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1 **Higher plasma oxidative damage and lower plasma antioxidant defences in an**  
2 **Arctic seabird exposed to perfluoroalkyl carboxylic acids**

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26 **ABSTRACT**

27 Perfluoroalkyl and polyfluoroalkyl substances (PFASs) may cause detrimental effects on  
28 physiological function and reproduction of Arctic animals. However, there is a paucity of  
29 information on the link between PFASs and oxidative stress, which can have potential  
30 detrimental effects on key fitness traits, such as cellular homeostasis or reproduction. We  
31 have examined the correlations between multiple blood-based markers of oxidative status  
32 and several perfluoroalkyl carboxylic acids (i.e., with 8 or more carbons) in male Arctic  
33 black-legged kittiwakes (*Rissa tridactyla*) during the pre-laying period. Higher protein  
34 oxidative damage was found in those birds having higher concentrations of  
35 perfluorododecanoic acid (PFDoA), perfluorotridecanoic acid (PFTriA) and  
36 perfluorotetradecanoic acid (PFTeA). Lower plasmatic non-enzymatic micro-molecular  
37 antioxidants was found in those birds having higher concentrations of  
38 perfluoroundecanoic acid (PFUnA), PFDoA and PFTeA. Effect size estimates showed  
39 that the significant correlations between PFASs and oxidative status markers were  
40 intermediate to strong. The non-enzymatic antioxidant capacity (including antioxidants  
41 of protein origin) was significantly lower in those birds having higher plasma  
42 concentration of linear perfluorooctanesulfonic acid (PFOSlin) only. In contrast, the  
43 activity of the antioxidant enzyme glutathione peroxidase in erythrocytes was not  
44 associated with any PFAS compounds. Our results suggest that increased oxidative stress  
45 might be one consequence of long-chain PFAS contamination. Given the correlative  
46 nature of our study, experimental work will be needed to demonstrate whether PFASs  
47 cause toxic effects on free-living vertebrates through increased oxidative stress.

48

## 49 INTRODUCTION

50 Ecotoxicological studies have so far extensively directed their attention toward legacy  
51 persistent organic pollutants (POPs) like organochlorine pesticides (OCPs) and  
52 polychlorobiphenyls (PCBs).<sup>1</sup> In contrast, less attention has been given to the  
53 environmental toxicity of other organic contaminants in the Arctic, such as chlorinated  
54 paraffins, phthalates, siloxanes or the perfluoroalkyl and polyfluoroalkyl substances  
55 (PFASs).<sup>2</sup> Among these, PFASs remain comparatively much less investigated.<sup>3</sup> PFASs  
56 are synthetically manufactured chemicals, produced since the 1950s, that are widely used  
57 for numerous industrial and commercial purposes as water repellents and surfactants (e.g.,  
58 impregnation agents for carpets, papers and textiles, fire-fighting foam, non-stick coating  
59 and waterproof clothing).<sup>4-5</sup> Chemically and thermally stable, PFASs are highly persistent  
60 in the environment and have been detected globally in both wildlife and humans.<sup>6-8</sup>  
61 Because of oceanic currents and atmospheric long-range transport, PFASs and their  
62 precursors and breakdown products can reach high latitudes, such as the Arctic Ocean.<sup>9-</sup>  
63 <sup>11</sup> Once deposited in the Arctic marine ecosystem, PFASs bio-accumulate in living  
64 organisms and bio-magnify along the food webs.<sup>12-16</sup> Importantly, (i) PFASs have long  
65 half-lives, which facilitates their bio-accumulation through the food webs depending on  
66 the species and PFAS congener<sup>8</sup>; (ii) the long and odd carbon-chain-length PFASs appear  
67 to be more bio-accumulative and toxic than the short and even-chain length PFASs in  
68 wildlife.<sup>17-20</sup>

69 It is worthwhile to note that while PFASs have been produced for over 50 years,  
70 it is only since late 1990s that their occurrence in the environment has come under  
71 scientific scrutiny. PFASs have raised recent concerns about their potential physiological  
72 disrupting properties and negative impacts on reproductive fitness in wildlife (multiple

73 species in <sup>21</sup>; lesser black-backed gull in <sup>22</sup>; northern fulmar in <sup>23</sup>; zebrafish in <sup>24</sup>; tree  
74 swallow in <sup>25</sup>; black-legged kittiwake and northern fulmar in <sup>26</sup>; black-legged kittiwake  
75 in <sup>27-28</sup>; black-legged kittiwake in <sup>29-30</sup>; glaucous gull in <sup>31</sup>; common eider, black guillemot,  
76 black-legged kittiwake, glaucous gull, arctic skua and great skua in <sup>32</sup>).

77         Increased molecular oxidative damage and disruption of antioxidant defences are  
78 suspected as important mechanisms through which PFASs could be detrimental for cell  
79 function and, possibly, for organism health. Experimental evidence on laboratory models  
80 found that PFASs may increase production of reactive oxygen species (ROS), increase  
81 molecular oxidative damage and up- or down-regulate antioxidant defences.<sup>24,33-34</sup>  
82 Further work found that the perfluoroundecanoic and perfluorododecanoic acids (PFUnA  
83 and PFDoA, respectively) are equally potent inducers of stress response genes relative to  
84 perfluorooctane sulfonic acid (PFOS) and perfluorononanoic acid (PFNA) and that the  
85 effect of carbon-chain-length was more important than the functional group in  
86 determining oxidative stress.<sup>35</sup> There is thus good reason to expect that long-chain PFASs  
87 might cause dysregulation of the oxidative homeostasis, leading to accumulation of  
88 oxidative damage to key biomolecules like proteins or nucleic acids. However, the effect  
89 of PFAS exposure on oxidative stress is almost unknown for wildlife. To the best of our  
90 knowledge, only two studies have addressed this topic in free-living animals. <sup>36</sup> did not  
91 find any significant relationship between plasma PFAS concentrations and the activity of  
92 the antioxidant enzyme superoxide dismutase in plasma of white-tailed eagle nestlings,  
93 while <sup>32</sup> did not find any significant relationship between PFASs and amount of DNA  
94 damage in lymphocytes.

95         Long-lived species, like many polar seabirds that occupy high trophic levels, are  
96 exposed to a greater risk of accumulation and sensitivity to high concentrations of

97 contaminants. In Svalbard (European Arctic), a number of studies showed that black-  
98 legged kittiwakes (*Rissa tridactyla*, hereafter “kittiwake”) are chronically exposed to a  
99 complex cocktail of organic contaminants and trace elements, which are known to  
100 correlate with physiological metrics, impaired individual fitness and population  
101 dynamics.<sup>27-30,37-39</sup> It is, however, unknown whether exposure of kittiwakes to PFASs is  
102 associated with markers of oxidative damage and antioxidant protection.

103         In this study, we have examined the correlations between blood-based markers of  
104 oxidative status and several PFAS compounds in male kittiwakes during the pre-laying  
105 period, while controlling for a number of potential confounding factors that might affect  
106 the oxidative status independently from PFASs (i.e., body condition, body size, both time  
107 and day of blood sampling, hormonal status; reviewed in <sup>40</sup>). As with the hormonal status,  
108 we measured plasma levels of testosterone, baseline corticosterone and luteinizing  
109 hormone because prior work found large individual variation among kittiwakes<sup>27-28,38</sup> and  
110 significant effects on organism’s oxidative status<sup>40</sup>, which could affect the relationships  
111 between PFAS and oxidative status markers. We focused on males because this  
112 investigation on oxidative stress is part of a larger project aiming at assessing the overall  
113 consequences (ornament coloration, fecundity, oxidative stress, sexual hormones) of  
114 PFASs exposure in males during the pre-laying stage (nest site defence, pair-bonding,  
115 copulation, nest building) a period during which males appear to be sensitive to  
116 pollutants.<sup>38</sup>

117         We have also examined whether the effect size of the association between each  
118 oxidative status marker and each PFAS compound varies according to their carbon-chain-  
119 length (C<sub>8-14</sub>) because the toxicity of PFASs may increase with carbon-chain-length.

120

## 121 **MATERIALS AND METHODS**

### 122 **Sampling**

123 Fieldwork was conducted in 2016 on a colony of Arctic kittiwakes at Kongsfjord (78° 54'  
124 N; 12° 13' E), Svalbard. Blood samples were collected from 50 adult males during the  
125 pre-laying period (courtship and mating period), from 25<sup>th</sup> May to 6<sup>th</sup> June. Birds were  
126 caught on their nest with a loop at the end of a long pole. Within 3 minutes since capture  
127 0.5 ml of blood were taken from the brachial vein using a heparinized syringe and a 25-  
128 gauge needle. This blood sample was used to measure oxidative status markers and  
129 hormones. Straightaway, a second sample of venous blood (ca. 2 ml) was collected using  
130 another syringe and this blood sample was used to assess the PFASs burden. Then, tarsus,  
131 skull (head + bill) and wing length were measured using a calliper (nearest 0.1 mm) and  
132 body mass was taken using a Pesola spring balance (nearest 5 grams). Blood samples  
133 were stored on ice in the field. On average, blood samples were stored on ice for 3h40min  
134 38s (min: 1h30; max 9h55) before being centrifuged and stored at -80°C. Plasma and red  
135 blood cells, obtained after centrifugation, were kept frozen separately, either at -80°C for  
136 subsequent oxidative status markers or at -20°C for PFAS analyses. All samples were  
137 analysed within 4 months since collection.

138

### 139 **Hormone assays**

140 Plasma levels of testosterone, baseline corticosterone and luteinizing hormone (LH) were  
141 measured by radioimmunoassay at the Centre d'Etudes Biologiques de Chizé following  
142 protocols previously validated. Briefly, testosterone and corticosterone were extracted  
143 using diethyl ether and ethyl ether, respectively. Plasma concentrations of all three  
144 hormones were measured by radioimmunoassay. As with corticosterone, a commercial

145 antiserum against corticosterone-3-(O-carboxy- methyl) oxime bovine serum albumin  
146 conjugate (Biogenesis, UK) was used. The lowest detectable quantities significantly  
147 different from zero at a 90% confidence level were 0.05 ng/ml for testosterone, 0.4 ng/ml  
148 for corticosterone and 1.7 ng/ml for LH. All samples were analysed in duplicate.  
149 Corticosterone and LH were analysed in a single run and the mean coefficient of variation  
150 was 4.9 and 12.0%, respectively. Testosterone was analysed in two runs and the  
151 coefficients of variation were 11.2 and 11.5%.

152

### 153 **Oxidative status markers**

154 One marker of plasma oxidative damage, one marker of plasma non-enzymatic  
155 antioxidant capacity and one red blood cell antioxidant enzyme were measured at the  
156 Centre d'Etudes Biologiques de Chizé using standard methods. Protein carbonyls (marker  
157 of oxidative protein damage) were measured using the Protein Carbonyl Colorimetric  
158 assay (Cayman Chemical Company, Ann Arbor, USA). This assay is based on the  
159 colorimetric method proposed by <sup>41</sup>. A same volume of plasma was used for all samples  
160 and the amount of carbonyls was standardised by the plasma protein concentration  
161 according to manufacturer's instructions. Protein carbonyls were derivatized to 2,4-  
162 dinitrophenylhydrazone by reaction with 2,4-dinitrophenylhydrazine (DNPH). The  
163 absorbance was read at 370 nm. The extinction coefficient for DNPH (0.022/μM/cm) was  
164 used to calculate the concentration of protein carbonyls, which was expressed as nmol/mg  
165 protein (amount of carbonyls generated per unit of protein) or as total nmol obtained by  
166 multiplying the concentration of carbonyls by the concentration of plasma proteins (i.e.,  
167 total amount of carbonyls in the sample, which is also dependent on the amount of  
168 substrates available, i.e., proteins). The mean coefficient of variation of duplicates was



169 11.5%. The metric expressed as nmol/mg indicates the amount of carbonyls that occurs  
170 in a same amount of protein, thus this is standardised by the amount of substrates (i.e.,  
171 proteins) that can be carbonylated. The second metric expressed as total amount of  
172 carbonyls indicates the total amount of carbonyls that occurs in the tissue, which is  
173 influenced by the amount of proteins available. This second metric is also important  
174 because accumulation of carbonyls is detrimental for the cells<sup>50</sup>. The OXY-Adsorbent test  
175 (Diacron International, Italy) was used to quantify the non-enzymatic antioxidant  
176 capacity of plasma against HOCl. Values were expressed as either mM of HOCl  
177 neutralised or as mM of HOCl neutralised/mg protein to estimate the antioxidant potential  
178 of micromolecular antioxidants (e.g., vitamins, carotenoids, glutathione) without the  
179 contribution of proteins (i.e., non-enzymatic micro-molecular antioxidant capacity). The  
180 correlation between OXY values and protein concentration was actually high and  
181 significant ( $r = 0.76$ ,  $p < 0.001$ ), which is to be expected because plasma proteins, such  
182 as albumin, are prone to react with HOCl. Although free-radical trapping properties vary  
183 among proteins, standardising OXY values by the concentration of total plasma proteins  
184 gave us some kind of control about the contribution of proteins to OXY. The mean  
185 coefficient of variation of duplicates was 9.4%. The Ransel assay (RANDOX  
186 Laboratories, UK) was used to measure the activity of the antioxidant enzyme glutathione  
187 peroxidase (GPX) in haemolysates (red blood cells diluted with distilled water). Values  
188 were expressed as Units of GPX/mg of protein of haemolysate. The mean coefficient of  
189 variation of duplicates was 8.1%. The Bradford protein assay (Bio-Rad Laboratories,  
190 Hercules, USA) with bovine albumin as a reference standard was used to measure the  
191 concentration of proteins in both plasma samples and haemolysates.

192

193 **PFAS analyses**

194 Perfluoroalkyl carboxylic acids (i.e., PFASs with 8 or more carbons) were analysed in  
195 plasma at the Norwegian Institute for Air Research (NILU) in Tromsø, Norway. The  
196 following compounds were analysed in each plasma sample: perfluorooctanesulfonamide  
197 (PFOSA), perfluorobutanesulfonic acid (PFBS), perfluoropropanesulfonic acid (PFPS),  
198 perfluorohexanesulfonic acid (PFHxS), perfluoroheptanesulfonic acid (PFHpS),  
199 perfluorooctane sulfonic acid (PFOSlin), branched perfluorooctane sulfonic acid  
200 (PFOSbr), perfluorononane sulfonic acid (PFNS), perfluorodecane sulfonic acid  
201 (PFDcS), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA),  
202 perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoate  
203 (PFDcA), perfluoroundecanoic acid (PFUnA), perfluorododecanoic acid (PFDoA),  
204 perfluorotridecanoic acid (PFTriA), perfluorotetradecanoic acid (PFTeA) and two  
205 precursor compounds, the fluorotelomer sulfonates (6:2 FTS and 8:2 FTS). PFASs with  
206 concentrations below the limit of quantification (LOQ) were replaced with a value equal  
207 to  $(\text{LOQ} \times \text{detection frequency})$  when the detection frequency (percentage of detection)  
208 was  $> 50\%$ . A 0.2 ml aliquot of plasma spiked with internal standards (carbon labeled  
209 PFAS)<sup>42</sup> was extracted in methanol (1 ml) by repeated sonication and vortexing. The  
210 supernatant was cleaned-up using ENVICarb graphitized carbon absorbent and glacial  
211 acetic acid. Extracts were analysed by UPLC/MS/MS. Recovery of the internal standards  
212 ranged between 86.3% and 120%. Results were validated with blanks (clean and empty  
213 glass tubes treated like a sample) and standard reference material (1957 human serum  
214 from NIST) run with every 10 samples. The deviation of the target concentrations in the  
215 SRMs were within the laboratory's accepted range (69 - 119%). Blanks varied between

216 concentrations below the instrument detection limits and 30 pg/g and were applied as the  
217 LOD in the form of 3 times the average concentration.

218

### 219 **Body size and body condition**

220 A body size index was estimated using the PC1 from a PCA of tarsus, skull and wing  
221 length (loadings, 0.73, 0.83 and 0.45, respectively). As far as the body condition, we did  
222 not use the ratio of body mass onto body size nor the residuals of a linear regression of  
223 body mass onto body size because both indices have been criticized.<sup>43</sup> Rather we included  
224 both body mass and body size index as factors in the models. In doing so, the coefficient  
225 estimate of body mass is calculated considering the effect of body size, thus the outcome  
226 reflects the effect of body condition on the given marker of oxidative status.<sup>43</sup>

227

### 228 **Statistical analyses**

229 Generalized linear models were performed using the software STATISTICA 10  
230 (StatSoft. Inc., Tulsa, OK, USA) to assess relationships between each oxidative status  
231 marker (protein carbonylation, non-enzymatic antioxidant capacity, non-enzymatic  
232 micro-molecular antioxidant capacity and glutathione peroxidase) and the following  
233 predictor variables: PFASs congener (PFOSlin, PFNA, PFDcA, PFUnA, PFDoA, PFTriA  
234 or PFTeA), body size index, body mass, hormonal status, day of blood sampling and time  
235 of blood sampling. All predictor variables were scaled to mean of 0 and standard deviation  
236 of 1. These predictor variables were included in all models because prior work showed  
237 that each of them can be significantly associated with oxidative status markers (reviewed  
238 in <sup>40</sup>). The two metrics of protein oxidative damage (protein carbonyls per mg of protein  
239 and total protein carbonyls) were combined using the PC1 from a Principal Components

240 Analysis (PCA) because they were highly correlated ( $r = 0.79$ ,  $p < 0.001$ ). The hormonal  
241 status was estimated using the PC1 from a PCA of corticosterone, testosterone and  
242 luteinizing hormone (loadings, 0.39, 0.61 and 0.84, respectively). A normal error function  
243 and an identity-link function were applied to models of non-enzymatic antioxidant  
244 capacity and GPX. A gamma error function and an identity-link function were applied to  
245 models of protein carbonyls and non-enzymatic micro-molecular antioxidant capacity.  
246 These functions were selected because the model had the best fitting to the dataset  
247 according to the Akaike Information Criterion. Preliminary analyses showed that the time  
248 elapsed from the collection of blood to its storage was not significantly correlated ( $p$ -  
249 value  $> 0.45$ ) with any marker of oxidative status nor hormones, thus it was not further  
250 considered in the analyses. Visual inspection of residuals, Q-Q plots and Cook's distance  
251 did not highlight any violation of normality and homogeneity of variance nor the presence  
252 of outliers (all samples were below a 0.5 Cook's distance). The variance inflation factor  
253 was always below 2, indicating that multicollinearity was low. The multicollinearity is  
254 thought to be high and problematic when the variance inflation factor is higher than 5.

255 The “compute.es package”<sup>44</sup> in R<sup>45</sup> was used to calculate the standardized effect  
256 size Hedges'  $g$  from test statistics of oxidative status markers that had significant  
257 associations with PFASs (i.e., protein oxidative damage and non-enzymatic micro-  
258 molecular antioxidant capacity). The “forestplots function” of the “metafor package” in  
259 R was used to visualise boxplots of effect size and 95% confidence interval. Effect sizes  
260 were considered to be small (Hedges'  $g = 0.2$ , explaining 1% of the variance),  
261 intermediate ( $g = 0.5$ , explaining 9% of the variance) or large ( $g = 0.8$ , explaining 25%  
262 of the variance) according to<sup>46</sup>.

263

## 264 RESULTS

265 Concentrations of detectable PFASs are reported in Table 1 together with other variables  
266 measured in kittiwakes. Six out of 20 PFASs (i.e. PFOSlin, PFNA, PFDcA, PFUnA,  
267 PFDoA, PFTriA) were detectable in all individuals, while one PFAS (i.e. PFTeA) was  
268 detectable in 33 out of 50 individuals. PFOSlin, PFUnA and PFTriA concentrations were  
269 the highest of all PFASs measured in the investigated samples, with a percentage  
270 contribution for each kittiwake ranging from 23.4 to 54.5%, from 20.3 to 34.8% and from  
271 11.8 to 35.2%, respectively. The percentage contribution of all other detected PFASs  
272 ranged between 0.1 and 10.6%. PFOSA, PFBS, PFPS, PFHxS, PFHpS, PFOSbr, PFNS,  
273 PFDcS, PFHxA, PFHpA, PFOA, and the two precursor fluorotelomer sulfonates (6:2 FTS  
274 and 8:2 FTS) were below the detection limit in all the investigated samples.

275 Protein oxidative damage was significantly higher in those birds having higher  
276 plasma concentration of PFDoA, PFTriA or PFTeA (Table 2). Effect size estimates  
277 increased with chain length of PFASs (indicating an increase of protein damage with  
278 chain length) and were significantly different from zero ) for PFDoA (95% confidence  
279 interval: 0.06 to 1.25), PFTriA (95% confidence interval: 0.01 to 1.20) and PFTeA (95%  
280 confidence interval: 0.29 to 1.54;Fig. 1). The non-enzymatic micro-molecular antioxidant  
281 capacity was significantly lower in those birds having higher plasma concentration of  
282 PFUnA, PFDoA or PFTeA (Table 2). There was also a near-significance tendency of the  
283 non-enzymatic micro-molecular antioxidant capacity to be lower in birds with higher  
284 plasma PFTriA (Table 2). Effect size estimates were larger for longer PFASs (indicating  
285 a decrease of micro-molecular antioxidants with chain length) and were significantly  
286 different from zero for PFUnA (95% confidence interval: -1.20 to -0.02), PFDoA (95%  
287 confidence interval: -1.35 to -0.14) and PFTeA (95% confidence interval: -1.23 to -0.04;

288 Fig. 2). The non-enzymatic antioxidant capacity including the contribution of antioxidant  
289 of protein origin was significantly lower in those birds having higher plasma  
290 concentration of PFOSlin, but it was not associated with any other PFAS congener (Table  
291 2). The activity of GPX was not associated with any PFAS compounds (Table 2).

292 Finally, our models showed that (i) kittiwakes in poorer body condition had more  
293 plasma protein carbonyls, (ii) the non-enzymatic antioxidant capacity was higher in  
294 kittiwakes sampled later in the day, and (iii) the non-enzymatic micro-molecular  
295 antioxidant capacity was higher in kittiwakes having lower concentrations of hormones.

296

## 297 **DISCUSSION**

298 Our results provide the first evidence in wild vertebrates that the correlation between  
299 oxidative status markers and PFASs is stronger for long-chain congeners. We found that  
300 male kittiwakes having higher plasma concentrations of long-chain PFASs had more  
301 protein oxidative damage and less plasma antioxidants after controlling statistically for  
302 potentially confounding variables. The non-enzymatic antioxidant capacity (including  
303 antioxidants of protein origin) was significantly lower in kittiwakes having higher plasma  
304 concentration of PFOSlin. On the other hand, the activity of glutathione peroxidase in  
305 erythrocytes was not related to any PFAS congener. Effect size estimates were  
306 intermediate to large, indicating that PFASs explained from 9 to more than 25% of the  
307 variance in protein oxidative damage and non-enzymatic micro-molecular antioxidant  
308 capacity of plasma.<sup>46</sup> Intermediate effect sizes are suggested to be biologically  
309 meaningful because average proportions of variance explained in ecological, evolutionary  
310 and physiological studies is usually below 7%.<sup>47</sup> Our effect size estimates were also larger

311 than those found in the comparison of oxidative status markers between animals living in  
312 polluted (e.g., air pollution, heavy metals) and unpolluted sites.<sup>48</sup>

313 PFOSlin, PFUnA and PFTrA concentrations were the highest of all PFASs  
314 measured in the investigated samples, with a percentage contribution for each kittiwake  
315 ranging from 11.8 to 54.5%. The concentration of PFOSlin was higher than that  
316 previously recorded in males from the same kittiwake population in 2012 (13.4 vs. 10.6  
317 pg/g ww in<sup>29</sup>). In contrast, the average concentrations of PFUnA (10.3 vs. 12.1 pg/g ww  
318 in<sup>29</sup>) and of PFTrA (8.6 vs. 11.6 pg/g ww in<sup>29</sup>) were both lower in our study than in prior  
319 work.<sup>29</sup> One reason for such differences might be because <sup>29</sup> measured PFASs of male  
320 kittiwakes caught during the chick rearing phase. Work on male glaucous gulls during the  
321 incubation period in Svalbard found levels of PFOSlin similar to ours, while those of  
322 PFUnA (4.4 pg/g ww) and of PFTrA (3.9 pg/g ww) were much lower than those we  
323 recorded.<sup>31</sup>

324 The strength of the correlation between oxidative status markers and PFASs  
325 increased with the chain length of the congener. While persistent in the environment,  
326 PFASs with fewer than eight carbons, such as PFHxA, and PFSAAs with fewer than six  
327 carbons, such as PFBS, are generally less toxic and bioaccumulative in wildlife and  
328 humans, while exposure of laboratory animals to long-chain congeners produces  
329 detrimental reproductive, developmental, and systemic effects.<sup>4,6,18,49</sup> For example, the  
330 toxic effects of PFASs on rat brain cells decreased with increasing carbon chain length.<sup>17</sup>  
331 Interestingly, the toxic effects of PFASs were attenuated by the antioxidant vitamin E,  
332 indicating a possible involvement of oxidative stress in the reduction of cell viability.<sup>49</sup>  
333 Further work showed that, compared to short-chain PFASs, long-chain PFASs are  
334 stronger inducers of the response of genes regulating the cell oxidative status.<sup>35</sup>

335           Effects of PFAS exposure on oxidative status have been moderately investigated  
336 in laboratory models and almost unexplored in wild animals. Thus, limited information  
337 is available for a comparison with our results and interpretation. Protein carbonylation  
338 arises from overproduction of ROS by metabolic reactions that use oxygen and shift the  
339 balance between oxidant/antioxidant statuses in favour of the oxidants.<sup>50</sup> Protein  
340 carbonylation also occurs when carbonyls are introduced into proteins through the  
341 reactions with lipid oxidative damage products (malondialdehyde and hydroxynonenal).<sup>50</sup>  
342 Carbonylation is mostly irreversible and results in alteration of protein structure and  
343 function. Only a small fraction of carbonylated proteins can be removed through  
344 proteasome-dependent proteolysis. Work carried out on laboratory models found  
345 evidence that exposure to PFASs may change expression of genes regulating proteasome  
346 activation and proteolysis.<sup>6,51-52</sup> It is, however, unclear, whether such changes in gene  
347 expression make proteins one important target of the pro-oxidant effects of PFASs. This  
348 is important because when protein carbonyls accumulate, they tend to aggregate leading  
349 to cell death, tissue injury and development of disorders. Several studies found higher  
350 amounts of plasma protein carbonyls in individuals affected by a given disease,<sup>53-54</sup>  
351 suggesting a potential role of protein carbonylation in disease progression.

352           Depletion of circulating non-enzymatic antioxidants might reflect increased  
353 oxidation due to reaction with ROS, reduced intake from diet or mobilisation of  
354 antioxidants from blood to other target tissues. Irrespective of the reason, prior work on  
355 other bird species found evidence that circulating antioxidants may be linked to important  
356 individual or population fitness-related traits. For example, <sup>55</sup> found that barn swallows  
357 (*Hirundo rustica*) with lower plasma non-enzymatic antioxidants had reduced probability  
358 of survival. <sup>56</sup> found that Gentoo (*Pygoscelis papua*) and Adélie (*Pygoscelis adeliae*)



359 penguins from increasing populations had higher plasma non-enzymatic antioxidant  
360 capacity than those from decreasing populations.

361 Metabolic activity is one important source of ROS production. Thus the  
362 association we found between PFASs and oxidative status markers might mirror an effect  
363 of PFASs on metabolism. Prior work on the same kittiwake population found a positive  
364 association between the long-chain PF<sub>TriA</sub> and resting metabolic rate in females but not  
365 in males.<sup>30</sup> Thus, the association between PFASs and oxidative status markers does not  
366 appear, at least for males, to be due to a dysregulation of general body metabolism. The  
367 lack of an effect on metabolic rate does not reject the hypothesis that ROS production  
368 might have been higher in the more contaminated birds. For example, PFASs might have  
369 localised effects on important ROS generators (e.g., mitochondria of red blood cells or of  
370 other target tissues) without compromising the metabolism of the whole organism. *In*  
371 *vitro* studies found that PFAS exposure may impair mitochondrial activity and lead to  
372 increased rates of ROS production.<sup>57</sup>

373 It is unclear why the activity of glutathione peroxidase was not associated with  
374 any PFAS compound. It might be that up-regulation of this enzyme might have been too  
375 costly for the birds given the imminent start of breeding activity or that any effects of  
376 PFASs on oxidative status did not come through the pathways involving glutathione  
377 peroxidase. The biochemical function of glutathione peroxidase is to reduce hydrogen  
378 peroxide to water and organic hydroperoxides to their corresponding alcohols.<sup>50</sup> Thus, we  
379 cannot exclude that results would have been different if another antioxidant enzyme with  
380 a different biochemical function would have been measured. There are, however, many  
381 discrepancies in the literature about the response of antioxidant enzymes to PFAS  
382 exposure. For example, prior work did not find any association between PFASs exposure

383 and whole-body catalase activity in the planktonic crustacean *Daphnia magna*<sup>58</sup> or  
384 plasma superoxide dismutase activity in white-tailed eagle (*Haliaeetus albicilla*)  
385 nestlings.<sup>36</sup> In contrast, exposure to PFASs affected catalase activity in hepatocytes of  
386 freshwater tilapia *Oreochromis niloticus*<sup>24</sup> and expression of antioxidant genes Sod1,  
387 Sod2, Gpx2 and Nqo1 in mouse pancreas.<sup>59</sup> Irrespective of the mechanisms involved, the  
388 activity of GPX in erythrocytes does not appear to be an informative marker about the  
389 impact on oxidative status of the PFASs we have measured in this work.

390 In conclusion, our work shows that higher protein oxidative damage was found in  
391 those birds having higher concentrations of PFDoA, PFTriA and PFTeA. Lower  
392 plasmatic non-enzymatic micro-molecular antioxidants was found in those birds having  
393 higher concentrations of PFUnA, PFDoA and PFTeA. Experimental work will be needed  
394 to ascertain whether the correlation between individual PFAS burden and oxidative status  
395 markers reflects a direct toxic effect of PFASs on oxidative homeostasis. It will also be  
396 important to determine whether increased oxidative damage or decreased antioxidant  
397 defences turn into a reduction in survival probability or lifetime reproductive success.  
398 Resistance against oxidative stress may also decrease with chronological age.<sup>60</sup> Thus, we  
399 highlight the importance of assessing in future studies whether the effects of PFASs on  
400 oxidative status markers are stronger in older individuals.

401

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417

#### 418 **COMPETING INTERESTS**

419 The authors declare no competing financial interests.

420

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- 601

602 Table 1. Descriptive statistics of all poly- and perfluorinated alkyl substances congeners,  
 603 oxidative status markers (i.e., response variables) and several individual metrics  
 604 (potential confounding factors) measured in 50 male black-legged kittiwakes from  
 605 Svalbard.

<b>Variable</b>	<b>Mean ± Standard deviation</b>
CONTAMINANT	
PFOSlin - Linear perfluorooctane sulfonic acid (ng/g ww)	13.4 ± 6.2
PFNA - Perfluorononanoic acid (ng/g ww)	2.0 ± 0.9
PFDcA - Perfluorodecanoic acid (ng/g ww)	2.9 ± 1.2
PFUnA - Perfluoroundecanoic acid (ng/g ww)	10.3 ± 3.7
PFDoA - Perfluorododecanoic acid (ng/g ww)	1.7 ± 0.8
PFTriA - Perfluorotridecanoic acid (ng/g ww)	8.6 ± 3.1
PFTeA - Perfluorotetradecanoic acid (ng/g ww)	1.0 ± 0.8
OXIDATIVE STATUS MARKERS – RESPONSE VARIABLES	
Protein carbonyls (nmol/mg protein)	1.8 ± 0.6
Total protein carbonyls (nmol)	22.5 ± 8.2
Glutathione peroxidase (Units/mg protein)	0.4 ± 0.2
Non-enzymatic antioxidant capacity (mM HOCl neutralised)	169.9 ± 37.1
Non-enzymatic micro-molecular antioxidant capacity (mM HOCl neutralised/mg protein)	13.0 ± 2.0
INDIVIDUAL TRAITS	
Body mass (g)	416.2 ± 23.1
Skull length (mm)	93.9 ± 1.6
Tarsus length (mm)	34.9 ± 1.0
Wing length (mm)	317.1 ± 6.3
Baseline corticosterone (ng/ml)	9.6 ± 7.4
Testosterone (ng/ml)	1.7 ± 1.4
Luteinizing hormone (ng/ml)	6.2 ± 2.4

606

607 Table 2. The table shows the outcomes of generalized linear models performed to test the  
608 effect of each PFASs congener on oxidative status markers. Each model also included a  
609 number of potential confounding factors that may affect oxidative status markers  
610 independently from PFASs. Significant effects are shown in bold type. Note that the  
611 coefficient estimate for the relationship between body mass and the dependent variable  
612 (i.e., oxidative status marker) is calculated considering the effect of body size, thus it  
613 actually indicates the covariation between a given marker and the individual body  
614 condition.<sup>43</sup> GPX = glutathione peroxidase; N-E Antioxs = non-enzymatic antioxidant  
615 capacity of plasma; N-E Micromol Antioxs = non-enzymatic micro-molecular  
616 antioxidant capacity of plasma; ce ± se = coefficient estimate ± standard error.

	Protein Oxidative Damage		GPX		N-E Antioxs		N-E Micromol Antioxs	
Main effect included in the model	ce ± se	P	ce ± se	P	ce ± se	P	ce ± se	p
PFOSlin	0.03±0.12	0.817	0.02±0.02	0.462	-13.7±5.0	<b>0.006</b>	-0.20±0.28	0.484
sampling date	0.11±0.13	0.406	0.03±0.03	0.309	-16.6±5.0	<b>0.001</b>	0.18±0.31	0.546
sampling time	-0.20±0.11	0.074	0.03±0.02	0.196	14.3±4.6	<b>0.002</b>	0.22±0.28	0.425
body mass	-0.29±0.12	<b>0.013</b>	-0.02±0.03	0.407	4.0±5.0	0.424	0.00±0.29	1.000
body size	-0.02±0.11	0.840	0.02±0.02	0.403	-5.5±4.7	0.236	0.10±0.27	0.704
hormonal status	-0.10±0.11	0.391	0.02±0.02	0.348	4.0±4.7	0.391	-0.94±0.28	<b>0.001</b>
PFNA	-0.03±0.13	0.852	0.01±0.03	0.764	-1.0±6.0	0.872	-0.09±0.34	0.804
sampling date	0.08±0.15	0.580	0.02±0.03	0.433	-10.7±6.0	0.077	0.22±0.35	0.534
sampling time	-0.20±0.11	0.074	0.03±0.02	0.208	14.2±5.0	<b>0.004</b>	0.22±0.28	0.423
body mass	-0.30±0.12	<b>0.011</b>	-0.02±0.03	0.395	5.2±5.4	0.336	0.01±0.29	0.977
body size	-0.01±0.11	0.896	0.02±0.02	0.353	-7.4±5.0	0.137	0.08±0.27	0.768
hormonal status	-0.08±0.11	0.460	0.03±0.02	0.262	0.28±4.9	0.953	-0.98±0.27	<b>&lt;0.001</b>
PFDcA	0.22±0.13	0.106	0.00±0.03	0.904	-0.65±5.8	0.910	-0.49±0.31	0.116
sampling date	0.22±0.14	0.104	0.02±0.03	0.493	-10.5±5.0	0.075	-0.02±0.33	0.950
sampling time	-0.18±0.11	0.088	0.03±0.02	0.195	14.1±4.9	<b>0.004</b>	0.21±0.27	0.438
body mass	-0.22±0.12	0.056	-0.02±0.03	0.407	5.1±5.6	0.357	-0.07±0.29	0.809
body size	-0.03±0.11	0.795	0.02±0.02	0.352	-7.4±5.0	0.140	0.09±0.27	0.723
hormonal status	-0.14±0.11	0.204	0.03±0.02	0.249	0.24±4.9	0.960	-0.92±0.27	<b>0.001</b>
PFOuA	0.14±0.13	0.268	0.00±0.03	0.970	-3.3±5.6	0.562	-0.62±0.29	<b>0.035</b>
sampling date	0.19±0.14	0.181	0.02±0.03	0.517	-12.0±5.8	<b>0.038</b>	-0.07±0.32	0.822
sampling time	-0.18±0.11	0.098	0.03±0.02	0.196	13.9±4.9	<b>0.005</b>	0.19±0.27	0.483
body mass	-0.24±0.12	<b>0.045</b>	-0.02±0.03	0.403	4.2±5.7	0.453	-0.13±0.29	0.659
body size	-0.03±0.11	0.807	0.02±0.02	0.348	-7.1±5.0	0.154	0.12±0.26	0.644
hormonal status	-0.13±0.11	0.237	0.03±0.02	0.247	0.86±4.9	0.861	-0.87±0.26	<b>0.001</b>
PFDaA	0.29±0.13	<b>0.024</b>	-0.01±0.03	0.761	-1.5±5.4	0.779	-0.73±0.29	<b>0.012</b>
sampling date	0.26±0.14	0.066	0.01±0.03	0.625	-10.9±5.6	0.052	-0.10±0.30	0.736
sampling time	-0.18±0.10	0.088	0.03±0.02	0.199	14.1±4.9	<b>0.004</b>	0.21±0.26	0.421
body mass	-0.20±0.11	0.076	-0.02±0.03	0.343	4.9±5.6	0.378	-0.16±0.28	0.563
body size	-0.02±0.11	0.833	0.02±0.02	0.325	-7.2±5.0	0.148	0.14±0.25	0.574
hormonal status	-0.14±0.10	0.176	0.03±0.02	0.220	0.21±4.8	0.965	-0.95±0.25	<b>&lt;0.001</b>
PFTriA	0.30±0.14	<b>0.036</b>	0.00±0.03	0.923	2.0±5.9	0.735	-0.58±0.32	0.064
sampling date	0.26±0.15	0.075	0.02±0.03	0.597	-8.8±6.1	0.153	-0.12±0.34	0.725
sampling time	-0.18±0.11	0.094	0.03±0.02	0.214	14.5±5.1	<b>0.004</b>	0.10±0.27	0.725
body mass	-0.26±0.11	<b>0.026</b>	-0.02±0.03	0.368	5.7±5.5	0.299	-0.08±0.28	0.763
body size	-0.06±0.12	0.632	0.02±0.02	0.348	-7.9±5.2	0.124	0.20±0.27	0.454
hormonal status	-0.14±0.11	0.181	0.03±0.02	0.226	-0.13±4.9	0.979	-0.92±0.26	<b>&lt;0.001</b>
PFTeA	0.33±0.11	<b>0.003</b>	-0.02±0.03	0.482	7.4±5.4	0.171	-0.65±0.29	<b>0.028</b>
sampling date	0.22±0.12	0.070	0.01±0.02	0.664	-7.4±5.1	0.145	0.05±0.29	0.852
sampling time	-0.27±0.11	<b>0.013</b>	0.03±0.02	0.149	12.3±5.0	<b>0.015</b>	0.40±0.27	0.144
body mass	-0.25±0.11	<b>0.018</b>	-0.03±0.03	0.317	6.4±5.3	0.227	-0.05±0.28	0.858
body size	-0.05±0.11	0.668	0.03±0.02	0.275	-9.0±5.0	0.072	0.19±0.26	0.461
hormonal status	-0.19±0.10	0.062	0.03±0.02	0.171	-1.9±4.9	0.704	-0.84±0.27	<b>0.001</b>

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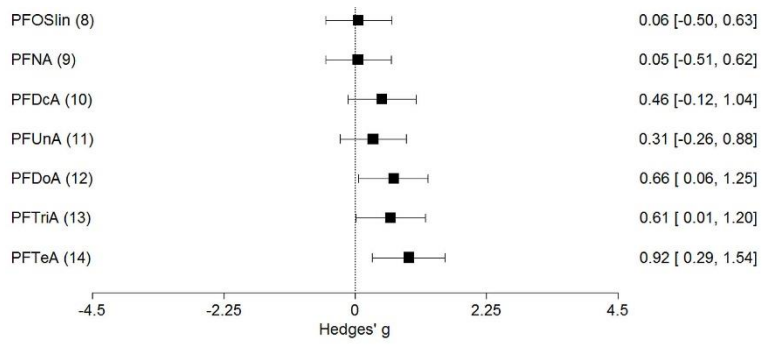
618 **Figure captions**

619 Figure 1. Mean estimates of effect size and 95% confidence interval are shown. These  
620 were calculated from the statistical outcomes showing the effect of each PFAS congener  
621 on protein oxidative damage. Estimates are positive when values of damage are higher in  
622 birds having higher plasma concentration of a given PFAS congener. Note that effect size  
623 estimates are significant when the 95% confidence interval does not include zero.  
624 Numbers in bracket indicate carbon-chain-length of each PFAS congener.

625

626 Figure 2. Mean estimates of effect size and 95% confidence interval were calculated from  
627 the statistical outcomes showing the effect of each PFAS congener on plasma non-  
628 enzymatic micromolecular antioxidant capacity. Estimates are negative when values of  
629 antioxidants are lower in birds having higher plasma concentration of a given PFAS  
630 congener. Note that effect size estimates are significant when the 95% confidence interval  
631 does not include zero. Numbers in bracket indicate carbon-chain-length of each PFAS  
632 congener.

633

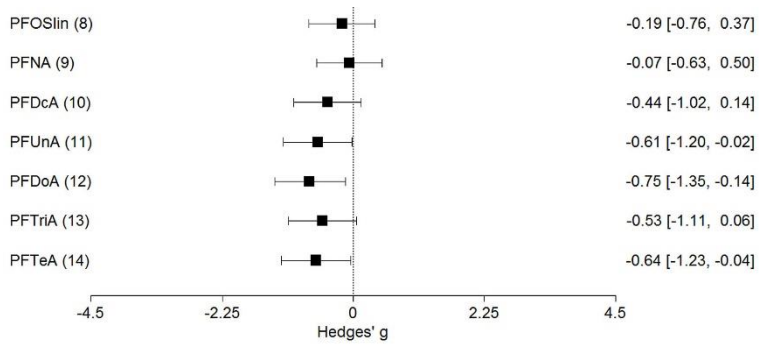


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636 Figure 1

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639 Figure 2

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