# Interplay between pollution and avian influenza virus infection risk in shorebirds and waterfowl

**Tobias A. Ross[1], Junjie Zhang[2], Michelle Wille[3,4,5], Alexandros G. Asimakopoulos[2], Veerle L. B. Jaspers[6], Marcel Klaassen[1,7]**

[1] Centre for Integrative Ecology, School of Life and Environmental Sciences, Deakin University, Geelong, VIC, 3216, Australia
[2] Department of Chemistry, Norwegian University of Science and Technology (NTNU), Trondheim-7491, Norway
[3] Sydney School for Infectious Diseases, School of Life and Environmental Sciences and School of Medical Sciences, The University of Sydney, Sydney, New South Wales, Australia.
[4] Department of Microbiology and Immunology, at the Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Melbourne, Victoria, Australia.
[5] WHO Collaborating Centre for Reference and Research on Influenza, at the Peter Doherty Institute for Infection and Immunity, Melbourne, Victoria, Australia.
[6] Department of Biology, Norwegian University of Science and Technology (NTNU), Trondheim 7491, Norway
[7] Victorian Wader Study Group, Thornbury, VIC, 3071, VIC, Australia

**Abstract**

Anthropogenic pollution may disrupt wildlife physiology and impact their behaviour and survival. Potentially compounding this threat are immunomodulatory properties of pollutants that 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. While many pollutants are known to have immunomodulative properties, little is known about how these pollutants affect AIV infection risk specifically. We examined the concentrations of per- and polyfluoroalkyl substances (PFASs), which are considered immunomodulatory, and assessed their influence on avian influenza infection status 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 ranging from <0.01 – 1290 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 any of our three species. This may be due to relatively low pollutant concentrations, but we cannot rule out the possibility of population culling through a synergy of pollution and infection stressors. We therefore recommend further studies on infection incidence in more polluted species.

## 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

68 (*Gallus gallus domesticus*) exposed to PFOS while in the egg, whereas Smits and Nain (2013) showed
69 a significant reduction of the T-cell dependent antibody response in Japanese quails (*Coturnix*
70 *japonica*). Effects on innate immunity in chickens include a downregulation in the expression of
71 immune genes nuclear factor kappa-light-chain-enhancer of activated B cells (*NF-κB),* interleukins 8
72 (*IL-8*) and 4 (*IL-4*), all of which are involved in the inflammation response (Castaño-Ortiz et al., 2019).
73 Migratory birds of the East-Asian Australasian flyway (EAAF) spending the non-breeding period in
74 Australia may experience exposure to low pathogenic avian influenza virus (AIV), but are potentially
75 also exposed to highly pathogenic avian influenza while in Asia (Wille et al., 2019). Highly pathogenic
76 avian influenza causes severe morbidity and mortality in wild birds, and is currently causing a
77 panzootic with profound impacts on birds, globally (Wille & Barr, 2022). AIV is endemic in Australian
78 wild birds (Wille et al., 2023), and is often associated with other co-infections (Wille et al., 2018), and
79 may thus function as an indicator of overall susceptibility to infection.
80 In this study, we investigated the effect of PFAS pollution on the infection risk of AIV in three of the
81 pathogen's prime Australian reservoir species. We focussed on three avian species: one species of
82 shorebird, red necked stint (*Calidris ruficollis*, order Charadriiformes), and two waterfowl species
83 (order Anseriformes): pacific black duck (*Anas superciliosa*) and grey teal (*Anas gracilis*). Long-term
84 AIV prevalence and AIV seroprevalence levels in these three species were established at 3% and 10%,
85 7% and 50%, and 6% and 55%, respectively, which are among the highest AIV prevalence values
86 measured in birds in Australia (Wille et al., 2023). Furthermore, evidence of highly pathogenic avian
87 influenza infection has previously been found in red-necked stint (Wille et al., 2019). This species also
88 migrates via an extremely polluted region (including PFASs), China's Yellow Sea (Muir & Miaz, 2021;
89 Xiao et al., 2017), highlighting the potential threat posed by the interplay between pollution and
90 disease. As PFASs (and PFOS in particular), have been shown to downregulate the innate immune
91 response (Castaño-Ortiz et al., 2019), we hypothesised that with increasing exposure to these
92 pollutants the incidence of both AIV and AIV antibodies would increase.
93
94 **Methods**
95 *Ethics statement*

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

100 *Sample collection*

101 Red-necked stint were captured on the coast of Victoria, at the Western Treatment Plant (WTP) and
102 in Western Port Bay, both near Melbourne, Australia. Pacific black ducks were sampled at the WTP,
103 and on farmland near Geelong, also in Victoria. Grey teal were also sampled at the WTP and
104 Innamincka Regional Reserve in South Australia. Catches of birds occurred from 2011-2020. Red-
105 necked stint were captured using cannon-nets in collaboration with the Victorian Wader Study Group
106 (VWSG), as part of ongoing shorebird banding work starting 1978 (Minton, 2006). Ducks were caught
107 using baited walk-in traps and mist nets. All birds were blood sampled and oropharyngeal and cloacal
108 swabs were taken. Of each bird, cloacal and oropharyngeal swab samples were collected using sterile
109 swabs, and immediately stored in virus transport medium (VTM, brain heart infusion [BHI] broth-
110 based medium [Oxoid] with 0.3 mg/ml penicillin, 5 mg/ml streptomycin, 0.1 mg/ml gentamicin, and
111 2.5 g/ml, amphotericin B), either separately (pre-March 2014) or together (March 2014 onwards).
112 Swab samples in VTM were kept refrigerated for up to a week, before being stored at below -80°C.
113 Blood samples (typically 200 µl) were collected from the brachial vein of each bird using Sarstedt
114 Microvette capillary tubes. Samples were refrigerated and left to clot, next centrifuged within 6-24
115 hrs after collection to separate red blood cells (RBC) from serum, after which they were stored at
116 below -20°C. Serum was used to test for the presence of AIV antibodies, while RBC was used for PFAS
117 analyses.
118 Across the entire influenza surveillance study, we collected 1512 sample sets from red necked stints,
119 446 from pacific black ducks, and 660 from grey teals. We selected the samples of all 41 AIV-positive
120 red-necked stints, 29 AIV-positive pacific black ducks and 29 AIV-positive grey teals. These samples
121 were matched with the samples from 70, 28 and 33 AIV negative birds, respectively. The AIV negative
122 sample sets were stratified-randomly selected, ensuring at least 10 AIV negative individuals were
123 included from each of the sites where AIV positive birds were sampled. Serum samples from the same
124 birds were also used for AIV antibody analyses with the exception of two pacific black ducks and three
125 grey teals for which we failed to collect serum. For red-necked stint we included an additional ten
126 serum samples for serological analysis. Total numbers of individuals involved across all samples were
127 121 red-necked stints, 57 pacific black ducks and 62 grey teals.

128 *AIV screening*

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

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

137 *Chemical analyses of blood samples*

138 All RBC samples were analysed for 12 different PFASs (full details provided in Table S1), which were
139 broadly grouped as carboxylates (PFPA, PFHxA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrA, PFTeA) and
140 sulfonates (PFBS, PFOS). We also calculated a total PFAS group, consisting of all carboxylates and
141 sulfonates listed above, as well as PFOSA. Analyses were conducted using methodology adapted from
142 Trimmel et al. (2021). In brief, ~ 50 mg of red blood cell samples was weighted in a 1.5 mL
143 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
144 methanol containing 1% ammonium formate, the sample was vortexed for 30 s and ultrasonicated for
145 30 min. Then the sample was centrifuged for 5 min at 3500 rpm. Finally, the supernatant was
146 transferred into a 1.5 mL injection vial with an insert vial after purification by Hybrid-SPE. The sample
147 extract was stored at -20 °C until analysis by UPLC-MS/MS.

148 *Statistical analysis*

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

157 We used generalised linear mixed effects models in R package lme4 (Bates, Mächler, Bolker, & Walker,
158 2015) (function glmer, family binomial) to investigate AIV status (positive or negative) in each species,
159 as a function of each of our 12 pollutants individually, as well as ΣPFCAs, ΣPFSAs, and ΣPFASs (i.e. 3 ×
160 15 = 45 models). These models included region as a random effect. We also ran this same structure of
161 models across all samples, including species as a second random effect alongside region (i.e. 15
162 models). Finally, we ran the same model across all samples combined (again with species and region
163 as random effects), in which ΣPFCAs and ΣPFSA concentrations were both entered simultaneously as
164 explanatory variables (i.e. 4 models). Overall, we thus ran a total of 64 models for AIV prevalence. We
165 ran the same set of models using AIV serostatus (positive or negative) instead of AIV status as

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

168 **Results**

169 Of the 12 individual pollutants that we targeted, only PFOS, PFDoA and PFUnA were detected in more
170 than 50% of our samples (91%, 73% and 59% respectively, Tables 1 and 2). When summed into overall
171 categories, ΣPFCAs, ΣPFSAs and ΣPFASs were detected in 87%, 91% and 97% of all samples
172 respectively, regardless of species. Across all the 241 samples tested, PFCAs were not detected in 31
173 samples, PFSAs were not detected in 21 samples, and total PFASs were not detected in only 8 samples.
174 ΣPFCAs concentrations were below 50 ng/g in all but 5 samples (2%), with samples from two red-
175 necked stints (71 and 98 ng/g) and three pacific black ducks (54, 70 and 74 ng/g) comprising the
176 exception. ΣPFSAs by contrast were higher in concentration, with 19 samples (8%) exceeding 200 ng/g
177 (Figure 1), comprising of 14 red-necked stints (205-1275 ng/g) and 5 pacific black ducks (208-503ng/g).
178 We found that all ΣPFCAs, ΣPFSAs and ΣPFASs were significantly higher at the WTP in all species
179 ($p$<0.05, see Table S2), with one exception where no significant differences between regions was
180 found for ΣPFCAs in pacific black duck. A complete range of detection rates and overall concentrations
181 by AIV status and AIV serostatus are shown in Table 1 and Table 2, respectively.

182 Across all 128 models, no significant effects were found for the influence of PFAS pollution on AIV
183 prevalence and seroprevalence, except for five models ($p$ values for each species, and overall sample
184 population provided in Tables 1 and 2). These significant effects were found for PFOSA on AIV
185 serostatus for all species combined, for PFTeA on AIV serostatus in red-necked stint only, and for PFOS,
186 ΣPFSAs and ΣPFASs on AIV infection status across all samples for all species combined. PFOSA was
187 found to be higher in birds that were seropositive (AIV seropositive median: 0.625 ng/g, range: <0.01-
188 36.5 ng/g; AIV seronegative median: <0.01 ng/g, range: <0.01-21.6 ng/g; slope: 0.058, $p$ = 0.040).
189 PFTeA was found to be higher in red-necked stint that were seropositive (AIV seropositive median: 1.3
190 ng/g, range: <0.01-20.4 ng/g; AIV seronegative median: <0.01 ng/g, range: <0.01-14 ng/g; slope =
191 0.144. $p$ = 0.043). PFOS concentrations were found to be slightly lower in birds that were actively
192 infected with AIV (AIV positive median: 29.4 ng/g, range: <0.01-270 ng/g; AIV negative median: 27
193 ng/g, range: <0.01-1280 ng/g; slope = -0.0036, $p$ = 0.039), as were ΣPFSAs when the group was
194 modelled by itself (AIV positive median: 29.4 ng/g, range: <0.01-270 ng/g; AIV negative median: 27
195 ng/g, range: <0.01-1280 ng/g; slope = -0.0036, $p$ = 0.040), and ΣPFASs (AIV positive median: 39.4 ng/g,
196 range: 1.7-292 ng/g, AIV negative median: 33.2 ng/g, range: <0.01-1290 ng/g; slope: -0.0030, $p$=0.047).

197 **Discussion**

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

206 We expect that the absence of a correlation between PFAS pollution and AIV in this study was because
207 our observed PFAS concentrations were too low to elicit immunomodulatory effects. In the *in vitro*
208 study by Castaño-Ortiz et al. (2019), exposure to PFOS was at 22mg/L, likely far higher than any
209 exposure our study birds experienced. Indeed, even though PFAS pollution often varied between
210 sample sites, the concentrations we observed were low relative to 'predicted no-effects
211 concentrations' in serum presented by Newsted, Jones, Coady, and Giesy (2005), the lowest of which
212 was 150 ng/g. PFAS concentrations in all but five individuals' (four red-necked stints and one pacific
213 black duck) were also low compared to 'lowest observable adverse effect levels' (LOAELs) of 400ng/g
214 in adult bird liver tissues proposed by Dennis et al. (2021). These LOAELs were calculated based on
215 decreased adult weight gain. Unfortunately, the disparity between sample matrices (liver vs. red blood
216 cells) means we cannot draw direct comparisons between our study and Dennis et al. (2021)'s
217 proposed toxicity thresholds. Nevertheless, concentrations of PFASs in liver tend to be higher than
218 those in blood (Gebbink & Letcher, 2012) and as such, we can consider Dennis et al. (2021)'s liver
219 LOAELs to be conservative estimates of toxicity in blood. The true number of our samples that exceed
220 the adult LOAEL from Dennis et al. (2021) is likely to be higher. Aside from the five individual
221 exceptions mentioned above, the low concentrations we observed may be considered remarkable for
222 birds that inhabit putatively polluted wastewater treatment plants (all focal species) or migrate via
223 highly polluted environments (red-necked stint migrating via e.g. the Yellow Sea) (Muir & Miaz, 2021).
224 Nevertheless, in 108 individuals, the total PFASs concentrations found were higher than the lowest
225 LOAELs posited by Dennis et al. (2021) (50 ng/g in liver for young birds, based on decreased successful
226 hatching), which suggested there may be some risk to juvenile birds in our population. The absence
227 of specific data on immunomodulation thresholds means we cannot definitively state whether our
228 concentrations are below levels to cause effect. However, our results did not yet suggest that this is
229 the case.

230 It is worth noting that some individuals had particularly elevated PFASs concentrations (e.g. one red-
231 necked stint was observed with 1275 ng/g of PFOS with the next highest at 674 ng/g, see Figure 1),

while being negative for both AIV and AIV antibodies. However, no conclusions can be based on such rare extreme cases due to of a) the very small sample size of individuals with elevated PFASs and b) the level of exposure of these red-necked stints to AIV (10% seroprevalence: Wille et al., 2023) increasing stochasticity in the occurrence of infection. It is clear though, that individuals can accrue a high PFAS load without necessarily incurring infections.

An alternative explanation for the low PFASs concentrations and no apparent interplay with AIV infection, is that the absence of an effect is due to population culling. Population culling is the phenomenon whereby sick, or in this case, polluted individuals do not survive and are thereby 'culled' from the population, such that we never or infrequently detect them when sampling. In our sample population, there was clearly the possibility for individuals to accrue elevated PFAS burdens, as demonstrated by the 19 birds with PFSAs concentrations greater than 200ng/g (see Figure 1). In the absence of additional stressors such as disease, birds may be able to withstand elevated concentrations, as evidenced by the highly polluted, AIV negative and seronegative individual red-necked stint (ΣPFSAs 1280 ng/g). Congruently, wild birds have limited documented signs of disease associated with low pathogenic avian influenza beyond a reduction in daily movement in mallards (*Anas platyrhynchos*) (Van Dijk et al., 2015) and poorer performance in foraging or migrating Bewick's swans (*Cygnus columbianus*) (Hoye et al., 2016; Van Gils et al., 2007). The limited extent of these signs are such that infection alone has little to no direct effect on the survival of its host (Maxted et al., 2012). However, both the negative effect of pollution in isolation as well as the potential combined effect of pollution and disease might even cause death, such that individuals with downregulated immune responses due to pollution may be unable to survive the combined effects of PFASs and the virus (Guruge et al., 2009). Such individuals may therefore have been lost to our study. Indeed, we found three significant models where birds with lower PFOS, ΣPFSAs and therefore ΣPFASs were more likely to be AIV positive. PFOS and thus ΣPFSAs were the most frequently detected compounds, also occurring in the highest concentrations. The significant model results could indicate that infected birds with higher concentrations are unable to survive the added pressures of both and therefore 'culled' from the population. Hence, it is possible that we did not sample highly polluted birds that also had avian influenza infections, because they did not survive. This is particularly pertinent for migrating shorebirds which also deal with the additional stressors of physiological effects of migration including immunomodulation (Buehler, Tieleman, & Piersma, 2010). Indeed, there is some evidence of migratory culling, whereby migration may remove sick individuals (McKay & Hoye, 2016). This effect could be more pronounced under pollution stress, which could become more toxic due to the thermal stress from exertion during migration (Gordon, Johnstone, & Aydin, 2014).

While possibly spurious given the large number of models that we tested (128 in total), there were five models that returned a significant result. The three significant models that found that birds with lower PFOS, ΣPFSAs and ΣPFASs were more likely to be AIV positive are discussed above, but without more information on the specific thresholds for immunomodulation we can only speculate on the culling hypothesis. The fourth significant model found that birds of any species with higher PFOSA concentrations were more likely to be seropositive. This was the trend we expected in our study and may suggest an immunomodulatory effect. However, given the lack of other significant results like this, such a result may be spurious. The only other result showing a positive relationship between a pollutant and AIV serostatus was our final significant model, which found that red-necked stint with higher concentrations of PFTeA were more likely to be seropositive. A possible, yet speculative explanation for this result is that PFTeA, having one of the longest carbon chains of any pollutant in our study (C14), also has the highest bioaccumulation potential (Lewis et al., 2022). As a result, this compound would remain in bird tissues (including blood) longer than other pollutants, allowing us continued detection of these pollutants long after the exposure occurred. The presence of anti-nucleoprotein antibodies are indicative of previous infection in these birds (Padilla-Quirarte, Lopez-Guerrero, Gutierrez-Xicotencatl, & Esquivel-Guadarrama, 2019). Being migratory, red-necked stint are exposed to a larger diversity of viruses and virus hotspots along the EAAF than the two duck species also in this study, both of which are year-round Australian residents. As such, the significant result of our model may reflect the occurrence of a prior, elevated exposure to both pollution and disease elsewhere along the flyway rather than exposure purely in Australia. If this is true, we hypothesize that if the same study were to be conducted in an AIV hotspot such as the Yellow Sea, we may not only see a relation between PFTeA and serostatus but also a relationship with active infection status.

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

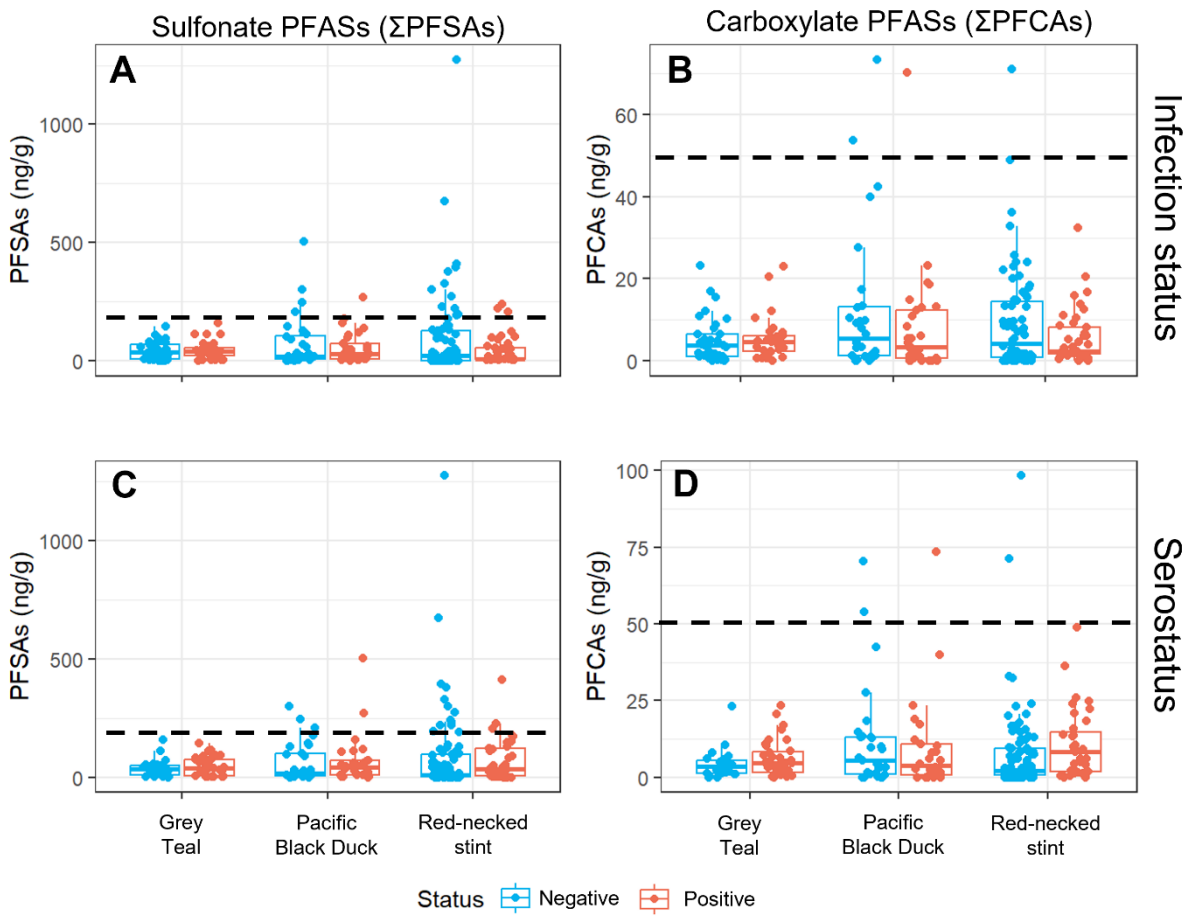


**Figure 1:** Concentrations (ng/g) of summed sulfonate (A,C) PFASs (ΣPFSAs) and summed carboxylate (B, D) PFASs (ΣPFCAs) in red blood cells from grey teal (*Anas gracilis, n*=62), pacific black duck (*Anas superciliosa,* n=57) and red-necked stint (*Calidris ruficollis,* n=121). A and B show infection status, while C and D show serostatus. Birds that were positive are represented in red, and negative in blue. 8% of samples exceeded 200ng/g (dashed line in A and C) of ΣPFSAs, while 2% of samples exceeded 50ng/g (dashed line in B and D) of ΣPFSAs (dashed line in B and D).

**Table 1** Median concentrations and range 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 detection limit. 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=70 | | 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 | 91.3 | **0.039** | 0.088 | 22 | (0-1275) | 7.3 | (2.45-235) | 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 | 0.301 | 0.487 | <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 | 1.2 | 0.98 | 0.998 | <0.01 | (<0.01-24.0) | <0.01 | (<0.01-<0.01) | 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 | 1.2 | 0.56 | 0.551 | <0.01 | (<0.01-7.60) | <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 | 32.4 | 0.891 | 0.547 | <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 | 41.1 | 0.528 | 0.882 | <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 | 26.1 | 0.317 | 0.409 | <0.01 | (<0.01-19.8) | <0.01 | (<0.01-5.43) | 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 | 59.3 | 0.638 | 0.723 | <0.01 | (0-13.2) | 0.34 | (<0.01-4.22) | 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 | 73.9 | 0.361 | 0.214 | 1.02 | (<0.01-15.8) | 0.77 | (<0.01-4.6) | 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 | 39.4 | 0.141 | 0.706 | <0.01 | (0-6.5) | <0.01 | (<0.01-5.22) | 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 | 34 | 0.18 | 0.375 | <0.01 | (0-20.4) | <0.01 | (<0.01-4.69) | 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 | 47.3 | 0.129 | 0.813 | <0.01 | (<0.01-18) | <0.01 | (<0.01-14.04) | 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 | | 91.3 | **0.04** | 0.089 | 22 | (0-1275) | 7.3 | (2.45-241) | 0.341 | 19 | (<0.01-504) | 30.1 | (<0.01-270) | 0.932 | 34.3 | (<0.01-143) | 38.5 | (0.81-159) |
| ΣPFCAs | | 87.1 | 0.083 | 0.135 | 4.25 | (0-71.2) | 2.35 | (<0.01-32.4) | 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 | | 96.7 | **0.047** | 0.088 | 27.4 | (0-1293) | 12.2 | (4.22-241) | 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 concentrations and range 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 serostatus of the birds. Groups of compounds have been indicated. Where <0.01 is noted, this means the concentration was below the detection limit. 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=86 | | AIV-seropositive n=35 | | | 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 | 91.3 | 0.852 | 0.695 | 11.5 | (0-1275) | 36 | (<0.01-411) | 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 | 0.444 | 0.458 | <0.01 | (<0.01-5.39) | <0.01 | (<0.01-<0.01) | 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 | 1.2 | 0.997 | 0.998 | <0.01 | (<0.01-24) | <0.01 | (<0.01-<0.01) | 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 | 1.2 | 0.628 | 0.65 | <0.01 | (<0.01-7.67) | <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 | 32.4 | 0.402 | 0.363 | <0.01 | (<0.01-12) | <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 | 41.1 | 0.443 | 0.643 | <0.01 | (<0.01-27.6) | <0.01 | (<0.01-7.9) | 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 | 26.1 | 0.953 | 0.294 | <0.01 | (<0.01-19.8) | <0.01 | (<0.01-16) | 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 | 59.3 | 0.468 | 0.524 | <0.01 | (0-20.5) | <0.01 | (<0.01-12) | 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 | 73.9 | 0.733 | 0.227 | 0.645 | (<0.01-15.8) | 1.8 | (<0.01-14) | 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 | 39.4 | 0.124 | 0.073 | <0.01 | (0-5.9) | <0.01 | (<0.01-6.5) | 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 | 34 | 0.107 | **0.043** | <0.01 | (0-14) | 1.34 | (<0.01-20.4) | 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 | 47.3 | **0.04** | 0.132 | <0.01 | (<0.01-14) | <0.01 | (<0.01-18) | 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 | | 91.3 | 0.849 | 0.693 | 11.5 | (0-1275) | 36 | (0-411) | 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 | | 87.1 | 0.435 | 0.175 | 2.2 | (0-98.2) | 8.2 | (0-48.9) | 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 | | 96.7 | 0.989 | 0.796 | 16.5 | (0-1293) | 45.4 | (0-460) | 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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