Interplay between pollution and avian influenza virus in shorebirds and waterfowl

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Abstract

Anthropogenic pollution may disrupt wildlife immune function and increase susceptibility to, and ability to withstand, infection. Of particular concern is avian influenza virus (AIV), which in its low-pathogenic form is endemic in many wild bird populations, notably waterfowl and shorebirds, and in its high-pathogenic form poses a threat to wildlife, livestock and people. Many pollutants have immunomodulative properties, yet little is known about how these pollutants affect AIV infection risk specifically. We examined concentrations of known immunomodulatory compounds, per- and polyfluoroalkyl substances (PFASs), and assessed their influence on AIV infection in three reservoir species, red-necked stint (*Calidris ruficollis,* n=121), pacific black duck (*Anas superciliosa,* n=57) and grey teal (*Anas gracilis*, n= 62). Using data on viral prevalence (cloacal/oropharyngeal swabs) and seroprevalence (AIV anti-nucleoprotein antibodies), we found no significant effect of PFASs pollution (total PFASs <0.01 – 470 ng/g in red-necked stint, <0.01 – 600 ng/g in pacific black duck and 0.3 – 200 ng/g in grey teal) on infection status in our three species. This may be due to relatively low pollutant concentrations, but we cannot rule out possible population culling through a synergy of pollution and infection stressors. We therefore recommend further studies on infection incidence in more polluted populations or species.

Keywords:

Avian influenza, shorebird, duck, PFASs, immunomodulation, wildlife health

Introduction

Pollution threatens wildlife species all around the globe through a diversity of effects. At high enough concentrations, chemical pollution can be acutely toxic, causing direct mortality (Katagi & Fujisawa, 2021). At sub-lethal levels, pollutants may cause physiological harm both individually (Dietz et al., 2019), and when interacting with one another. These interactions can result in other additive or even synergistic harmful effects (Dennis et al., 2020). Physiological effects of pollutants are diverse and may include disruption of both endocrine systems (Dietz et al., 2019; Sebastiano et al., 2021) and reproductive function (Dietz et al., 2019), an increase in oxidative stress (Valavanidis, Vlahogianni, Dassenakis, & Scoullos, 2006), and immunomodulation (Castaño-Ortiz, Jaspers, & Waugh, 2019; Vallverdú-Coll, Mateo, Mougeot, & Ortiz-Santaliestra, 2019). Immunomodulation can change the way organisms interact with pathogens in their environment, thus impacting individuals' susceptibility to and recovery from disease with knock-on effects on the epidemiology of pathogens.

Interactions between pollution and pathogens can be complex. Direct effects have been studied in a range of pollutants; lead, for example, increases susceptibility to infections by altering T-lymphocyte response in domestic chickens (*Gallus gallus*) (Vallverdú-Coll et al., 2019). The same effects have also been reported from air contaminants from oil sands (including ozone, sulphur dioxide, and polycyclic aromatic hydrocarbons) in wild tree swallows (*Tachycineta bicolor*) (Cruz-Martinez et al., 2015). Mercury pollution has been shown to cause a reduction in natural antibodies in barnacle goslings (*Branta leucopsis*) (Han, van den Berg, Loonen, Mateo, & van den Brink, 2022). Expression of different immune genes in embryonic chicken fibroblasts can be reduced by both polychlorinated biphenyls (PCBs: Badry, Jaspers, & Waugh, 2020) and per-/poly-fluoroalkyl substances (PFASs: Castaño-Ortiz et al., 2019). Indirect interactions with immune function can also emerge with pollution potentially reducing an animal's body condition resulting in limited availability of resources to mount an immune response to combat infection (Teitelbaum et al., 2022). While immunomodulatory effects of many pollutants have been well studied *in vitro* and in captive birds, studies of both pollution and disease simultaneously in wild birds have rarely been conducted (Teitelbaum et al., 2022).

PFASs are a growing immunomodulatory contaminant group that consist of partially or fully fluorinated carbon chains. This structure lends them both hydro- and lipophobic properties and high stability (Buck et al., 2011). These properties have led to the use of PFASs in many products, including firefighting foams, textiles and kitchen utensils. PFASs are rapidly increasing in diversity with some estimates suggesting over 14,000 are currently in circulation (US Environmental Protection Agency, 2022) with little regulation in place (UNEP, 2022). The immunomodulative properties of PFASs can affect both adaptive and innate immunity of wildlife. In birds, Peden-Adams et al. (2008) showed a reduction of the adaptive response in response to the phytohemagglutinin (PHA) test in chickens

91 (*Gallus gallus domesticus*) exposed to PFOS while in the egg, whereas Smits and Nain (2013) showed
92 a significant reduction of the T-cell dependent antibody response in Japanese quails (*Coturnix*
93 *japonica*). Effects on innate immunity in chickens include a downregulation in the expression of
94 immune genes nuclear factor kappa-light-chain-enhancer of activated B cells (*NF-κB),* interleukins 8
95 (*IL-8*) and 4 (*IL-4*), all of which are involved in the inflammation response (Castaño-Ortiz et al., 2019).
96 Migratory birds of the East-Asian Australasian flyway (EAAF) spending the non-breeding period in
97 Australia may experience exposure to low pathogenic avian influenza virus (AIV), but are potentially
98 also exposed to highly pathogenic avian influenza while in Asia (Wille et al., 2019). Highly pathogenic
99 avian influenza causes severe morbidity and mortality in wild birds, and is currently causing a
100 panzootic with profound impacts on birds, globally (Wille & Barr, 2022). AIV is endemic in Australian
101 wild birds (Wille et al., 2023), and is often associated with other co-infections (Wille et al., 2018), and
102 may thus function as an indicator of overall susceptibility to infection.
103 In this study, we investigated the effect of PFAS pollution on the infection risk of AIV in three of the
104 pathogen's prime Australian reservoir species. We focussed on three avian species: one species of
105 shorebird, red necked stint (*Calidris ruficollis*, order Charadriiformes), and two waterfowl species
106 (order Anseriformes): pacific black duck (*Anas superciliosa*) and grey teal (*Anas gracilis*). Long-term
107 AIV prevalence and AIV seroprevalence levels in these three species were established at 3% and 10%,
108 7% and 50%, and 6% and 55%, respectively, which are among the highest AIV prevalence values
109 measured in birds in Australia (Wille et al., 2023). Furthermore, evidence of highly pathogenic avian
110 influenza infection has previously been found in red-necked stint (Wille et al., 2019). This species also
111 migrates via an extremely polluted region (including PFASs), China's Yellow Sea (Muir & Miaz, 2021;
112 Xiao et al., 2017), highlighting the potential threat posed by the interplay between pollution and
113 disease. As PFASs (and PFOS in particular), have been shown to downregulate the innate immune
114 response (Castaño-Ortiz et al., 2019), we hypothesised that with increasing exposure to these
115 pollutants the incidence of both AIV and AIV antibodies would increase.
116
117 **Methods**
118 *Ethics statement*

119 All birds were captured and sampled under approval of Deakin University Animal Ethics Committee
120 (permit numbers A113-2010, B37-2013, B43-2016, B39-2019), Wildlife Ethics Committee of South
121 Australia (2011/1, 2012/35, 2013/11), and Philip Island Nature Park Animal Ethics Committee
122 (SPFL20082).

123 *Sample collection*

124 Red-necked stint were captured on the coast of Victoria, at the Western Treatment Plant (WTP) and
125 in Western Port Bay, both near Melbourne, Australia. Pacific black ducks were sampled at the WTP,
126 and on farmland near Geelong, also in Victoria. Grey teal were also sampled at the WTP and
127 Innamincka Regional Reserve in South Australia. Catches of birds occurred from 2011-2020. Red-
128 necked stint were captured using cannon-nets in collaboration with the Victorian Wader Study Group
129 (VWSG), as part of ongoing shorebird banding work starting 1978 (Minton, 2006). Ducks were caught
130 using baited walk-in traps and mist nets. All birds were blood sampled and oropharyngeal and cloacal
131 swabs were taken. Of each bird, cloacal and oropharyngeal swab samples were collected using sterile
132 swabs, and immediately stored in virus transport medium (VTM, brain heart infusion [BHI] broth-
133 based medium [Oxoid] with 0.3 mg/ml penicillin, 5 mg/ml streptomycin, 0.1 mg/ml gentamicin, and
134 2.5 g/ml, amphotericin B), either separately (pre-March 2014) or together (March 2014 onwards).
135 Swab samples in VTM were kept refrigerated for up to a week, before being stored at below -80°C.
136 Blood samples (typically 200 µl) were collected from the brachial vein of each bird using Sarstedt
137 Microvette capillary tubes. Samples were refrigerated and left to clot, next centrifuged within 6-24
138 hrs after collection to separate red blood cells (RBC) from serum, after which they were stored at
139 below -20°C. Serum was used to test for the presence of AIV antibodies, while RBC was used for PFAS
140 analyses.
141 Across the entire influenza surveillance study, we collected 1512 sample sets from red necked stints,
142 446 from pacific black ducks, and 660 from grey teals. We selected the samples of all 41 AIV-positive
143 red-necked stints, 29 AIV-positive pacific black ducks and 29 AIV-positive grey teals. These samples
144 were matched with the samples from 68, 28 and 33 AIV negative birds, respectively. The AIV negative
145 sample sets were stratified-randomly selected, ensuring at least 10 AIV negative individuals were
146 included from each of the sites where AIV positive birds were sampled. Serum samples from the same
147 birds were also used for AIV antibody analyses with the exception of two pacific black ducks and three
148 grey teals for which we failed to collect serum. For red-necked stint we included an additional 12
149 serum samples for serological analysis. Total numbers of individuals involved across all samples were
150 121 red-necked stints, 57 pacific black ducks and 62 grey teals.

151 *AIV screening*

152 Samples were screened for AIV following established methods, described in Wille et al. (2023). Briefly,
153 RNA was extracted from swab samples, which were assayed for AIV using quantitative reverse
154 transcriptase real time PCR targeting a short fragment of the matrix gene (Spackman et al., 2002). A
155 cycle threshold (Ct) cut-off of 40 was used. In cases where oropharyngeal and cloacal swabs were
156 collected separately (pre-March 2014), results were combined and a bird was deemed AIV-positive if
157 either swab returned a positive result. Blood serum samples were assayed for anti-nucleoprotein

158 antibodies of AIV using the Multi Screen Avian Influenza Virus Antibody Test Kit (IDEXX, Hoppendorf,
159 The Netherlands) following manufacturer's instructions.

160 *Chemical analyses of blood samples*

161 All RBC samples were analysed for 12 different PFASs (full details provided in Table S1), which were
162 broadly grouped as carboxylates (PFPA, PFHxA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrA, PFTeA) and
163 sulfonates (PFBS, PFOS). We also calculated a total PFAS group, consisting of all carboxylates and
164 sulfonates listed above, as well as PFOSA. Analyses were conducted using methodology adapted from
165 Trimmel et al. (2021). In brief, ~ 50 mg of red blood cell samples was weighted in a 1.5 mL
166 polypropylene tube and 10 µL of PFOA-$^{13}C_8$ and PFOS-$^{13}C_8$ (1000 µg/L) was added. After adding 0.3 mL
167 methanol containing 1% ammonium formate, the sample was vortexed for 30 s and ultrasonicated for
168 30 min. Then the sample was centrifuged for 5 min at 3500 rpm. Finally, the supernatant was
169 transferred into a 1.5 mL injection vial with an insert vial after purification by Hybrid-SPE. The sample
170 extract was stored at -20 °C until analysis by UPLC-MS/MS.

171 *Statistical analysis*

172 All statistical analyses were conducted using R Version 4.2.0, in RStudio 2022.02.0 Build 443. All PFASs
173 were summed into carboxylate (ΣPFCAs), sulfonate (ΣPFSA), and total PFASs (ΣPFAS) categories as
174 outlined above. All non-detected values of individual compounds were replaced with 0.015 ng/g,
175 equivalent to half the minimum quantification limit of detection for all compounds. When summed
176 categories were calculated, any totals where no compounds were detected were also set at 0.015
177 ng/g. For each of the three focal species, we first ran two-sample t-tests on the grouped PFAS data
178 (ΣPFCAs, ΣPFSAs and ΣPFASs) from each region, to determine if there were pollution differences
179 between regions.

180 We used generalised linear mixed effects models in R package lme4 (Bates, Mächler, Bolker, & Walker,
181 2015) (function glmer, family binomial) to investigate AIV status (positive or negative) in each species,
182 as a function of each of our 12 pollutants individually, as well as ΣPFCAs, ΣPFSAs, and ΣPFASs (i.e. 3 ×
183 15 = 45 models). These models included region as a random effect. We also ran this same structure of
184 models across all samples, including species as a second random effect alongside region (i.e. 15
185 models). Finally, we ran the same model across all samples combined (again with species and region
186 as random effects), in which ΣPFCAs and ΣPFSA concentrations were both entered simultaneously as
187 explanatory variables (i.e. 4 models). Overall, we thus ran a total of 64 models for AIV prevalence. We
188 ran the same set of models using AIV serostatus (positive or negative) instead of AIV status as

189 dependent variable, culminating in a grand total of 128 models testing the effect of PFASs on AIV
190 status and serostatus.

191 **Results**

192 Of the 12 individual pollutants that we targeted, only PFOS, PFDoA and PFUnA were detected in more
193 than 50% of our samples (94%, 72% and 60% respectively, Tables 1 and 2). When summed into overall
194 categories, ΣPFCAs, ΣPFSAs and ΣPFASs were detected in 90%, 95% and 98% of all samples
195 respectively, regardless of species. Across all the 240 samples tested, ΣPFCAs were not detected in 24
196 samples, ΣPFSAs were not detected in 13 samples, and ΣPFASs were not detected in only six samples.
197 ΣPFCAs concentrations were below 50 ng/g in all but 7 samples (3%), with samples from four red-
198 necked stints (56, 71, 93 and 98 ng/g) and three pacific black ducks (54, 70 and 74 ng/g) comprising
199 the exception. ΣPFSAs by contrast were higher in concentration, with 11 samples (5%) exceeding 200
200 ng/g (Figure 1), comprising of 6 red-necked stints (223-423 ng/g) and 5 pacific black ducks (208-
201 503ng/g). We found that all ΣPFCAs, ΣPFSAs and ΣPFASs were significantly higher at the WTP in all
202 species (p<0.05, see Table S2 for summary concentrations), with one exception where no significant
203 differences between regions was found for ΣPFCAs in pacific black duck. A complete range of detection
204 rates and overall concentrations by AIV status and AIV serostatus are shown in Table 1 and Table 2,
205 respectively.

206 Across all 128 models, no significant effects were found for the influence of PFAS pollution on AIV
207 prevalence and seroprevalence, except for five models (p values for each species, and overall sample
208 population provided in Tables 1 and 2). Significant effects were found for PFDA, PFTrA and ΣPFCAs on
209 AIV infection status for all species combined, and for ΣPFCAs on AIV infection status for red-necked
210 stints when modelled individually. Finally, ΣPFCAs were also found to have a significant effect on AIV
211 infection status of red-necked stints when ΣPFSAs were also included in the same model. No significant
212 effect of pollution on serostatus was found in any model. PFDA was found to be lower in birds that
213 were infected with AIV (AIV positive median: <0.01 ng/g, range: <0.01-13.71 ng/g; AIV negative
214 median: <0.01 ng/g, range: <0.01-19.8 ng/g; slope: -0.146, p = 0.044), as was PFTrA (AIV positive
215 median: <0.01 ng/g, range: <0.01-13.6 ng/g; AIV negative median: <0.01 ng/g, range: <0.01-28.3 ng/g;
216 slope = -0.129, p = 0.023) The same pattern was observed for all three models in which ΣPFCAs were
217 found to have a significant effect, across all species modelled together (AIV positive median: 3.44 ng/g,
218 range: <0.01-98.2 ng/g; AIV negative median: 5.25 ng/g, range: <0.01-93.2 ng/g; slope = -0.028, p =
219 0.021), and in red-necked stints when ΣPFCAs were modelled individually (AIV positive median: 2.36
220 ng/g, range: <0.01-49.2 ng/g; AIV negative median: 9.40 ng/g, range: <0.01-93.2 ng/g; slope = -0.037,
221 p = 0.041) and with ΣPFSAs (slope = -0.048, p = 0.049).

Discussion

Our results, encompassing concentrations of 12 per-/polyfluorinated compounds, and both AIV infection and serostatus in three different species, did not suggest that infection of AIV in these birds is influenced by their PFAS burdens. Of the 128 models run, only five significant correlations were detected with *p* values just below 0.05, which could well be spurious given the number of models that were run. Our general finding of no (or few) significant correlations is in contrast to a previous *in vitro* study where PFASs were demonstrated to have immunomodulatory effects in birds (Castaño-Ortiz et al., 2019). If such effects were present in the current study, we would have expected actively AIV-infected or previously AIV-infected birds to have higher concentrations of PFASs in their system.

We expect that the absence of a correlation between PFAS pollution and AIV in this study was because our observed PFAS concentrations were too low to elicit immunomodulatory effects. In the *in vitro* study by Castaño-Ortiz et al. (2019), exposure to PFOS was at 22mg/L, likely far higher than any exposure our study birds experienced. Indeed, even though PFAS pollution often varied between sample sites, the concentrations we observed were low relative to 'predicted no-effects concentrations' in serum presented by Newsted, Jones, Coady, and Giesy (2005), the lowest of which was 150 ng/g. PFAS concentrations in all but three individuals' (two red-necked stints and one pacific black duck) were also low compared to 'lowest observable adverse effect levels' (LOAELs) of 400ng/g in adult bird liver tissues proposed by Dennis et al. (2021). These LOAELs were calculated based on decreased adult weight gain. Unfortunately, the disparity between sample matrices (liver vs. red blood cells) means we cannot draw direct comparisons between our study and Dennis et al. (2021)'s proposed toxicity thresholds. Nevertheless, concentrations of PFASs in liver tend to be higher than those in blood (Gebbink & Letcher, 2012) and as such, we can consider Dennis et al. (2021)'s liver LOAELs to be conservative estimates of toxicity in blood. The true number of our samples that exceed the adult LOAEL from Dennis et al. (2021) is likely to be higher. Aside from the three individual exceptions mentioned above, the low concentrations we observed may be considered remarkable for birds that inhabit putatively polluted wastewater treatment plants (all focal species) or migrate via highly polluted environments (red-necked stint migrating via e.g. the Yellow Sea) (Muir & Miaz, 2021). Nevertheless, in 105 individuals, the total PFASs concentrations found were higher than the lowest LOAELs posited by Dennis et al. (2021) (50 ng/g in liver for young birds, based on decreased successful hatching), which suggested there may be some risk to juvenile birds in our population. The absence of specific data on immunomodulation thresholds means we cannot definitively state whether our concentrations are below levels to cause effect. However, our results did not yet suggest that this is the case.

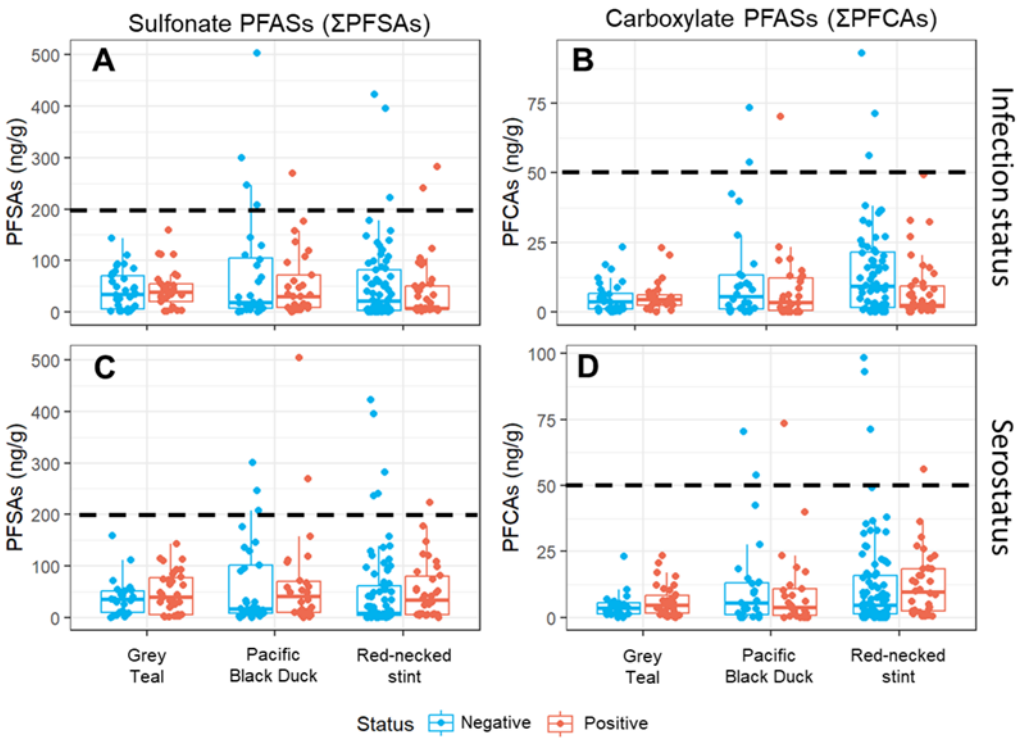
255 It is worth noting that some individuals had particularly elevated PFASs concentrations (e.g. one pacific
256 black duck was observed with 599 ng/g of PFOS, with the next highest concentration observed in a
257 red-necked stint at 467 ng/g, see Figure 1), while being negative for both AIV infection. However, no
258 conclusions can be based on such rare extreme cases due to of a) the very small sample size of
259 individuals with elevated PFASs and b) the level of exposure of these pacific black ducks and red-
260 necked stints to AIV (10% seroprevalence: Wille et al., 2023) increasing stochasticity in the occurrence
261 of infection. It is clear though, that individuals can accrue a high PFAS load without necessarily
262 incurring infections.

263 An alternative explanation for the low PFASs concentrations, and minimal significant effects (aside
264 from 5 out of the 128 models suggesting a negative relationship between AIV infection and pollution
265 burden), is that the absence of an effect is due to population culling. Population culling is the
266 phenomenon whereby sick, or in this case, polluted individuals do not survive and are thereby 'culled'
267 from the population, such that we never or infrequently detect them when sampling. In our sample
268 population, there was clearly the possibility for individuals to accrue elevated PFAS burdens, as
269 demonstrated by the 11 birds with PFSAs concentrations greater than 200ng/g (see Figure 1). In the
270 absence of additional stressors such as disease, birds may be able to withstand elevated
271 concentrations. Congruently, wild birds have limited documented signs of disease associated with low
272 pathogenic avian influenza beyond a reduction in daily movement in mallards (*Anas platyrhynchos*)
273 (Van Dijk et al., 2015) and poorer performance in foraging or migrating Bewick's swans (*Cygnus*
274 *columbianus*) (Hoye et al., 2016; Van Gils et al., 2007). The limited extent of these signs are such that
275 infection alone has little to no direct effect on the survival of its host (Maxted et al., 2012). However,
276 both the negative effect of pollution in isolation as well as the potential combined effect of pollution
277 and disease might even cause death, such that individuals with downregulated immune responses due
278 to pollution may be unable to survive the combined effects of PFASs and the virus (Guruge et al.,
279 2009). Such individuals may therefore have been lost to our study. Indeed, in all five models where
280 we found significant effects of pollution on AIV infection status, birds with lower pollution were more
281 likely to be AIV positive. The significant model results could indicate that infected birds with higher
282 concentrations are unable to survive the added pressures of both and therefore 'culled' from the
283 population. Hence, it is possible that we did not sample highly polluted birds that also had avian
284 influenza infections, because they did not survive. This is particularly pertinent for migrating
285 shorebirds which also deal with the additional stressors of physiological effects of migration including
286 immunomodulation (Buehler, Tieleman, & Piersma, 2010). Indeed, there is some evidence of
287 migratory culling, whereby migration may remove sick individuals (McKay & Hoye, 2016). This effect
288 could be more pronounced under pollution stress, which could become more toxic due to the thermal

289 stress from exertion during migration (Gordon, Johnstone, & Aydin, 2014). This is a possible
290 explanation as to why the only significant single-species models found were models involving data
291 from red-necked stints, as these are the only migratory species included in the study.

292 Overall, our birds were all sampled in environments with relatively low PFASs concentrations, with the
293 most polluted being processed wastewater at the WTP. In other regions of the world, PFAS pollution
294 may be higher (Muir & Miaz, 2021), as is the prevalence of AIV and therefore the likelihood of a birds'
295 exposure to the virus (Wille & Barr, 2022). In these regions, the effects of pollution-mediated
296 immunomodulation may be much stronger. The consequences of widespread immunomodulation
297 could be severe, for populations of wild birds, and for human health and poultry industries. Our
298 findings of no clear evidence of PFAS-mediated immunomodulation in Australia cannot discount
299 effects in more heavily polluted regions of the world where exposure to more dangerous high
300 pathogenicity AIV strains is more prevalent. The current global circumstances of increased highly
301 pathogenic avian influenza transmission amongst wild birds in the northern hemisphere highlight the
302 necessity to continue monitoring potential drivers of host susceptibility such as pollution, so that we
303 may garner a better understanding of the threats the interactions of these stressors pose to wild birds.

304



305

306 **Figure 1:** Concentrations (ng/g) of summed sulfonate (A,C) PFASs (ΣPFSAs) and summed carboxylate (B, D) PFASs (ΣPFCAs)
307 in red blood cells from grey teal (*Anas gracilis, n*=62), pacific black duck (*Anas superciliosa,* n=57) and red-necked stint
308 (*Calidris ruficollis,* n=121). A and B show infection status, while C and D show serostatus. Birds that were positive are
309 represented in red, and negative in blue. 4.5% of samples exceeded 200ng/g (dashed line in A and C) of ΣPFSAs, while 3.9%
310 of samples exceeded 50ng/g (dashed line in B and D) of ΣPFCAs (dashed line in B and D).

311

Table 1 Median and range concentrations of PFASs (in ng/g) in red blood cells of red-necked stint (*Calidris ruficollis*), pacific black duck (*Anas superciliosa*) and grey teal (*Anas gracilis*), based on the AIV infection status of each individual. Groups of compounds have been indicated. Where <0.01 is noted, this means the concentration was below the limit of quantification. Emboldened and shaded *p*-values indicate statistical significance between AIV negative and positive individuals. Where a value is recorded as NA, this compound was not detected in the species.

Compounds		All Samples		Red-necked stint					Pacific black duck					Grey teal				
					AIV-negative n=68		AIV-positive n=41			AIV-negative n=28		AIV-positive n=29			AIV-negative n=33		AIV-positive n=29	
	Group	%	P_{all}	P_{stint}	Median	Range	Median	Range	P_{duck}	Median	Range	Median	Range	P_{teal}	Median	Range	Median	Range
PFOS	Sulfonate	94.6	0.246	0.348	20.6	(<0.01-396)	7.17	(1.26-282)	0.34	19	(<0.01-504)	30.1	(<0.01-270)	0.932	34.3	(<0.01-143)	38.5	(0.81-159)
PFBS	Sulfonate	2.1	0.305	0.491	<0.01	(<0.01-0.737)	<0.01	(<0.01-5.39)	0.947	<0.01	(<0.01-<0.01)	<0.01	(<0.01-1.48)	NA	<0.01	(<0.01-<0.01)	<0.01	(<0.01-<0.01)
PFPA	Carboxylate	1.25	0.990	0.997	<0.01	(<0.01-24.0)	NA	NA	NA	<0.01	(<0.01-<0.01)	<0.01	(<0.01-<0.01)	NA	<0.01	(<0.01-<0.01)	<0.01	(<0.01-<0.01)
PFHxA	Carboxylate	1.3	0.553	0.553	<0.01	(<0.01-7.6)	<0.01	(<0.01-0.67)	NA	<0.01	(<0.01-<0.01)	<0.01	(<0.01-<0.01)	NA	<0.01	(<0.01-<0.01)	<0.01	(<0.01-<0.01)
PFOA	Carboxylate	31.7	0.710	0.288	<0.01	(<0.01-12.8)	0.89	(<0.01-8.17)	0.695	<0.01	(<0.01-1.49)	<0.01	(<0.01-1.35)	0.86	<0.01	(<0.01-2.04)	0.41	(<0.01-2.36)
PFNA	Carboxylate	41.7	0.441	0.956	<0.01	(<0.01-15.5)	<0.01	(<0.01-8.49)	0.228	0.52	(<0.01-3.36)	<0.01	(<0.01-4.27)	0.591	0.48	(<0.01-3.05)	0.54	(<0.01-1.54)
PFDA	Carboxylate	37.9	**0.044**	0.069	0.66	(<0.01-19.8)	<0.01	(<0.01-9.14)	0.669	<0.01	(<0.01-6.49)	<0.01	(<0.01-13.7)	0.664	<0.01	(<0.01-1.31)	<0.01	(<0.01-2.81)
PFUnA	Carboxylate	60.4	0.325	0.351	<0.01	(<0.01-18.5)	0.37	(<0.01-6.79)	0.272	1.39	(<0.01-25.8)	0.74	(<0.01-9.79)	0.475	0.91	(<0.01-6.32)	1.84	(<0.01-9.65)
PFDoA	Carboxylate	72.5	0.541	0.749	0.515	(<0.01-16.2)	0.77	(<0.01-13.8)	0.582	1.29	(<0.01-20.5)	1.06	(<0.01-40.7)	0.978	0.93	(<0.01-6.85)	1.15	(<0.01-6.33)
PFTrA	Carboxylate	42.9	**0.023**	0.129	<0.01	(<0.01-28.3)	<0.01	(<0.01-13.6)	0.137	0.278	(<0.01-17.4	<0.01	(<0.01-5.51)	0.119	0.52	(<0.01-3.73)	<0.01	(<0.01-2.71)
PFTeA	Carboxylate	39.6	0.084	0.194	0.192	(<0.01-23.4)	<0.01	(<0.01-10.9)	0.802	<0.01	(<0.01-10.4	<0.01	(<0.01-12.7)	0.444	<0.01	(<0.01-2.93)	<0.01	(<0.01-2.96)
PFOSA	Total PFASs	50	0.244	0.579	<0.01	(<0.01-26.1)	<0.01	(<0.01-14.0)	0.669	0.56	(<0.01-17.9)	1.39	(<0.01-36.5)	0.351	4.86	(<0.01-26.9)	5.83	(<0.01-21.6
ΣPFSAs		94.6	0.215	0.304	21.4	(<0.01-423)	7.17	(1.26-282)	0.341	19	(<0.01-504)	30.1	(<0.01-270)	0.932	34.3	(<0.01-143)	38.5	(0.81-159)
ΣPFCAs		90	**0.021**	**0.041**	9.40	(<0.01-93.2)	2.36	(<0.01-49.2)	0.311	5.45	(<0.01-73.5)	3.41	(<0.01-70.4)	0.963	3.68	(<0.01-23.3)	4.56	(<0.01-23)
ΣPFASs		97.5	0.186	0.221	38.3	(<0.01-467)	10.5	(1.84-331)	0.353	27.9	(<0.01-599)	36.6	(1.98-292)	0.847	50.3	(0.3-174)	54.2	(1.7-205)

Table 2: Median and range concentrations of PFASs in red blood cells (in ng/g) of red-necked stint (*Calidris ruficollis*), pacific black duck (*Anas superciliosa*) and grey teal (*Anas gracilis*), based on AIV serostatus of each individual. Groups of compounds have been indicated. Where <0.01 is noted, this means the concentration was below the limit of quantification. Emboldened and shaded *p*-values indicate statistical significance between seropositive and seronegative individuals. Where a value is recorded as NA, this compound was not detected in the species.

Compounds		All Samples		Red-necked stint					Pacific black duck					Grey teal				
					AIV-seronegative n=87		AIV-seropositive n=34			AIV-seronegative n=29		AIV-seropositive n=26			AIV-seronegative n=24		AIV-seropositive n=35	
	Group	%	P_{all}	P_{stint}	Median	Range	Median	Range	P_{duck}	Median	Range	Median	Range	P_{teal}	Median	Range	Median	Range
PFOS	Sulfonate	94.6	0.564	0.825	12.0	(<0.01-396)	17.9	(<0.01-282)	0.8	17.5	(<0.01-300)	40.7	(<0.01-504)	0.374	34.9	(<0.01-159)	40.2	(0.81-143)
PFBS	Sulfonate	2.1	0.580	0.511	<0.01	(<0.01-0.73)	<0.01	(<0.01-5.39)	0.943	<0.01	(<0.01-1.48)	<0.01	(<0.01-<0.01)	NA	<0.01	(<0.01-<0.01)	<0.01	(<0.01-<0.01)
PFPA	Carboxylate	1.25	0.981	0.999	<0.01	(<0.01-24)	<0.01	(<0.01-8.07)	NA	<0.01	(<0.01-<0.01)	<0.01	(<0.01-<0.01)	NA	<0.01	(<0.01-<0.01)	<0.01	(<0.01-<0.01)
PFHxA	Carboxylate	1.3	0.631	0.653	<0.01	(<0.01-7.6)	<0.01	(<0.01-7.67)	NA	<0.01	(<0.01-<0.01)	<0.01	(<0.01-<0.01)	NA	<0.01	(<0.01-<0.01)	<0.01	(<0.01-<0.01)
PFOA	Carboxylate	31.7	0.349	0.322	<0.01	(<0.01-11.1)	<0.01	(<0.01-12.8)	0.153	<0.01	(<0.01-1.49)	<0.01	(<0.01-0.44)	0.09	<0.01	(<0.01-0.93)	<0.01	(<0.01-2.36)
PFNA	Carboxylate	41.7	0.416	0.613	<0.01	(<0.01-15.5)	<0.01	(<0.01-27.6)	0.176	0.54	(<0.01-4.27)	0.092	(<0.01-3.36)	0.515	0.5	(<0.01-1.54)	0.62	(<0.01-3.05)
PFDA	Carboxylate	37.9	0.141	0.452	0.700	(<0.01-19.8)	<0.01	(<0.01-10)	0.199	0.46	(<0.01-13.7)	<0.01	(<0.01-6.49)	0.731	<0.01	(<0.01-2.81)	<0.01	(<0.01-1.9)
PFUnA	Carboxylate	60.4	0.909	0.358	<0.01	(<0.01-18.5	<0.01	(<0.01-20.5)	0.934	1.59	(<0.01-19.5)	1.37	(<0.01-25.8)	0.258	0.92	(<0.01-9.65)	1.88	(<0.01-8.49)
PFDoA	Carboxylate	72.5	0.607	0.524	0.510	(<0.01-16.2)	0.56	(<0.01-13.8)	0.632	1.53	(<0.01-40.7)	1.26	(<0.01-20.5)	0.382	0.815	(<0.01-6.33)	1.08	(<0.01-6.85)
PFTrA	Carboxylate	42.9	0.562	0.669	<0.01	(<0.01-28.3)	<0.01	(<0.01-25.5)	0.656	0.54	(<0.01-9.79)	<0.01	(<0.01-17.4)	0.283	0.505	(<0.01-2.71)	0.47	(<0.01-3.73)
PFTeA	Carboxylate	39.6	0.271	0.144	<0.01	(<0.01-16.7)	1.21	(<0.01-23.4)	0.794	<0.01	(<0.01-12.7)	<0.01	(<0.01-10.4)	0.626	<0.01	(<0.01-2.04)	<0.01	(<0.01-2.96)
PFOSA	Total PFASs	50	0.180	0.836	<0.01	(<0.01-26.1)	<0.01	(<0.01-14.0)	0.191	0.47	(<0.01-15)	1.31	(<0.01-36.5)	0.782	5.31	(<0.01-21.6)	5.47	(<0.01-26.9)
ΣPFSAs		94.6	0.600	0.870	12.0	(<0.01-423)	18.7	(<0.01-282)	0.801	17.5	(<0.01-300)	40.7	(<0.01-504)	0.374	34.9	(<0.01-159)	40.2	(0.81-143)
ΣPFCAs		90	0.845	0.994	8.0	(<0.01-93.1)	4.69	(<0.01-98.2)	0.645	5.46	(<0.01-70.4)	3.78	(<0.01-73.5)	0.27	3.66	(<0.01-23)	4.56	(<0.01-23.3)
ΣPFASs		97.5	0.609	0.887	24.0	(<0.01-467)	22.3	(<1.32-343)	0.815	25.4	(<0.01-373)	45.5	(<0.01-599)	0.364	50.6	(0.3-205)	55.4	(1.2-174)

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