Developmental toxicity of perfluorooctane sulfonate (PFOS) and its chlorinated polyfluoroalkyl ether sulfonate alternative F-53B in the domestic chicken

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Abstract

The chlorinated polyfluoroalkyl ether sulfonate F-53B is used as a mist suppressant in the Chinese electroplating industry. Due to the regulations on perfluorooctane sulfonate (PFOS), its use is expected to increase. Until now, F-53B toxicity data have been scarce and are, to our knowledge, lacking for birds. This study therefore investigated the effects of PFOS and F-53B, separately and as mixtures, on the development of the chicken (*Gallus gallus domesticus*). Compounds were injected *in ovo*, before incubation, at 150 and 1500 ng/g egg. At embryonic day 20, a significantly lower heart rate was observed in all treated groups compared to the control group and hatchlings exposed to the high dose of F-53B had a significantly enlarged liver (8 %). Embryonic survival was not affected and no significant effects on hatchling body mass or oxidative stress parameters were found. Our results suggest that these compounds likely have different toxicity thresholds for the investigated endpoints, and/or different modes of action. This study thereby underlines the potential developmental toxicity of PFOS and F-53B at environmentally relevant concentrations. Assessment of PFOS alternatives should therefore continue, preferably prior to their large scale use, as they should be ensured to be less harmful than PFOS itself.

Introduction

Perfluorooctane sulfonate (PFOS) was in production for approximately 40 years before its ubiquity in the environment became apparent by the turn of the century.[1] A voluntary phase-out of PFOS by 3M, the major manufacturer, followed from 2000 to 2002, which started a series of measures to restrict the production and use of the compound. In 2009, PFOS and its salts were listed in Annex B of the Stockholm Convention.[2] However, due to a lack of readily available alternatives, one of the exemptions of the Convention for the production and use of PFOS is the electroplating industry.[2] To avoid the formation of highly toxic chromium vapors during the electroplating process, mist suppressants are added to the metal bath, which increases safety for the workers. These mist suppressants are commonly based on PFOS and its salts.[3]

In China, where the electroplating industry is well developed, the chlorinated polyfluoroalkyl ether sulfonate 6:2 Cl-PFESA (trade name F-53B) has been used as a mist suppressant since the 1970s. Similar to PFOS, the environmental presence and potential hazard of F-53B have been overlooked for decades,[3] and it is likely that due to the reduction in the use of PFOS, the demand for fluorinated alternatives, such as F-53B, will increase. Until now, F-53B has been unregulated and toxicity data are scarce and scattered,[4] however, literature on the topic seems to be quickly expanding. F-53B was first reported in Chinese river water in 2013[3] and was subsequently found in sediment,[5] aquatic organisms from China,[6,7] and even in human serum and placenta.[8,9] A worrisome observation was made by Gebbink et al. (2016) when F-53B was detected in liver of polar bears (*Ursus maritimus*), killer whales (*Orcinus orca*) and ringed seals (*Pusa hispida*) from Greenland, although in relatively low concentrations, indicating long-range transport.[10] Potential for long-range transport similar to PFOS has now been estimated, suggesting that F-53B can potentially reach remote regions and distribute on a global scale.[11] Like PFOS, F-53B was also found in the liver of killer whale fetuses,[10] showing similar

maternal transfer rates for both compounds. The esterification of the fluorocarbon chain of F-53B was thought to make the molecule more degradable than the environmentally persistent PFOS. Nonetheless, current literature suggests similar persistence[3] and even higher bioaccumulative potential of F-53B in comparison to PFOS.[6,12]

Due to its high persistence, PFOS is still one of the most dominating poly- and perfluoroalkyl substances (PFASs) detected in environmental samples. It is commonly found in bird eggs, as a high load of PFOS can be deposited into the egg by the mother.[13–16] The embryonic stage is highly sensitive to xenobiotic compounds and prenatal exposure can affect the birds, even later in life.[17] PFOS has been found to affect survival, morphometrics and immunology, as well as brain asymmetry and alterations in cognitive behavior of avian embryos among others (see Table 1 for an overview of the available literature). Although few studies are available, F-53B has already been shown to exert an effect on the nervous system of rodents,[18,19] and besides its detection in human placenta and killer whale fetuses, it has also been suggested to be maternally transferred in frogs,[12] increasing concerns about its developmental toxicity. These concerns have recently been confirmed in zebrafish (*Danio rerio*) embryos, where malformations and cardiac malfunction were observed,[20] and in mouse embryonic stem cells where neural differentiation was disrupted.[19]

To our knowledge, the toxicity of F-53B in birds has not yet been assessed. Birds are key organisms in many ecosystems, they can occupy multiple trophic levels within the food chain and contribute to a variety of ecosystem services. Therefore, they are a valuable tool in ecotoxicological studies and risk assessment.[21] In addition, due to the accessibility and the relative isolation of the avian embryo, they can be used to investigate the embryotoxicity of environmental contaminants.[22]

74 The aim of this experiment was therefore to investigate the avian developmental toxicity of the
75 largely uninvestigated compound F-53B in relation to, and in combination with, exposure to
76 the well-investigated PFOS. By exposing fertilized chicken eggs to environmentally relevant
77 concentrations prior to incubation, maternal transfer was mimicked and developmental effects
78 of these two compounds on survival, heart rate, liver mass, body mass and oxidative stress
79 parameters in the hatchlings were investigated.

Table 1: Overview of the available studies on *in ovo* exposure to PFOS in birds and comparison of the applied concentrations, injection method and endpoints. For clarity, non-significant trends are not mentioned in this table.

Authors	Species	Doses	Injection method	Endpoints	Day	Effects
Molina et al. 2006 [23]	Chicken	0.1, 1.0, 10, 20 µg/ g egg	Air cell at ED0	Hatching success	1	Reduced in all treatments (dose dep)
				Body and organ weight	7	No effect
				Liver histopathology	7	From 1.0 µg/g egg
				LOAEL	1	0.1 µg/g egg
O'Brien et al. 2009 [24]	Chicken	0.1, 5.0, 100 µg/g egg	Air cell at ED0	Pipping success	Pipping	Reduced at 100 µg/g egg (dose dep)
				Gene expression	1	No effect (PPARα-regulated genes)
				LD50	1	93 µg/g egg
Peden-Adams et al. 2009 [25]	Chicken	1, 2.5, 5 µg/g egg	Air cell at ED0	Hatching success	1	No effect
				Organ mass	14	Increased spleen mass in all treatments
						Increased liver mass from 2.5 µg/g egg
				Body mass (change)	1,7,14	No effect
				Body length	14	Increased at 5 µg/g egg
				Limb measurements	14	Right wing shorter in all treatments
				Brain asymmetry	14	Increased frequency in all treatments
				WBC counts	14	No effect
				Blood chemistry	14	ALT, LDH, CK decreased
				Immune function	14	Total SRBC-specific Ig decreased in all treatments
						Increased plasma lysozyme activity in all treatments
Pinkas et al. 2010 [26]	Chicken	5, 10 µg/g egg	Chorioallantois end at ED0	Embryo survival	ED19	Reduced
				Hatching success	1	Reduced
				Body weight	1	No effect
				Morphological and functional score	1	No effect
				Imprinting behavior	1	Reduced
				Protein kinase C	1	Reduced
Strömqvist et al. 2012 [27]	Chicken	20, 100 µg/g egg	Air cell at ED15	Embryo survival	ED18	No effect
				Gene expression		No effect (PPARα-regulated genes)

Table 1 continued: Overview of the available studies on *in ovo* exposure to PFOS in birds and comparison of the applied concentrations, injection methods and endpoints. For clarity, non-significant trends are not mentioned in this table.

Authors	Species	Doses	Injection method	Endpoints	Day	Effects
Nordén et al. 2012 [28]	Chicken	0.03, 0.1, 0.3, 1.0, 2.0 µg/g egg	Air cell at ED4	Embryo survival Liver mass Palmitic acid β-oxidation LOEL	ED10	No effect No effect Induced from 0.1 µg/g egg 0.1 µg/g
Nordén et al. 2016 [29]	Chicken Great cormorant (GC) Herring gull (HG)	0.1, 0.3, 1, 3, 10 µg/g egg	Air cell at ED4 Corresponding stage for GC and HG	Embryo survival Body and organ weight LD50 NOEL LOEL BMD_{10} $BMDL_{10}$	ED19 (chicken) Pipping (GC, HC)	Chicken: reduced at 10 µg/g egg GC: no effect HG: reduced at 10 µg/g egg Chicken: No effect GC: increased liver mass at 10 µg/g egg HG: increased body mass at 10 µg/g egg 8.5 µg/g egg 2.7 µg/g egg 0.9 µg/g egg 1.3 µg/g egg 0.4 µg/g egg
Parolini et al. 2016 [30]	Yellow-legged gull	0.1, 0.2 µg/g egg	Albumen at ED1	Pipping success Body mass (change) Tarsus length Liver and brain mass Total antioxidant capacity Total oxidant status Protein carbonyl content DNA fragmentation	Pipping	No effect
This study	Chicken	0.15, 1.5 µg/g egg	Yolk sac at ED0	Pipping success Hatching success Embryonic heart rate Body mass Liver mass Enzyme activity Oxidative damage Gene expression	Pipping 1 ED14,17,20 1 1 1 1 1	No effect No effect Reduced at 0.15 µg/g egg (ED20) No effect No effect No effect No effect No effect

Material and methods

This study was approved by the Norwegian Food Safety Authority (Mattilsynet; FOTS ID 9134) and was conducted in the animal laboratory facilities at the Department of Biology at NTNU, Norway, according to the appropriate regulations and protocols.

Potassium perfluorooctane sulfonate (PFOS; CAS no. 2795-39) was purchased from Sigma-Aldrich (St. Louis, MO, USA), with a stated purity of ≥ 98 %. However, our analysis showed a purity of 90 %, with PFHpS (7.6 %) and PFNS (1.4 %) as main impurities. Potassium 2-[(6-chloro-1,1,2,2,3,3,4,4,5,5,6,6-dodecafluorohexyl)oxyl]-1,1,2,2-tetrafluoroethanesulfonate (6:2 Cl-PFESA or F-53B; CAS no. 73606-19-6) was kindly donated by Dr. Thanh Wang (Örebro University) and had a stated purity of ≥ 98 %.[31]

Vehicle preparation

An emulsion of peanut oil and water, using lecithin as an emulsifier, was used as a vehicle to transfer the compounds into the egg yolk, as the majority of PFOS in bird eggs is found in the yolk, and not in the albumen.[14] The emulsion was prepared in accordance to Brunström and Örberg (1982) by dissolving lecithin (L-α-phosphatidylcholine from egg yolk, Sigma-Aldrich, St. Louis, MO, USA) in dichloromethane (DCM; Merck, Darmstadt, Germany) and peanut oil (Sigma-Aldrich, St. Louis, MO, USA).[32] Then, DCM was evaporated at 35 °C and 300 mbar using a rotary evaporator (RV 10 digital, IKA). The compounds, dissolved in ethanol, were then added to the lecithin/peanut oil mixture and the ethanol was evaporated at 40 °C and 175 mbar. After autoclave sterilization (20 min, 121 °C), two parts (1.6 mL) of the mixture were added to three parts (2.4 mL) of sterile distilled water to form an emulsion.[32] All emulsions were sonicated (ultrasonic processor GEX 400 connected to a four-element probe) for 30 seconds prior to injection. For each compound separately, an emulsion was prepared in a low and high concentration of 75 and 750 ng/µL to achieve final exposure doses of 150 and 1500

ng/g egg, respectively. In addition, mixtures of the two compounds in all four possible combinations of the two concentrations were prepared in order to achieve a 3 x 3 full factorial study design (Fig.1). Both nominal and actual doses in this study reflect the PFOS anion only, not the salt. The exposure doses of 150 and 1500 ng/g egg correspond to 0.30 and 3.0 nmol/g egg for PFOS and to 0.28 and 2.8 nmol/g egg for F-53B, respectively and represent the wide range of PFOS concentrations found in wild bird eggs.

Embryonic exposure and chick sampling

Broiler chicken (*Gallus gallus domesticus*) eggs from the hybrid breed 'Ross 308' were kindly donated by a local hatchery (Soknedal, Norway) and were kept at 18 °C until injection (two to three days). Out of 160 eggs, 122 eggs were randomly divided over eight treatment groups and 38 were divided over three control groups to investigate the potential effect of the injection (needle puncture and/or emulsion; Fig. 1). Fifteen eggs were selected as untreated control (not injected, not punctured) and another fifteen eggs were injected with a control emulsion without the compounds (vehicle control). In addition, eight eggs were only punctured with a needle, without being injected (Fig. 1).

The injected volume was 2 µL/g egg, hence the total injected volume was adjusted according to the individual egg mass (mean ± SD: 62.3 ± 3.4 g). Eggs were injected in the yolk sac at embryonic day (ED) zero, before the start of incubation. The entire injection procedure was conducted in a laminar flow cabinet. Prior to injection, the blunt segment of each egg was cleaned with ethanol (70 % *v/v*). Using a round shaped dentist drill bit mounted on an electrical drill (Robust 140W 9922 (GS), Hong Kong, P.R. China), a 0.6 mm hole was made in the eggshell until visibility of the inner shell membrane. The solution was injected using a Hamilton syringe (250 µL) and disposable needles (BD Microlance™ 3, 25 G, 0.5 × 16 mm) and the hole was sealed with paraffin. The eggs were divided over three incubators (type 180,

America A/S, Thisted, Denmark and J. Hemel, Verl, Germany) in which they were placed horizontally and kept at 37.5 - 38 °C and 60 % humidity). Eggs in incubator 2 and 3 were turned continuously (90° per hour) by an automatic egg-turning device, but due to a failure in the turning mechanism, eggs in incubator 1 required manual turning twice per day. To avoid effects related to the incubators such as variations in temperature, humidity or egg rotation, eggs were randomly divided between the three incubators, as well as between the shelves within the incubators, following a randomized block design with incubator as a blocking factor.

In the initial stage of incubation, eggs were regularly candled to monitor the embryonic development. The heart rate of embryos was determined using a digital egg monitor (Buddy, Avitronics, Cornwall, UK) at multiple time points, with systematic measurements on ED14, 17 and 20. Eggs that showed no or an arrested development were removed from the incubator and frozen at -20 °C. These eggs were opened later to determine embryonic viability and developmental status.

The last three to five days before hatching (ED16 - 18), eggs were transferred to hatching boxes at the bottom of the same incubator and relative humidity was increased to 70 - 80 %. After 20 days of incubation, the first eggs started to hatch. When dry, chicks were taken out of the incubator, weighed, and then euthanized by decapitation using scissors. The liver was dissected, weighed, divided for chemical and oxidative stress analyses, and snap frozen at -80 °C. Pipping and hatching success was determined by calculating the percentage of fertile eggs that were able to pip (externally) and hatch, respectively. External pipping is defined as the stage at which the chick breaks the outer egg shell membrane to commence hatching and a pipping star appears.

Chemical analysis

Analysis of the targeted compounds, PFOS and F-53B, was performed at the Norwegian Institute for Air Research (NILU) in Tromsø, Norway. The internal and recovery standards for chemical analysis consisted of ^{13}C-labelled PFOS in MeOH and perfluoro-3,7-dimethyloctanoic acid in MeOH, respectively (Wellington Laboratories, Guelph, Canada). The extraction and clean-up procedure was based on the Powley method,[33] and was adapted from Herzke et al. (2012)[34] for emulsions, and from Ahrens et al. (2011)[16] for livers. Detailed information on these procedures is given in section 1 of the Supporting Information (SI), and results of the emulsion and liver analyses are shown in Table S1 and S2, respectively. Compounds were analyzed by ultrahigh pressure liquid chromatography triple-quadrupole mass-spectrometry (UHPLC-MS/MS). Analysis was performed as described in detail by Hanssen et al. (2013) (also described in the SI section 1.2).[35] Method blanks and standard reference material (SRM; Pike perch sample QM03-2, QUASIMEME) samples were processed with every batch of ten samples. No blank contamination was encountered. The measured concentrations of the SRM were within the acceptable range, except for PFNA with an average recovery of 185 % due to levels close to the limit of detection. Recoveries of the internal standards varied in all samples between 73 and 113 % with the exception of 6:2 FTS and PFTeA with average recoveries of 128 and 140 %, respectively.

Oxidative stress

Expression of a suite of genes involved in the oxidative stress pathway was investigated in chicken liver samples using real-time quantitative PCR (qPCR). Target genes were catalase (CAT), glutathione reductase (GR), superoxide dismutase (SOD) 2, glutathione S-transferase (GST) α2, glutathione peroxidase (GPx) 4, nuclear factor erythroid 2 like 2 (NFE2L2), glutamate-cysteine ligase catalytic subunit (GCLC) and glutamate-cysteine ligase modifier subunit (GCLM). Target and reference genes, including their primer details, can be found in

Table S3. Details on primer design, sample preparation, qPCR protocol, quality control and data treatment can be found in section 2 of the SI.

Oxidative damage and enzymatic activity were investigated in chicken liver samples using a spectrophotometer. All reagents for the CAT, GPx and GR assays and kits for the determination of GST activity, lipid peroxidation (malondialdehyde; MDA) and protein carbonyl content were purchased from Sigma-Aldrich (St. Louis, MO, USA). Detailed information on the sample preparation, analytical methods (excluding assays using kits) and quality control can be found in the SI section 3 and Table S5.

Statistical analysis

Statistical analysis was performed using R (version 3.4.0).[36] Details of the performed calculations and statistical tests can be found in section 4 of the SI. In brief, to test the effect of the compounds on the parameters of interest, a two-way analysis of variance (ANOVA), followed by Tukey's or Dunnett's post-hoc test, was performed on a linear model that included both compounds separately and in interactions (corresponding to the mixtures). To test the effect of all treatment groups separately, a one-way ANOVA was performed, followed by a Dunnett's post-hoc test. To account for possible variation caused by the incubators, 'incubator' was added as a blocking factor to the model. When the residuals of the model were not normally distributed, a Kruskal-Wallis test was performed instead, followed by a Dunn's post-hoc test. Survival curves were plotted and tested for effects of the treatment using the Tarone-Ware test. Binomial generalized linear models were used to investigate effects of the compounds on pipping and hatching success. Body mass was normalized for egg mass, and the hepatosomatic index (HSI) was calculated to obtain a liver mass normalized for body mass. The hepatic fraction (%) of the total injected concentrations was calculated as described in the SI (section 4) and was used to estimate the distribution of the compounds to the liver.

Based on the statistical analysis between the three types of control groups (details in SI, section 5), the injected vehicle control was found to be the most relevant for comparison with the treated groups. Therefore, in all analyses, treated groups were only compared with the injected vehicle control.

Results and discussion

Concentrations of F-53B and PFOS in the liver of the hatchlings

Actual concentrations detected in the liver can be found in Table S2 and ranged from 1.08 – 2.17 mg/g, 11.6 – 28.4 mg/g, 1.04 – 2.20 mg/g and 7.87 – 23.4 mg/g, for PFOS LD, PFOS HD, F-53B LD and F-53B HD, respectively. The percentage of the total injected amount (based on the nominal concentration) found in the liver ranged from 15 - 19 % and 10 - 16 % for PFOS and F-53B, respectively (Table S2), indicating that chicks distribute both PFOS and F-53B to the liver. The liver can therefore be considered a target organ for these compounds in birds.

When comparing the isomeric composition in the liver with that in the PFOS standard (37 % branched, 63 % linear), chicken embryos from this study preferentially accumulated the linear isomer (75 %) over the branched isomer (25 %) in the liver. This is in agreement with the findings of O'Brien et al. (2011), who also found an enrichment of the linear isomer relative to the *in ovo* injected technical mixture.[37]

Effect on embryonic heart rate

Although eggs and treatments were randomized between and within the three incubators (randomized block design), the heart rate of the embryos measured at ED20 was affected by the incubator they developed in ($F_{2,98} = 4.77$, $p = 0.011$). A post-hoc Tukey test showed that the heart rate of embryos from incubator 1 was significantly lower than those of incubator 2 ($p = 0.04$) and 3 ($p = 0.01$), potentially due to the manual rotation of the eggs. It is also known that the embryonic heart rate is sensitive to changes in relative humidity and temperature within

the incubator.[38] However, these parameters were monitored twice daily and no aberrant values were observed. To focus on the effect of the compounds, only incubator 2 and 3 were considered for further statistical investigation. No effect of the incubator on heart rate was found at ED14 or 17.

While no effect of the compounds was found on the heart rate measured at ED14 or 17, a decreased heart rate was detected at ED20, the day before hatching. The heart rate of control embryos at ED20 was 298 ± 23 beats per minute (bpm), while the heart rate in exposed embryos was lower, ranging from 247 ± 19 bpm in the PFOS low dose group, to 279 ± 39 bpm in the F-53B low dose group (Table 2). Compared to the control group, all exposed groups had a significantly lower heart rate (one-way ANOVA: $F_{8,61} = 2.06$, $p = 0.054$), especially F-53B HD (Dunnett's test: t = 3.11, $p = 0.017$) and PFOS LD (Dunnett's test: t = 3.06, $p = 0.019$). Moreover, a significant interaction was observed between the two compounds in mixture (two-way ANOVA: $F_{4,61} = 3.54$, $p = 0.012$). This interaction shows that F-53B HD and PFOS LD and HD lower the heart rate more when occurring alone, than when occurring as mixtures (i.e. PFOS LD + F-53B HD and PFOS HD + F-53B HD; Table 2). This suggests a possible antagonistic reaction between the two compounds at these concentrations.

A decreased heart rate could be related to a decreased body mass (e.g. due to a developmental delay) and metabolism. In the current study, body mass at hatching was not correlated with heart rate (Spearman's rho = 0.11, $p = 0.30$). Reductions in heart rate can also be related to morphological alterations that can occur during cardiogenesis. Data on this are lacking for birds, but cardiac defects were reported in exposed rodent fetuses,[39] and heart malformations and altered heart rate were observed in zebrafish and medaka (*Oryzias melastigma*) embryos exposed to PFOS.[40–42] Similar to our results in chicken embryos, PFOS only altered the heart rate of medaka embryos in the late developmental stages.[41] In zebrafish embryos, F-53B induced cardiac toxicity by decreasing the heart rate, in combination with the incidence of

pericardial edema (also seen as an effect of PFOS) and downregulation of several genes related to cardiac development.[20] Cardiac development is a critical phase in embryogenesis and disruptions might ultimately affect survival, warranting further investigation in birds.

Survival, pipping and hatching success

Pipping and hatching success of the treated groups ranged between 75 – 93 % and 64 – 93 %, respectively (Table 2). No statistical differences in pipping success (χ^2_{10} = 5.4, p = 0.86), hatching success (χ^2_{10} = 7.9, p = 0.64), nor in embryo survival (Tarone-Ware test, p = 0.92; Figure S2) were found between treatments. Interestingly, all chicks that pipped in the present study also subsequently hatched, except for three out of eleven chicks exposed to a low dose of PFOS, who pipped externally and did not survive the hatching process. Although not statistically significant, this observation could be biologically relevant, indicating the potential of PFOS to affect hatchability at low doses.

The lowest PFOS dose that has previously been reported to affect hatching success after *in ovo* injection was 0.1 µg/g egg.[23] This result could not be confirmed by other similar studies (including the current study) and has therefore been thought to be due to the vertical placement of the egg in the incubator. It has been shown that a vertical egg orientation increases the toxicity of the compound, as opposed to a more natural, horizontal position.[43] Another study on chickens observed reduced embryo survival and pipping success at 10 µg/g egg when PFOS was injected in the air cell at ED4.[29] Critical developmental phases (such as organogenesis) occur within the first four days of incubation, and it was found that, compared to injection at ED4, embryos exposed prior to incubation exhibited a higher sensitivity to the investigated endpoints.[44] Finally, O'Brien et al. (2009) injected PFOS prior to incubation, positioned the eggs horizontally in the incubator and observed reduced pipping success at 100 µg/g egg.[24] In contrast to the present study, PFOS in the study of O'Brien et al. (2009) was injected in the air

277 cell instead of in the yolk sac. Even though a higher sensitivity of chicken embryos to the
278 compound can be expected when exposed via the yolk,[45] the effect concentration found by
279 O'Brien et al. (2009) is much higher than the environmentally relevant doses used in our study.

280 In contrast to the lack of effects in experimental studies, Custer et al. (2014) found a negative
281 association between hatching success and PFOS concentration in eggs of free-living tree
282 swallows (*Tachycineta bicolor*), with decreasing hatching success from 150 – 200 ng/g egg.
283 These levels corresponded to the low PFOS dose in the current study.[46] It is therefore important
284 to consider the discrepancy between laboratory studies and field studies at all times, as many
285 extrinsic factors can contribute to xenobiotic toxicity in biota.

286 Similar to PFOS, F-53B did not affect pipping, hatching or survival of the chicken embryos at
287 the investigated concentrations. In zebrafish, F-53B did not affect the hatching success of
288 exposed embryos either, but it did significantly delay their hatching.[20] F-53B also decreased
289 the zebrafish embryos' survival, be it at higher concentrations than found in the environment.
290 In the present study, the exact time of hatching was not recorded, however, this could be
291 assessed in future experimental work on the effects of F-53B.

Table 2: The amount of viable eggs, pipping and hatching success and embryonic heart rate (mean ± SD) at ED14, 17 and 20 per treatment group.

Treatment group		Total viable eggs (*n*)	Pipping success (%)	Hatching success (%)	Heart rate at ED14 (bpm)	Heart rate at ED17 (bpm)	Heart rate at ED20 (bpm) all inc	Heart rate at ED20 (bpm) inc 2 + 3
Controls	Non-injected	14	93	93	249 ± 28	253 ± 14	258 ± 31	269 ± 21
	Punctured	7	86	86	263 ± 9	257 ± 12	276 ± 23	284 ± 18
	Vehicle-injected	9	78	78	259 ± 16	263 ± 25	290 ± 30	298 ± 23
PFOS	LD	11	91	64	242 ± 28	244 ± 20	248 ± 22	247 ± 19
	HD	13	85	85	248 ± 22	253 ± 19	250 ± 34	267 ± 14
F-53B	LD	12	75	73 (*n*=11)*	251 ± 23	246 ± 20	263 ± 48	279 ± 39
	HD	11	91	91	253 ± 23	266 ± 17	257 ± 33	248 ± 37
Mixtures	PFOS LD + F-53B LD	14	93	93	258 ± 23	256 ± 19	265 ± 30	267 ± 20
	PFOS HD + F53B HD	16*	81	80 (*n*=15)	241 ± 44	256 ± 21	272 ± 30	278 ± 28
	PFOS LD + F-53B HD	11	82	82	261 ± 21	256 ± 20	265 ± 26	266 ± 28
	PFOS HD+ F-53B LD	12	83	83	234 ± 35	251 ± 23	249 ± 41	261 ± 26

* one egg was opened after pipping and was not included when calculating hatching success

Effect on body mass

A significant effect of the incubator on hatchling body mass was found (type II ANOVA for unbalanced data: $F_{2,84} = 7.45$, $p = 0.001$). Further, no significant effect of the compounds on the hatchling body mass was found.

In concordance with our study, no effect on body mass has been found in other avian *in ovo* studies on PFOS (Table 1). Only in herring gulls was an increased body mass observed at exposure concentrations of 10 µg/g egg.[29] Studies on mammals have found postnatal growth delay in rat pups following *in utero* exposure to PFOS, which was associated with hypothyroxinemia,[47] and a decreased thyroid hormone level by PFOS has been found in adult rats, mice and monkeys.[39,48,49] Thyroid hormones are known to be important for growth and development, and it has been hypothesized that PFOS competes for target binding to the hormone receptor. However, the PFOS concentrations in the present study did not exert an effect on the body mass of newly hatched birds at the investigated concentrations.

Limited data exist on the effects of F-53B. However, a recent study on zebrafish larvae showed that F-53B, at environmentally relevant concentrations, caused a significant decrease in the body weight of the larvae, which was also related to a disruption in the thyroid hormone system.[50] In contrast, no effect of F-53B on the body mass of the hatchlings was found in the current study, which likely reflects interspecific and experimental differences.

Effect on the hepatosomatic index (HSI)

Chicks exposed to a high dose of F-53B (including mixture groups) showed a significant 8 % increase in their HSI ($H = 5.9$, $df = 2$, $p = 0.053$; Dunn's post-hoc test: $z = 2.4$; $p = 0.026$) compared to chicks not exposed to this compound (Fig. 2). No such increase was observed in chicks exposed to PFOS. A high dose of F-53B and PFOS (including single and mixture

317 treatments) corresponded to an actual concentration range of 7.87 – 23.4 mg/g and 11.6 – 28.4
318 mg/g detected in the liver, respectively (Table S2).

319 The HSI is considered a measure of the body condition of an animal, as it reflects both the
320 metabolic energy demands and the short-term nutritional status.[51] It is sensitive to
321 environmental contaminants, and hepatic enlargement is therefore a common symptom in
322 exposure studies. The effect of F-53B on the HSI has, to our knowledge, not been investigated
323 yet. However, in similar avian exposure studies, PFOS has been found to increase the liver
324 mass of chicken hatchlings at exposure concentrations from 2.5 µg/g egg.[25] Further, the HSI in
325 great cormorants (*Phalacrocorax carbo sinensis)* increased by 18 % following exposure to 10
326 µg PFOS/g egg.[29] Also in northern bobwhite quails (*Colinus virginianus*), an increase in the
327 HSI was observed after dietary PFOS exposure.[52] Considering the comparatively low doses
328 used in our study, it is not surprising that PFOS did not exert an effect on HSI. The finding that
329 F-53B, in contrast to PFOS, did have an effect at 1500 ng/g egg may therefore indicate a higher
330 hepatotoxicity potential in birds compared to PFOS.

331 Hepatotoxicity of PFOS has been mostly linked to PPARα-mediated pathways, as PFOS
332 structurally resembles fatty acids, the endogenous PPAR ligands. Interestingly, it was recently
333 shown that F-53B has an even higher binding affinity to PPARs than PFOS.[53] An increased
334 HSI could be related to changes in lipid metabolism (PPAR-dependent), as well as to
335 histopathological changes and hepatic injuries as found in PFOS-treated rats.[54] This highlights
336 the need for further investigations on these endpoints in birds. Finally, together with the
337 concentrations detected in the liver of the hatched chicks, the increased HSI confirms that the
338 liver is a target organ for F-53B exposure.

Effects on the oxidative stress response

Exposure to certain environmental pollutants is known to increase production of reactive oxygen species (ROS) and hence trigger an oxidative stress response in birds,[55] potentially leading to a detrimental effect on the embryonic development.[56] In this study, a significant interaction effect of PFOS and F-53B was found on the gene expression of SOD2 ($F_{4,45} = 3.34$, $p = 0.018$) and GR ($F_{4,44} = 2.70$, $p = 0.043$), as shown in Figure S1 and Table S4. Both compounds when exposed separately, in both low and high dose, triggered a mild increase (1.43 - 1.85 fold) in SOD2 expression compared to the control group. This indicates an oxidative stress response by increasing the production of the SOD2 enzyme. However, when the compounds occurred in a mixture of different doses (PFOS HD + F-53B LD and PFOS LD + F-53B HD), the expression was similar to the control group (Figure S1A and B and Table S4). This result shows an antagonistic effect for the latter mixtures. For GR on the other hand, no effect was observed in chicks exposed to a low dose of PFOS or a high dose of F-53B. Yet, when the compounds occurred in a mixture and in the respective doses, GR expression decreased twofold (Figure S1C and Table S4). Although not significant, a corresponding trend was observed in the enzyme activity of GR in chicks exposed to that mixture (PFOS LD + F-53B HD). GR is an essential enzyme in the oxidative stress pathway, catalyzing the reduction of glutathione,[57] hereby providing the substrate for several anti-oxidative stress enzymes (e.g. glutathione-S-transferase and glutathione peroxidase). A decrease in the expression and activity of this enzyme could therefore cause a hampered defense against ROS.

Activity and expression of other investigated enzymes and genes, respectively, were not significantly affected by the treatments. In concordance with the lack of an oxidative stress response, no lipid or protein damage, potentially related to oxidative stress, could be detected. Therefore, it can be assumed that embryonic exposure of chickens to PFOS and F-53B at environmentally relevant concentrations did not trigger any major changes in the oxidative

stress response and did not cause oxidative damage to lipids or proteins. Other pathways could therefore be more relevant to the toxic mode of action of these compounds.

In conclusion, environmentally relevant concentrations of PFOS and its alternative F-53B were found to significantly decrease the heart rate of avian embryos immediately before hatching. Further, F-53B significantly increased the liver mass of the hatchlings. Further investigations on liver metabolizing enzymes and - histopathology might therefore elucidate the mechanisms of this compound. Based on our results, the oxidative stress pathway is unlikely to be a target pathway at these concentrations. Although no effects were observed on survival, pipping/hatching success or body mass, the sublethal effects observed could potentially lead to fitness consequences later in life,[17] especially in the context of multiple stressors. Therefore, potential effects on survival cannot be ruled out, as e.g. hatching delay and postnatal survival were not assessed in the current study, and further investigations are necessary. F-53B is increasingly detected in the environment, and interest in the toxicity of this compound is therefore also increasing. The current study contributes to the knowledge gap that exists regarding the toxicity of F-53B in birds, and biota in general. The results of this study show that there is potential for this alternative compound to exceed the toxicity of PFOS at environmentally relevant concentrations. In combination with other available toxicity data, the current study highlights that the chlorinated polyfluoroalkyl ether sulfonate F-53B can be a compound for future concern and should not be overlooked.

Acknowledgements

This work is part of the NEWRAPTOR project (grant number 230465/F20), funded by the Norwegian Research Council and NTNU. We would like to acknowledge A. Ullah and I.B. Kroglund for help during the experiment and sampling; C. Waugh for help with sampling and English language revision; P. Winge for the primer design; T. Sparstad for help with the qPCR

analysis and L. Hanssen for help with the chemical analysis. G.S. Eggen is greatly acknowledged for her help during the oxidative stress assays.

Supporting information

Details on extraction and clean-up (chemical analysis); details on quantification (chemical analysis); concentrations in emulsions (Table S1); hepatic concentrations (Table S2); primer design; sample preparation for qPCR analysis; qPCR protocol; quality control of the qPCR analysis; qPCR data treatment; primer sequences (Table S3); interaction effects on gene expression (Figure S1 and Table S4); homogenization procedure for oxidative stress assays; CAT assay protocol; GR assay protocol; GPx assay protocol; normalization of assay results; intra- and interplate variation for oxidative stress assays (Table S5); statistical analysis; comparison of control groups; survival plot (Figure S2).

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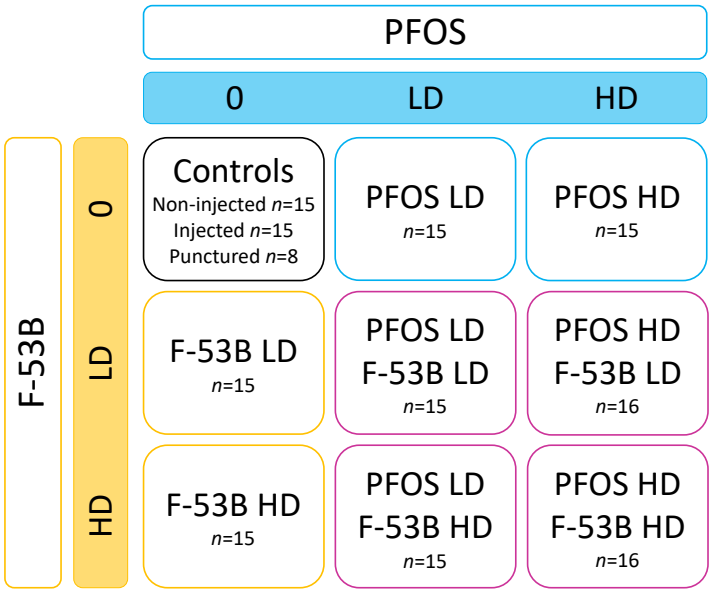
Figure captions

Figure 1: The 3 x 3 full factorial design of this study. All control and treatment groups are represented with their respective sample size. LD: low dose (150 ng/g egg) and HD: high dose (1500 ng/g egg).

Figure 2: The effect of F-53B on the mean hepatosomatic index (HSI; liver mass / body mass) of the hatchlings. A high dose of F-53B increases the liver mass with 8 % compared to groups (including mixtures) not exposed to F-53B ($p = 0.026$, as signified by the asterisk). Error bars represent the standard error of the mean.

598 **Figures**

599 Figure 1



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601 Figure 2