOS as other PFSAs were not detected. Egg numbers 6, 7 and 8 were excluded from the statistical analysis as these data were missing from some clutches.*

	Pearson correlation coefficient							
Egg-laying sequence	**PFOA**	**PFNA**	**PFDA**	**PFDoA**	**PFTrA**	**∑ PFCAs**	**PFOS**	**∑ PFAAs**
1 vs 2	0.43	0.12	0.46	-0.13	-0.5	-0.58	-0.49	0.05
1 vs 3	-0.36	**0.6***	**0.67****	0.04	**0.74****	**0.83****	**0.66****	**0.71***
1 vs 4	-0.07	**-0.92***	0.48	0.09	-0.21	0.31	-0.27	-0.2
1 vs 5	0.17	0.33	-0.18	0.12	-0.14	0.25	0.51	0.46
2 vs 3	**-0.84***	0.06	0.49	0.61	-0.48	-0.11	-0.02	0.26
2 vs 4	0.24	0.11	**0.73****	-0.53	0.3	0.32	-0.04	0.47
2 vs 5	0.5	0.01	**-0.83***	-0.02	0.26	0.1	-0.06	-0.07
3 vs 4	-0.58	-0.49	0.38	-0.15	-0.11	-0.05	-0.34	-0.27
3 vs 5	-0.3	0.2	-0.01	0.15	0.19	**0.66***	0.51	0.53
4 vs 5	-0.55	-0.52	**-0.79***	-0.05	-0.63	-0.61	-0.64	-0.65

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793 **9. Supplementary information**

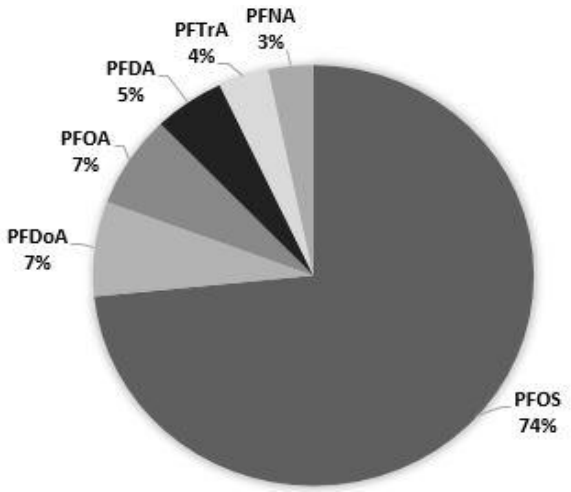

Fig. S1*: Relative mean contribution of each PFAA compound, expressed in %, to the total amount of PFAAs in great tit eggs.*

Table S1: *The multiple reaction monitoring (MRM) transitions of the target compounds and the used isotopically mass-labelled internal standards (ISTD) to quantify the compounds. The TQD tandem quadrupole mass spectrometer (MS) conditions, including collision energy (eV) and cone voltage (V) were adjusted to optimize detection of each compound. Adopted from Groffen et al. (submitted).*

Compound	Precursor ion (m/z)	Product ion (m/z)		Cone Voltage (V)	Collision energy (eV) for diagnostic transition 1	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
PFUnA	563	519	169	18	30	35	[1,2-$^{13}C_2$]PFUnA
PFDoA	613	569	319	22	21	30	[1,2-$^{13}C_2$]PFDoA
PFTrA	663	619	319	26	21	30	[1,2-$^{13}C_2$]PFDoA
PFTeA	713	669	169	28	21	21	[1,2-$^{13}C_2$]PFDoA
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-$^{13}C_2$]PFUdA	565	520	170	18	32	35	

[1,2-$^{13}C_2$]PFDoA	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

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802 ***Table S2****: Transfer percentage of PFOS concentrations according to the egg laying sequence of each clutch. NA = data not*
803 *available.*

	Transfer percentage PFOS (%)							
Clutch ID	1	2	3	4	5	6	7	8
1	31.2	14.4	21.4	7.7	16.0	9.4	NA	NA
2	28.8	32.5	25.8	NA	13.0	NA	NA	NA
3	26.9	20.9	22.2	18.6	5.6	5.7	NA	NA
4	0.2	15.2	11.2	12.4	10.4	13.2	7.9	13.4
5	25.9	8.5	31.4	19.7	14.5	NA	NA	NA
6	NA	NA	35.7	20.3	15.6	NA	12.4	16.0
7	9.5	20.6	15.7	7.0	6.4	11.6	9.5	19.7
8	15.9	23.3	26.6	17.1	17.2	NA	NA	NA
Mean ± SE	19.7 ± 2.7	16.4 ± 2.9	24.9 ± 2.1	12.6 ± 2.0	12.1 ± 1.5	4.9 ± 1.3	3.6 ± 1.4	5.9 ± 2.0

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805 ***Table S3****: Overview of studies in which the laying order patterns and/or clutch variation (within-clutch variation (WCV) and*
806 *among-clutch variation (ACV)) in concentrations of perfluorooctane sulfonic acid (PFOS) or other persistent organic pollutants*
807 *(polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs)) were reported. NA = data not available.*

Publication	**Species**	**Sampling year**	**Pollutant(s)**	**Laying order effects**	**WCV (%)**	**ACV (%)**	**N clutches**
Present study	Great tit (*Parus major*)	2016	PFOS	Yes	67.2	32.8	8
Vicente et al. (2015)	Audouin's gull (*Larus audouinii*)	2009	PFOS	Yes	NA, but ± 3.5-fold difference within clutch	NA	10
Custer et al. (2012)	Tree swallow (*Tachycineta bicolor*)	2008-2009	PFOS	NA	NA, but 4-fold difference within clutch	NA	2
Van den Steen et al. (2009b)	Blue tit (*Cyanistes caeruleus*)	2006	PCBs	Yes	40	60	10
			PBDEs	Yes	39	61	10
Van den Steen et al. (2009a)	Great tit (*Parus major*)	2006	PCBs	Yes	40	60	8
			PBDEs	Yes	30	70	8
Van den Steen et al. (2006)	Great tit (*Parus major*)	2000	PCBs	No	7	93	10
			PBDEs	No	3	97	10

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