Understanding PFAAs exposure in a generalist seabird species breeding in the vicinity of a fluorochemical plant : influence of maternal transfer and diet

Understanding PFAAs exposure in a generalist seabird species breeding in the vicinity of a fluorochemical plant: influence of maternal transfer and diet

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ABSTRACT

Perfluoroalkyl acids (PFAAs) are a focus of scientific and regulatory attention nowadays. However, PFAAs dynamics in the environment and the factors that determine wildlife exposure are still not well understood. In this study we examined PFAAs exposure in chicks of a generalist seabird species, the lesser black-backed gull (*Larus fuscus*), breeding 49 km away of a PFAAs hotspot (a fluorochemical plant in Antwerp, Belgium). In order to study the pathways of PFAAs exposure, we measured how chicks' PFAAs burden varied with age, sex, and body condition. In addition, we related PFAA concentrations to chicks' diet using stable isotope signatures. For this purpose, we studied plasma PFAA concentrations in 1-week and 4-week-old gull chicks. Only 4 (PFOS, PFOA, PFDA and PFNA) out of the 13 target PFAA compounds were detected. Measured concentrations of PFOS and PFOA were generally high compared to other seabird species but were highly variable between individuals. Furthermore, our results suggest that maternal transfer plays a significant role in determining chicks' PFAAs burden, and that there are variable sources of exposure for PFOS and PFOA

during post-hatching development. The association between PFOS and specific stable isotopes (i.e. $\delta^{15}N$ and $\delta^{13}C$) suggests a higher exposure to PFOS of birds with a predominantly marine diet. We also found that males' condition was positively associated with PFOS plasmatic concentration, probably due to the indirect effect of being fed a high quality (marine) diet which appears PFOS rich. Yet, exact exposure source(s) for PFOA remain(s) unclear. Given that PFOS concentrations measured in some chicks surpassed the toxicity reference value calculated for top avian predators, continued monitoring of exposure and health of this gull population, and other wildlife populations inhabiting the area, is highly recommended.

Capsule: High concentrations of PFAAs were found in lesser black-backed gull chicks in the vicinity of a 3M plant. Uptake likely occurs via the diet, as e.g. marine diet increased PFOS exposure risk.

Keywords: PFAS, maternal transfer, stable isotopes, PFOS, PFOA.

INTRODUCTION

Perfluoralkyl acids (PFAAs) are anthropogenic substances that have been produced and widely used for more than 50 years. The two main classes of PFAAs are perfluoroalkyl carboxylic acids (PFCAs) and perfluoroalkyl sulfonic acids (PFSAs; Buck et al. 2011).

Scientific interest in PFAA compounds has grown dramatically since the early 2000s, when it became evident that most known PFAAs (i.e. perfluorooctanoic acid (PFOA, C7F15COOH) and perfluorooctane sulfonic acid (PFOS, C8F17SO3H)) were globally contaminating the environment and biomagnifying in the food chain (Giesy and Kannan 2001; Routti et al. 2015; Yamashita et al. 2005). This stimulated regulators and the industry to act in order to reduce the release of these compounds. In 2002, 3M, the main PFOS producer, voluntarily phased out the production of this compound 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.

Despite restrictions, LC-PFAAs remain ubiquitous and are still found in high concentrations in wildlife (Custer et al. 2019; Sedlak et al. 2017; Sun et al. 2019) and humans (Calafat et al. 2007; Song et al. 2018), while the intrinsic (sex, age, body condition) and extrinsic (food resources) factors that determine wildlife exposure are not yet well understood (Kannan 2011; Land et al. 2018; Prevedourus et al. 2006; Carravieri et al. 2020), especially in highly contaminated areas where the exposure profile is different from that found in remote areas (Armitage et al. 2009b).

Maternal transfer and dietary exposure are known to be two important exposure pathways to PFAAs in wild bird chicks (Bertolero et al. 2015; Custer et al. 2014; Gebbink et al. 2011; Lopez Antia et al. 2019), but the relative importance of these pathways for PFAA congeners has hardly been studied. Moreover, while long-chain PFAAs are known to biomagnify in the food web (Ballutaud et al. 2019; D'Hollander et al. 2014; Haukas et al. 2007), the association between diet and PFAAs exposure has rarely been demonstrated at the individual level (Bustnes et al. 2013; Loseth et al. 2019; Vicente et al. 2015), probably due to the complexity of the factors that govern the exposure to PFAAs in wildlife.

In addition, contradictory results have been reported when the relationship between PFAA compounds, age and condition of wildlife was investigated. Although some PFAAs compounds are known to be bioaccumulative (Haükas et al. 2007), there is no clear pattern how PFAA concentrations increase with age (Bustnes et al. 2013; Carravieri et al. 2020; Dauwe et al. 2006; Holmstrom and Berger 2008; Loseth et al. 2019; Route et al. 2014). Regarding condition, studies have found negative (Barghi et al. 2018; Van den Vijver et al. 2004), positive (Barghi et al. 2018; Tartu et al. 2014) or no associations (Hoff et al. 2005; Svendsen et al. 2018; Tartu et al. 2017) between several PFAA compounds and body condition. This could relate to the fact that a higher food intake could produce a concurrent increase in both body condition and PFAAs concentration, when the contamination of food resources used by the individual is rather homogeneous (i.e. an individual's food resources are all equally contaminated). On the other hand, an increase in body weight could produce a dilution effect, and thus a reduction of PFAAs concentration, if the contamination of food resources used by the

individual is rather heterogeneous (e.g. only a few resources are contaminated but very highly). Finally, negative effects of PFAAs on the body condition, associated with their toxicity, cannot be ruled out and may also be species specific.

Several studies have demonstrated the presence of a PFAAs contamination hotspot in Antwerp (Belgium) related to a fluorochemical plant (Dauwe et al. 2007; Groffen et al. 2017, 2019; Hoff et al. 2005; Lasters et al. 2019; Lopez-Antia et al. 2017). Even in recent days, years after the plant has stopped producing PFOS, concentrations of this compound, and also of other PFAAs, in wild birds in the vicinity of the plant are among the highest ever registered in wildlife (Groffen et al. 2019; Lopez-Antia et al. 2019) and no clear temporal trend has been observed (Groffen et al. 2017, 2019; Lopez-Antia et al. 2019). Thus, it is of vital importance to continue monitoring wildlife exposure around this contamination hotspot.

In the current study, we examined exposure patterns in a seabird species, the lesser black-backed (LBB) gull (*Larus fuscus*), breeding in The Netherlands, in the neighbourhood (49 km away) of the above-mentioned fluorochemical plant. This breeding area is connected to the fluorochemical plant by the Scheldt river, which may transport the contamination downstream (Figure S1). LBB gulls are generalists using a wide range of resources and show a high level of individual variation in diet, with individuals having an almost exclusively terrestrial diet to individuals with an almost exclusively marine diet (Camphuysen 2011, Camphuysen et al. 2015). This makes the LBB gull a suitable species to study how dietary preferences may shape the exposure to contaminants. A common way of characterizing diet is to use stable isotopes (SI) of nitrogen (δ15N) and carbon (δ13C) as proxies of trophic position and carbon source (e.g. terrestrial vs marine) respectively (Kelly 2000). A previous study performed in a LBB gull breeding colony in Belgium, close to that of our study population (Santos et al. 2017), found that chicks with a marine signature (i.e. mainly fed with marine preys) tended to present higher concentrations of mercury compared to those with a terrestrial signature. However, associations between SI signature and PFAA concentrations have rarely been found in birds (Bustnes et al. 2013; Gomez-Ramirez et al. 2017; Haukås et al. 2007; Leat et al. 2013; Loseth et al. 2019; Vicente et al. 2015; Carravieri et al. 2020).

In the present study, we study contamination in chicks, which is especially relevant due to their greater susceptibility to PFAAs during early stages of development (Houde et al. 2006; Lau et al. 2004; Vasseur and Cossu-Legille 2006). We examined how intrinsic factors (sex, age, body condition) and the feeding behaviour regulate PFAAs accumulation with increasing age. Due to the proximity of the colony to a fluorochemical plant, we expect to find high levels of PFAAs on average, but we hypothesize that variation in PFAAs between chicks will be found, which we expect to depend on their predominant diet. Moreover, we expect that PFAAs concentrations will increase with age and that negative effects on growth and body condition may occur on heavily exposed chicks. However, negative effects of PFAAs may be counteracted by the positive effects of diet if PFAAs are associated with higher quality food resources.

MATERIAL AND METHODS

Field work

Lesser black-backed gulls were monitored from mid-April until mid-July in 2015 in the colony of Vlissingen-Oost, the Netherlands (51°27′N, 3°42′E; Figure S1). The colony hosts approximately 4500 ground-breeding pairs. While the population occupies a wide ecological niche and makes use of marine, anthropogenic and terrestrial resources, most individuals in this population are highly specialized foragers that consistently use the same resources (Kavelaars 2020). Egg laying starts by the end of April, and the modal clutch size is 3 eggs. Subsequently, both parents jointly raise their chicks (Kavelaars et al. 2019; Kavelaars 2020). However, in the current study, pairs were subjected to either low demand (1 chick) or high demand (3 chicks; natural brood size in this study population is 1.7 at fledging), as part of a brood size manipulation experiment (for more details see Kavelaars et al. 2019). All broods only contained unrelated foster nestlings, that hatched from first or second laid eggs and that were of the same age. Nests were checked every other day (laying interval is about 48h) and each egg was marked with the laying date and laying position using a non-toxic marker. Enclosures (built using chicken wire) were built around each nest (circa 2 x 2 m in size, and 0.3 m high) to ensure that the chicks stayed close to the nest during the entire developmental period so measurements could be taken. PVC tubes were added to provide shelter. On

147 the day of hatching, chicks were individually marked with colour-tape and down feathers
148 were collected for molecular sexing. Chicks show a logistic growth curve and they are
149 fully grown by day 30 (Camphuysen 2011). Chick mortality was recorded every 2-3 days
150 until fledging on day 30, and on day 7±1 (hereafter called 1-week-old chicks; n=15
151 chicks from 7 low demand and 5 high demand nests) and day 28±1 (hereafter called 4-
152 week-old chicks; n=44 chicks from 14 low demand and 15 high demand nests) blood
153 samples were taken and body mass (precision of 0.1g), tarsus and head length
154 (precision of 0.01 mm) were measured. Some chicks were sampled at both ages (n=9
155 chicks). Samples were kept refrigerated using freezer blocks in the field and upon return
156 in the laboratory, the plasma was separated from the red blood cells and stored at -18
157 ˚C.

158 Body condition was calculated using tarsus length to correct body mass according to
159 the scaled mass index proposed by Peig and Green (2009). This method has proven to
160 be a good indicator of relative size of energy reserve.

161 ***Stable isotope analysis***

162 In the lab, plasma samples were homogenised before taking 60 µl subsamples for
163 further processing. Stable isotope analyses of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) were
164 measured in continuous flow by mass spectrometry (Isoprime 100, Isoprime, UK)
165 coupled to an elemental analyser (Vario Microcube, Elementar, Germany). Analyses
166 were conducted at the Laboratory of Oceanology, MARE Centre at the University of
167 Liège following the method described in Thiebot et al. (2015).

168 Lipid concentration (C:N ratio) was >4 for all plasma samples (range: 4.6 to 11.3)
169 indicating a high lipid content. Lipids are depleted in $\delta^{13}C$ relative to proteins and
170 carbohydrates (Post et al. 2007; Tieszen et al. 1982). Thus, in order to avoid bias in
171 $\delta^{13}C$, values were corrected for lipid content for all samples according to Post et al.
172 (2007).

173 The half-life of these isotopes is estimated to be 2.6 days in plasma (Hobson and Clark
174 1993), and therefore the isotopic measurement in the blood plasma samples reflects the
175 diet that the chicks received over a week.

PFAAs analysis

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.

Samples were extracted by using the solid-phase extraction technique described by Groffen et al. (2019), a technique optimized to be able to measure PFAAs in small amounts of plasma (≈10 µL). Briefly, plasma samples were vortex-mixed for 1 minute and 10 µL were transferred to a new tube. Then, 80 µL of isotopically mass-labelled internal standard (IS) mixture (Wellington Laboratories (Guelph, Canada)), and 10 mL of acetonitrile (ACN) were added to the plasma sample. Tubes were sonicated 3 consecutive times (10 minutes each time) with vortex-mixing in between and samples were left overnight on a shaking plate. Tubes were centrifuged and the supernatant was transferred into a 14 mL tube and loaded on HR-XAW columns. The eluent was completely dried using a rotational-vacuum-concentrator at 30°C (Eppendorf concentrator 5301, Hamburg, Germany), reconstituted with 200 µL 2% ammonium hydroxide in ACN and vortex-mixed for at least 1 minute. Prior to the analysis, samples were filtrated through an Ion Chromatography Acrodisc 13 mm Syringe Filter with 0.2 µm Supor (PES) Membrane (VWR International, Leuven, Belgium).

To separate PFAAs, an ACQUITY BEH C18 column (2.1 x 50 mm; 1.7 µm, Waters, USA) was used. Mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid in ACN (B). Solvent gradients were 65% A to 0% A in 3.4 min and 65% A at 4.7 min. The injection volume was 10 µL at a flow rate of 450 µL/min, with a total run time of 6.7 min. An ACQUITY BEH C18 pre-column (2.1 x 30 mm; 1.7 µm, Waters, USA) was inserted between the solvent mixer and injector, to retain any PFAAs contamination originating from the system. Identification and quantification of individual PFAAs was based on multiple reaction monitoring (MRM) of two diagnostic transitions per analyte or IS.

Calibration was performed by adding a constant amount of IS to varying amounts of non-labelled standards, ACN and water, to construct calibration curves. Individual PFAAs were quantified using their corresponding IS with exception of PFPeA,

PFHpA, PFTrDA, PFTeDA, PFBS and PFDS, which were all quantified using the ISTD of the compound closest in terms of functional group and size. We obtained good recoveries (> 90 %) for all compounds except for PFBS and PFHxS, for which recoveries were too low and therefore they were excluded from further analysis

The quality of the method was assured by regular analysis of procedural blanks (one per batch of 10 samples) and contained no contamination. The limit of quantification (LOQ) was determined, based on a signal-to-noise ratio of 10 and ranged from 1.4 to 8.4 pg μL^{-1} for all compounds with the exception of PFOS (46.6 pg μL^{-1}) and PFPeA (52.4 pg μL^{-1}) which had considerably higher LOQs due to high noise.

Modelling and statistical analysis

To perform statistical analyses, we used JMP Pro 14 statistical software. As the sources of exposure may differ in 1-week and 4-week-old chicks (maternal influence will be higher in 1-week-old chicks), we analyzed data from both age groups separately. The significance threshold level was set at $p \leq 0.05$. When necessary, values below the LOQ were replaced by a randomly assigned value >0 and <LOQ.

To estimate the impact of growth dilution on PFAA concentrations we followed the method used by Bustnes et al. (2013). For this, we assumed that blood volumes remain a constant fraction of body weight over the whole growth period and that PFAAs in plasma rapidly valances with the rest of the body (Tarazona et al. 2015). We divided the concentrations found in 1-week-old chicks by the relative body mass increase (body weight at 4 weeks/body weight at 1 week) to calculate the theoretical concentration that would be found in the 4-week-old chicks after growth dilution (in absence of any other exposure). This was done for 9 chicks that were sampled at both ages.

PFOS had detection frequencies >70% at both ages and thus, for statistical analyses, values below the LOQ were replaced as described previously. PFOS concentrations did not follow a normal distribution so data was Ln-transformed. Mixed models, with the nest as random effect, were used to study how PFOS concentrations depended on sex, the number of chicks in the nest (i.e. low demand or high demand), body condition and SI (fixed factors). Since the effect of body condition on PFOS may differ between males

and females, the sex*body condition interaction was included in the model. Variance Inflation Factors (VIF) for all predictor variables were ≤4.0, thus collinearity was not considered an issue in the models.

In birds measured twice, the correlation between PFOS concentrations at two different ages was tested using a non-parametric Spearman's test ρ. To compare concentrations in the same bird at different ages and to compare concentrations found in 4-week-old chicks with the theoretical concentrations that would be found after growth dilution (in absence of any other exposure) paired sample tests (with PFOS concentrations Ln-transformed) were used. A standard least square test was performed to test for associations between PFOS concentrations (at both ages) and relative mass increase.

PFOA had detection frequencies ≤50% at both ages and therefore, methods for left censored data (Shoari and Dube 2018) were used for most of the analyses (see the paragraph below). For a few analyses, it was not possible (or we considered it was not advisable) to use methods for left censored data, this was the case for: i) correlation between PFOS and PFOA concentrations (Spearman's test ρ was used), ii) comparison of the PFOA concentrations in the same bird at different ages (paired sample test was used), iii) comparison of the PFOA concentrations found in 4-week-old chicks and the theoretical concentrations that would be found after growth dilution (paired sample test was used); in these analyses, values below the LOQ were replaced as previously described.

Left censored data were analyzed in JMP using a Generalized Regression personality where the left censored Y response (in this case, PFOA concentration) was represented by two response columns (variables): in the first Y column values below the LOQ were missing, in the second Y column these values below LOQ were replaced by the LOQ (for more information see the JMP Help link in the reference list). Left censored data methods do not support the inclusion of random effects so we cannot control for the effect of the common rearing environment in these models. A potential solution for this would be to use mean values per nest but that would imply to disregard sex as fixed factor. Moreover, we observed a high variation within nests in PFOA concentrations for

both ages (see Figure S2), so we decided to analyze every chick as an independent sample.

Generalized regression models were constructed to study the association between PFOA concentration and the different variables (i.e. age, sex, body condition and SI). Models were built as above described for PFOS, but with the previously explained particularities (i.e. no random effect of the nest, and PFOA concentration was represented by two response columns that were computed as censored data).

In birds measured twice, we used generalized regressions to study associations of PFOA (at both ages) with mass increase.

In 4-week-old chicks, there was an extreme outlier for PFOA concentration (=38.9 pg μL^{-1}). This chick was included in descriptive statistics, but it was removed for all other statistical analyses.

RESULTS AND DISCUSSION

PFAAs concentrations and comparison with other bird species.

From the 13 target PFAA compounds (two out of the 15 initial ones were left out because they presented very low recoveries), we detected four with at least one value above the LOQ: PFOS, PFOA, PFDA and PFNA. For each compound, mean and median concentrations, range and detection frequency are shown in Table 1. PFNA was only detected in two 1-week-old chicks. PFDA was detected in three 1-week-old, and two 4-week-old chicks. All subsequent statistical analyses were therefore restricted to PFOS (detection frequency=81%) and PFOA (42%). PFOS ($F_{1,51.9}$=1.5; p=0.22) and PFOA (Wald χ^2=0.003; n=58; p=0.95) concentrations were not significantly different according to age, and their concentrations were not significantly correlated in 1-week-old chicks (Spearman's test p=0.13; n=15; p=0.64), but were correlated in 4-week-old chicks (Spearman's test p=0.43; n=43; p=0.004). Chicks' PFOS (1-week-old: $F_{1,12.2}$=2.83; p=0.118. 4-week-old: $F_{1,18.5}$=0.26; p=0.62) and PFOA (1-week-old: Wald χ^2=0.739; p=0.39. 4-week-old: Wald χ^2=1.41; p=0.23) concentrations were not affected by sex in either age group.

PFOS and PFOA concentrations found in this study were high in comparison to the concentrations found in other seabird species elsewhere (Table 2). PFOS concentrations are only surpassed by those found in bald eagle (*Haliaeetus leucocephalus*) nestlings from upper Midwestern United States (Route et al. 2014), an area highly contaminated by the presence of a 3M fluorochemical plant. PFOA concentrations were higher in LBB gull chicks (geometric mean=3.54 pg μL^{-1}) than those found in bald eagle nestlings (max geometric mean=1.0 pg μL^{-1}; Route et al. 2014) but were lower (mean±SD=7.6±9.2 pg μL^{-1}) than those found in adult European shags (*Phalacrocorax aristotelis;* mean±SD in males=27±8.8 pg μL^{-1}) breeding in a Scottish estuary (Carravieri et al. 2020). PFDA and PFNA concentrations (in the two chicks that presented concentrations above the LOQ) were in the same range as those found in the Scottish shags (Carravieri et al. 2020) and were only surpassed by the concentrations found in bald eagle nestlings in the USA (Route et al. 2014). On the other hand, the number of compounds detected was low. All previous studies included in table 2 detected a minimum of six PFAA compounds. Also, the detection frequencies were quite low when compared with other seabird studies (Table 2). These differences are probably due to differences in detection limits between studies, with our method, which allows us to work with very small amounts of plasma, presenting a higher LOQ for several compounds (Table 2). In addition to this, different profiles of PFAAS between these bird species could also be due to differences in the origin of the contamination (Armitage et al. 2009b); while in the case of the LBB gulls from our study, the source of contamination was probably direct emitted PFAA compounds (during manufacturing and use), in the case of arctic birds, indirect sources (degradation of precursors such us fluorotelomer alcohols) probably played a more important role (Armitage et al. 2009a; Ellis et al. 2004; Pickard et al. 2018). Our results also indicate that a high spatial heterogeneity in PFAAs distribution exists in our study area, as some animals were exposed to very high concentrations while others were almost not exposed (i.e. 23% of the 4-week-old chicks presented concentrations <LOQ for all the compounds, Table 1). This could be related to the kind of diet the chicks received (terrestrial or marine) and/or to the areas used for foraging (highly contaminated versus less contaminated areas) by the parents.

A previous study performed in LBB gulls breeding in Norway found PFOS concentrations from 8.3 to 37.7 pg μL^{-1} in whole blood from adults (Bustnes et al. 2008), with a prevalence of 100%. That study did not find detectable concentrations of PFOA, PFDA or PFNA but did detect five PFAA compounds in addition to PFOS. Mean ∑PFAA concentration found in Norwegian gulls was 42.4 pg μL^{-1}, while we found a mean ∑PFAA concentration of 199 and 167 pg μL^{-1} in plasma of 1-week and 4-week-old chicks respectively. In order to compare these two studies, we must consider that concentrations in whole blood are estimated to be 2 to 5-fold lower than in plasma (Kannan et al. 2001), which would mean that our values are similar or slightly lower than those found in the LBB gulls from Norway ten years earlier.

The highly variable plasma concentrations found in the current study (i.e. few individuals being highly contaminated while others presenting concentrations above the LOQ), are probably due to the fact that the breeding colony is located in the proximity of the fluorochemical plant (3M) in Antwerp (Belgium). This area has demonstrated to be one of the main hotspots for PFAAs pollution in Europe, and wildlife of this area present the highest concentrations ever reported for most PFAA compounds analyzed (e.g. D'Hollander et al. 2014; Groffen et al. 2019; Lopez-Antia et al. 2017, 2019). Two different seabird species, breeding somewhat closer to the plant, were also investigated previously. Here, concentrations of PFOS (mean ± SD) measured in adult individuals of the Mediterranean gull (*Ichthyaetus melanocephalus*) breeding in 2006 in Zandvliet (Belgium, 14.5 km away from the plant; Lopez Antia et al. 2017) were 422 ± 275 pg μL^{-1}. Mean PFOS concentrations in common terns (*Sterna hirundo*) breeding in 2007 in Terneuzen (The Netherlands, 33 km away from the plant; Van den Brink et al. 2007) were 376 ± 119 pg μL^{-1}. These concentrations are higher than the ones found in the current study (Figure S3). For an among-species/studies comparison, it must be considered that 3M phased out the production of PFOS in 2002 (3M, 2000), which should have resulted in a lower exposure of wildlife to PFOS over time (D'Hollander et al. 2014). However, whether this has been achieved is currently unclear (Groffen et al. 2017, 2019; Lopez-Antia et al. 2019).

Exposure vs growth dilution: age effect in chicks sampled repeatedly

A repeated sampling at consecutive time points was possible for 9 chicks. The results for each chick are presented in Figures 1a (PFOS) and 1b (PFOA). There was no correlation between the concentrations of PFOS (Spearman's test p=-0.02; p=0.97) or PFOA (Spearman's test p=-0.08; p=0.83) measured at two different ages in the same chick. Concentrations of PFOS (t_8=0.31; p=0.76) and PFOA (t_8=1.59; p=0.15) did not differ significantly between different ages (i.e. there is not age effect). In the previous section, when all chicks were considered, we also did not detect any age effect on PFOS and PFOA concentrations. Previous studies performed in chicks of several raptor species detected both positive effects of age on PFOS (Bustnes et al. 2013) and PFAAs concentrations (Loseth et al. 2019) and no age effect on PFOS and PFOA concentrations (Route et al. 2014). It is important to highlight that our study, together with Bustnes et al. (2013), are the only ones that used a longitudinal approach, which makes data much more valuable. However, the small sample size in our study prevents us from drawing strong conclusions.

PFOS and PFOA (together with other PFAA compounds) are known to be transferred from the mother to the chicks through the egg (Bertolero et al. 2015; Custer et al. 2014; Gebbink and Letcher 2012; Lasters et al. 2019; Lopez-Antia et al. 2019), which suggests that concentrations found in chicks are to some extent reflecting maternal exposure. However, if this would have been the only source of exposure, concentrations found in the plasma of 4-week-old chicks would have been lower than the ones found one week after hatching due to growth dilution. We estimated the mean theoretical concentrations that would be found in the 4-week-old chicks after growth dilution (in absence of any other exposure). These theoretical concentrations were significantly lower (PFOS: 3.6-fold lower, t_8=4.4; p=0.002. PFOA: 7.9-fold lower, t_8=3.1; p=0.015) than the ones found (Figure 2a, 2b). This indicates that, in addition to the maternal transfer of PFOS and PFOA, there was an additional influx of contaminants post-hatching. A study performed in goshawks (*Accipiter gentilis*) and white-tailed eagle (*Haliaeetus albicilla)* nestlings found a negative relationship between growth rate and PFOS, indicative of growth dilution, and a strong increase in PFOS plasmatic concentrations with time, indicative of the existence of an alternative (to maternal transfer) route of exposure (Bustnes et al. 2013).

On the other hand, the temporal individual patterns found in the chicks sampled at both sampling times, where PFOA levels increased and PFOS decreased in most of the chicks (Figure 1a, 1b), suggest that the relative importance of the alternative source (or sources) of exposure, compared with maternal transfer, was higher for PFOA than for PFOS. Similarly, a recent study that analysed plasmatic PFAAs concentrations in great tit mothers, eggs and nestlings (2-week-old) breeding in Antwerp (Belgium), showed that the main exposure of nestlings to PFOS occurred through maternal transfer and/or the diet provided by the parents, while this was not the case for PFOA, for which exposure to volatile precursor substances (i.e. fluorotelomers) was suggested as a possible exposure route (Lopez-Antia et al. 2019). It is important to consider that there was a limited number of individuals sampled at both sampling times and that the experimental design, a cross-fostering study, may have disrupted the expected correlation between concentrations in week one and four.

Association between PFAAs exposure, body condition and diet

Values for all biometrical parameters are shown in Table S1. The effect of the number of chicks in the nest on all biometrical parameters in 4-week-old chicks are shown in Figure S4.

No associations were found between mass increase and PFOS concentration (1-week old (t_7=1.2; p=0.27). 4-week-old (t_7=-0.12; p=0.90)) or PFOA concentration (1-week-old (Wald χ^2=0.03; p=0.87). 4-week-old (Wald χ^2=1.04; p=0.31)) in chicks sampled twice. We must consider that the number of chicks sampled twice (n=9) was limited.

There were no differences in plasma stable isotopes values according to age, sex, or brood size. $\delta^{13}C$ and $\delta^{15}N$ were highly correlated (R=0.74; p<0.0001; N=59; Figure 3). SI plasmatic values show high inter-individual variation that extends continuously from a predominantly terrestrial diet (dots closer to the origin) to a predominantly marine diet (dots farthest from the origin), which is in accordance with what was previously described for this species (Camphuysen 2011, Camphuysen et al. 2015; Kavelaars 2020; Santos et al. 2017).

In 1-week-old chicks, no associations were found between PFOS concentration and body condition ($F_{1,9}$=0.27; p=0.62), $\delta^{13}C$ ($F_{1,5.4}$=0.36; p=0.57) or $\delta^{15}N$ ($F_{1,6.3}$=1.1; p=0.34) . In 4-week-old chicks, $\delta^{15}N$ ($F_{1,34.6}$=18.7; p=0.001) and $\delta^{13}C$ ($F_{1,34.1}$=9.6; p=0.004) were significantly associated with PFOS concentration (Figure S5) and a significant interaction existed for body condition*sex ($F_{1,35.8}$=5.1; p=0.03). Model parameter estimates indicated that PFOS and body condition were positively and significantly related in males (slope ± SE= +0.0034 ± 0.0021) but not in females (-0.0012 ± 0.0021). The association between body condition and PFOS in 4-week-old male chicks is shown in Figure 4.

PFOA concentrations were not associated with body condition (Wald χ^2=0.04; p=0.84), $\delta^{13}C$ (Wald χ^2=0.25; p=0.62) or $\delta^{15}N$ (Wald χ^2=0.06; p=0.81) in 1-week-old chicks (n=15). In 4-week-old chicks (n=41), PFOA was negatively and significantly associated with $\delta^{13}C$ (Wald χ^2=4.4; p=0.03), positively and significantly associated with body condition (Wald χ^2=4.1; p=0.04) and not associated with $\delta^{15}N$ (Wald χ^2=2.5; p=0.12), but a significant interaction existed for body condition*sex (Wald χ^2=5.5; p=0.02). A separate analysis for each sex was also performed for this age group. For males (n=17), body condition (Wald χ^2=8.8; p=0.003) and $\delta^{13}C$ (Wald χ^2=10.1; p=0.001) were positively and negatively associated with PFOA respectively (no association was found with $\delta^{15}N$ (Wald χ^2=2.8; p=0.10)) , while for females (n=24), no significant association were found with $\delta^{13}C$ (Wald χ^2=2.5; p=0.11), $\delta^{15}N$ (Wald χ^2≤2.9; both p≥0.08) or body condition (Wald χ^2=1.8; p=0.18).

Marine environments are enriched with $\delta^{15}N$ and δ13C compared to terrestrial environments (Kelly 2000; Schoeninger and DeNiro 1984) and thus, the positive association we found between PFOS concentration and $\delta^{15}N$ / $\delta^{13}C$ points to a higher exposure to PFOS of birds with a predominantly marine diet. Previous studies demonstrated that PFAAs concentrations are normally higher in fish-eating birds than in those with a terrestrial diet (Gomez Ramirez et al. 2017; Lau et al. 2007; Meyer et al. 2009; Yoo et al. 2008), but this association between SI signature and PFAAs has rarely been found. To the best of our knowledge, only one study performed in birds has found a significant association between $\delta^{15}N$ / $\delta^{13}C$ and PFAAs (Gebbink et al. 2011). In

442 marine mammals, positive associations have been found in harbour porpoise
443 (*Phocoena phocoena;* Van de Vijver et al. 2004) and polar bear (*Ursus maritimus;* Tartu
444 et al. 2017). Yet, most bird studies did not find any correlation (Bustnes et al. 2013;
445 Carravieri et al. 2020; Gomez-Ramirez et al. 2017; Haukås et al. 2007; Leat et al. 2013;
446 Loseth et al. 2019; Vicente et al. 2015).

447 On the other hand, we did not find an association between PFOS and SI in 1-week-old
448 chicks. A possible explanation for this could be that both PFOS concentrations
449 (Bertolero et al. 2015; Gebbink and Letcher 2012; Lopez-Antia et al. 2019) and SI
450 signature (Hobson 1995; Sears et al. 2009) could be highly affected by maternal (egg)
451 transfer in 1-week-old chicks. Moreover, it has been demonstrated that growth can
452 affect $\delta^{15}N$ in an ecologically meaningful way (i.e. $\delta^{15}N$ is negatively correlated with
453 growth rate due to a more efficient use of nitrogen in growing birds; Sears et al. 2009;
454 Williams et al. 2007). These factors, together with the low sample size of 1-week-old
455 chicks, may be obscuring an association between PFOS and SI in very young chicks.

456 In the case of PFOA, the main source of exposure to this compound is not clear. Yet,
457 PFOS and PFOA concentrations were correlated in 4-week-old chicks, suggesting
458 some relationship in the sources of exposure to both compounds. However, no
459 associations were found between PFOA and SI signatures unless body condition is
460 considered, and in that case only males presented an association in which body
461 condition and $\delta^{13}C$ seemed to be positively and negatively associated with PFOA
462 respectively. Thus males with better condition and a more terrestrial diet presented
463 higher concentrations of PFOA. One possible explanation for these confounding results
464 could be that a high spatial or prey specific heterogeneity exists in PFOA distribution,
465 which would result in a weak relationship with the diet (Elliot et al. 2009). Our findings
466 that some individuals presented very high concentrations while others were below the
467 LOD and the high heterogeneity found even within nests (Figure S2), support this
468 hypothesis. This pattern would be reinforced with the short depuration half-life of PFOA
469 in birds (=4.6 days in chickens; Yoo et al. 2009), which means that plasmatic
470 concentrations of this compound only reflect the exposure in the last few days. In any

471 case, these results should be considered cautiously due to the low frequency of values
472 above the LOD (e.g. 5 out of 17 for males) detected for PFOA.

473 Body condition was positively and significantly associated with the PFOS concentrations
474 in 4-week-old male chicks. Males grow to a larger size than females, and are hence
475 more sensitive to food limitations (Müller et al. 2005), which could emphasize the
476 relationship of a high-quality diet (e.g. marine diet) with growth, while this diet also has
477 higher PFOS concentrations. Then potentially toxic effects of PFOS (Newsted et al.
478 2005) are counteracted by the positive effects of more nutritious food. In accordance
479 with our results, a recently published study also detected a positive relationship
480 between PFOS and body mass in breeding European male shags, but not in females
481 (Carravieri et al. 2020). Previous studies either found no association (Aas et al. 2014;
482 Haukas et al. 2007; Hoff et al. 2005; Lopez-Antia et al. 2019; Sletten et al. 2016;
483 Svendsen et al. 2018; Tartu et al. 2017) or found a negative association (Barghi et al.
484 2018; Van den Vijver et al. 2004) between these two parameters. If we consider other
485 PFAA compounds, positive associations were found between PFNA and body condition
486 again in males, but not in females, of Black-legged Kittiwakes breeding in Svalvard
487 (Tartu et al. 2014).

488 In addition to the greater influence of diet on males' condition, another not exclusive
489 explanation for the lack of association between PFOS and body condition in females
490 would be a higher sensitivity to PFOS in females (i.e. beneficial effect of consuming
491 more profitable (and contaminated) prey would be counteracted by the negative effects
492 of the pollutant). The Toxicity Reference Value (TRV) estimated for PFOS in female top
493 predators in serum (240 pg µL-1) is lower than that estimated for males (3900 pg µL-1 ;
494 Newsted et al. 2005). These TRVs are based on acute and chronic exposures,
495 considered endpoints being mortality, growth, food consumption and histopathology (for
496 acute exposure) or egg production, fertility, hatchability and offspring growth and
497 survival (for chronic exposure). Although it is not clear whether this lower TRV in
498 females is due to a greater sensitivity to the toxic or to other physiological reasons (the
499 study was carried out on breeding females; Newsted et al. 2005), the possibility of sex
500 differences in toxicokinetics and toxicity of PFOS exists (Lau et al. 2007; Lopez Antia et

al. 2019). Moreover, other sexual differences such as differences in blood chemistry (i.e. females commonly present higher level of total plasma proteins and thus PFAAs binding potential; Carravieri et al. 2020) may also influence PFOS concentrations and their association with body condition.

Toxicity Reference Value for PFOS in avian top predators, regardless of the gender, has been estimated to be 1700 pg µL-1 (Newsted et al. 2005). None of the individuals sampled in this study reached this value, but a greater susceptibility during early stages of development (Houde et al. 2006; Lau et al. 2004; Vasseur and Cossu-Legille 2006) must be considered to rule out any risk. Moreover, TRV estimated for females (240 pg µL-1) was surpassed by 10 female chicks (5 in each age group), pointing at potential long-term problems, which could range from lower reproductive success to higher mortality (Newsted et al. 2005), for these individuals.

CONCLUSIONS

PFOS and PFOA concentrations detected in LBB gull chicks that hatched 49 km away from a fluorochemical plant are high compared with other (sea)bird species elsewhere and are also highly variable between individuals. In the case of PFAAs, exposure occurred both via maternal transfer to the egg, and then again post-hatching via food. We found an association between PFOS and stable isotopes signature, which indicates that the dietary exposure risk increases with a more marine diet. However, the exposure source(s) for PFOA remain unclear. For PFOS and PFOA, the concentrations that were measured in some gull chicks could be a matter of concern and so continued monitoring of exposure and health of this gull population, together with other wildlife populations is highly recommended.

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Table 1. *Mean and median concentrations (pg μL-1), range and detection frequencies (%) of PFAA compounds in plasma of 1-week and 4-week-old lesser black-backed gull chicks sampled at colony of Vlissingen-Oost (the Netherlands).*

Compound	1-week-old (n=15)				4 -week-old (n=44)			
	Mean	Median	Range	Freq %, (n)	Mean	Median	Range	Freq %, (n)
PFOS	189	169	<LOQ–337	94, (14)	160	127	<LOQ–781	77 (34)
PFOA	6.1	<LOQ	<LOQ–20.4	50, (7)	7.6	<LOQ	<LOQ–38.8	41 (18)
PFDA			<LOQ–18.5	20, (3)			16–17	4.5 (2)
PFNA			<LOQ–12.2	13, (2)				0
∑PFAAs	195	170	31–349	100	166	135	25–783	77

<LOQ: Values below the limit of quantification. These values are: PFOS 46.6 $pg\,\mu L^{-1}$, PFOA 2.6 $pg\,\mu L^{-1}$, PFDA 5.5 $pg\,\mu L^{-1}$, PFNA 4.15 $pg\,\mu L^{-1}$

PFOS (perfluorooctane sulfonic acid); PFOA (perfluorooctanoic acid); PFDA (Perfluorodecane acid); PFNA (perfluorononane acid); ∑PFAAs: summatory of all quantified Perfluoroalkyl acids.

Table 2. *Concentrations (range or mean±SD; $pg\,\mu L^{-1}$) and detection frequency of PFAA compounds and ∑PFAAs found in the plasma of scavenging-predatory aquatic bird species (only the results for the 4 PFAAs compounds found in current study have been included). Values below the LOQ are indicated as "<LOQ for that compound on that study".*

Species	Age[1] (N/A)	place	PFOS range (Freq)	PFOA range (Freq)	PFDA range (Freq)	PFNA range (Freq)	∑PFAAs	Study
Glaucus gull	A	Norway	48–349 (100)	nd	3.1–15 (100)	<2.3–6.3 (40)	90–613	Verreault et al. 2005
European shag	A	Norway	30±8.4 (100)	4.7±1.5 (100)	4.6±2.4 (100)	nd	44±15.4	Herzke et al. 2009
Great skua	A	Scotland	9.5–78 (100)	0.01–0.5 (100)	0.–3.4 (100)	0.4–1.6 (100)	19–140	Leat et al. 2013
Black-legged Kitiwakes	A	Svalbard	6.8–15 (100)	<0.03–0.2 (20)	1.2–3.1 (100)	0.9–1.2 (100)	31–86	Tartu et al. 2014
Bald eagle	N	US	6.6–2400 (100)	<0.12–14.6 (86)	<0.12–85 (98)	<0.12–160 (100)	14–7370	Route et al. 2014
White tailed eagle	N	Norway	16–60 (100)	0.4–2.0 (100)	1.0–3.2 (100)	1.6–6.5 (100)	28–79	Gomez-Ramirez et al. 2017
European shag	A	Scottland	63–396 (100)	<1.0–53 (98)	5.7–24 (100)	<1.0–44 (93)	180–600	Carravieri et al. 2020
White tailed eagle	N	Norway	<0.18[2]–249 (99)	<002[2]–7.8 (78)	Not given (81)	<0.09[2]–22.8 (95)	8.8–156	Jouanneau et al. 2020
LBB gulls	N	The Netherlands	<46.6–781 (85)	<2.6–38.8 (45)	<5.5–18.5 (12)	<4.1–12.2 (6)	25–783	Current study

[1] N=nestlings, A=adults

[2] The lower LOQ for all years is given.

PFOS (perfluorooctane sulfonic acid); PFOA (perfluorooctanoic acid); PFDA (Perfluorodecane acid); PFNA (perfluorononane acid); ∑PFAAs: summatory of all quantified Perfluoroalkyl acids.

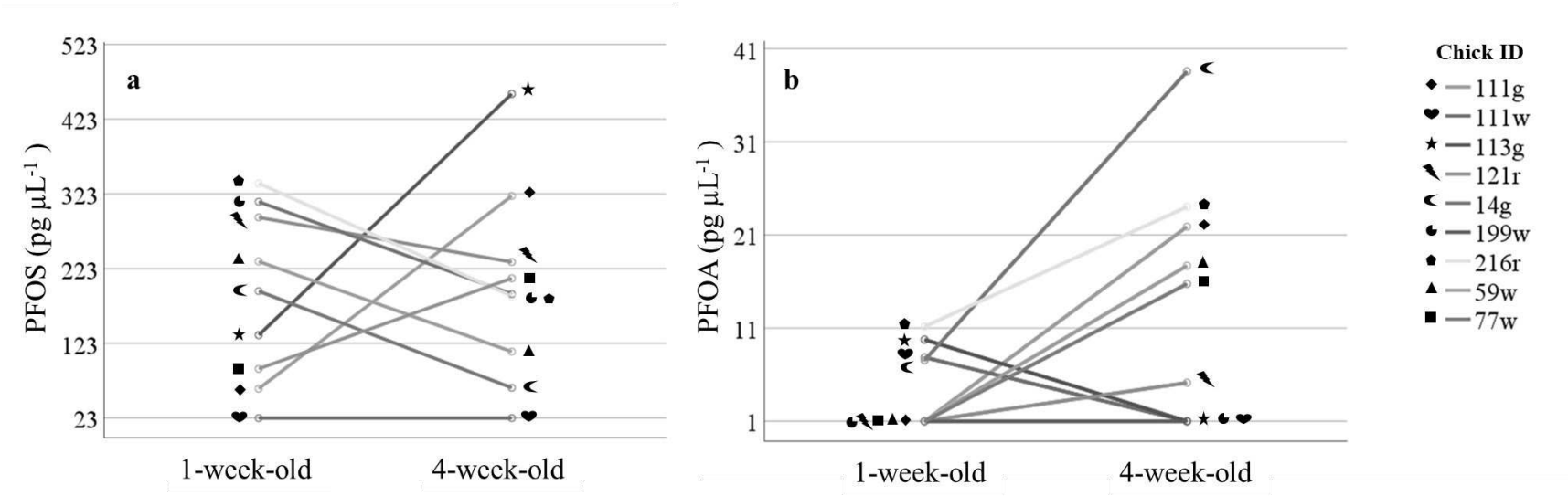

Figure 1a, 1b*. PFOS (1a) and PFOA (1b) concentrations found in 9 chicks sampled 1 and 4 weeks after hatching.* PFOS (perfluorooctane sulfonic acid); PFOA (perfluorooctanoic acid).

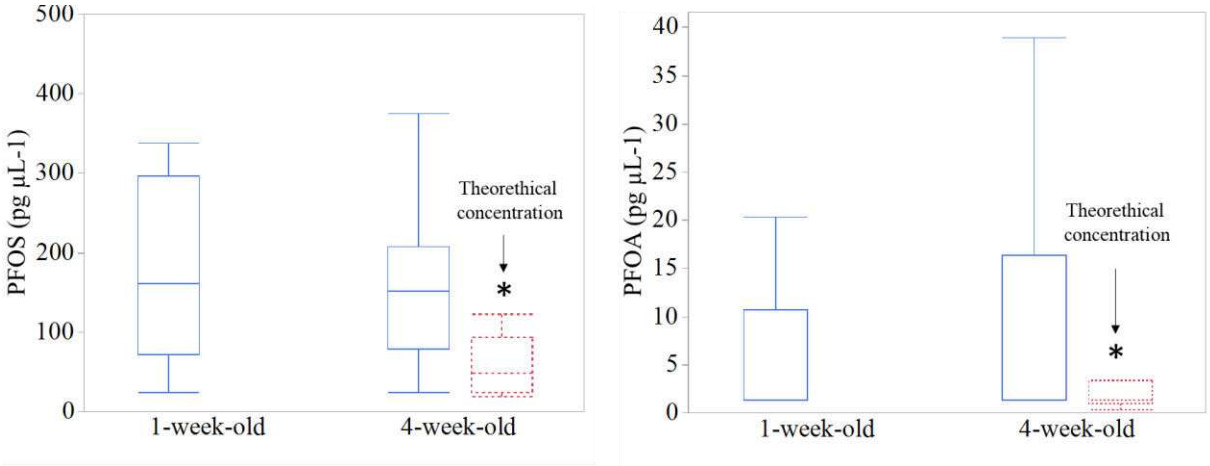

***Figure 2a, 2b**. Box plot of PFOS (perfluorooctane sulfonic acid) and PFOA (perfluorooctanoic acid). concentrations found in the plasma of the nestlings sampled 1 and 4 weeks after hatching and the theoretical concentration that would be found, due to growth dilution, in the absence of additional exposure (dashed line). Theoretical concentrations were calculated dividing concentrations in 1-week-old chicks by the relative body mass increase in each chick (mass at second sampling / mass at first sampling). N=9, only chicks sampled at the both sampling times were used. *Indicates that the theoretical concentration is significantly different from the real concentration. P level ≤ 0.05.*

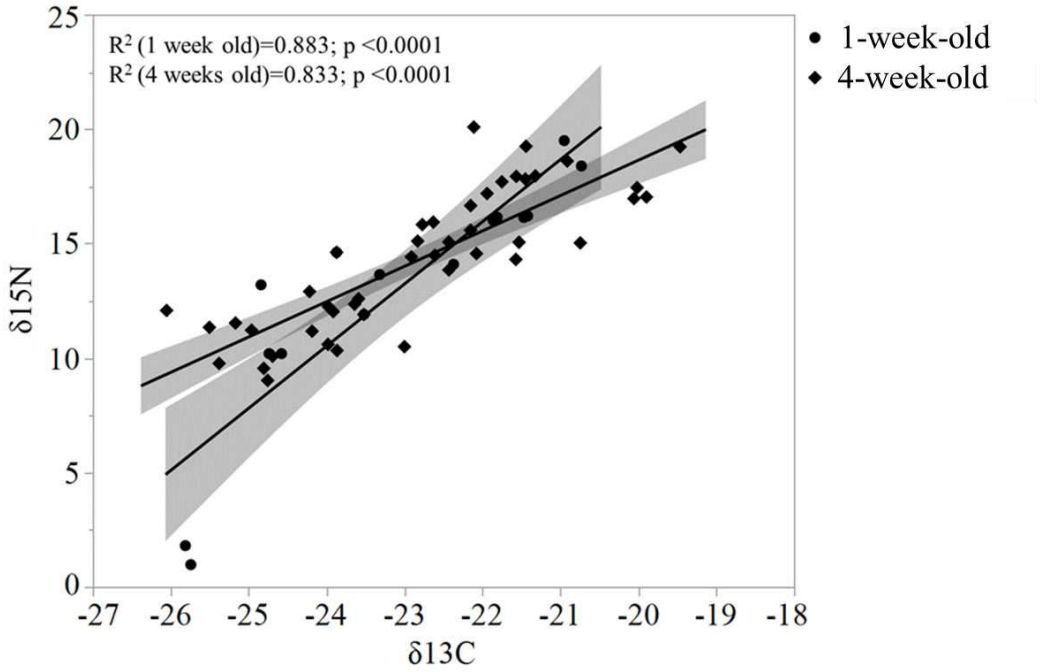

Figure 3. *$\delta^{13}C$ (corrected for lipid content) and $\delta^{15}N$ values measured in 1- and 4-week-old lesser black-backed gulls chicks' plasma. Regression lines are shown with 95% confidence bands shaded.*

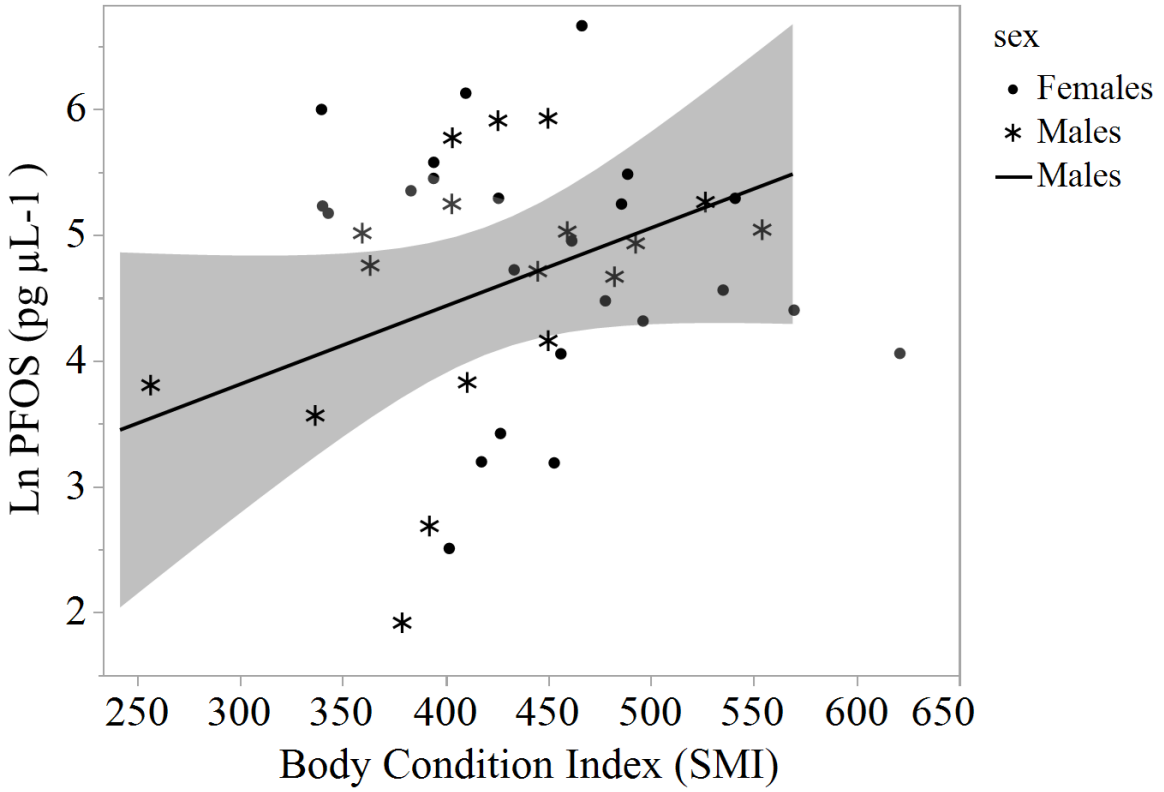

Figure 4. *Association between the Body Condition Index (SMI: Scaled Mass Index) and PFOS (perfluorooctane sulfonic acid) concentration in 4-week-old lesser black-backed gulls chicks separated by sex. Regression line for males is shown with 95% confidence bands shaded.*