Influence of perfluoroalkyl acids and other parameters on circulating thyroid hormones and immune-related microRNA expression in free-ranging nestling peregrine falcons.

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Abstract

Exposure to certain perfluoroalkyl acids (PFAAs) can have considerable effects on the endocrine and immune systems, although such effects remain largely uncharacterized in wildlife. Using an apex avian predator, we investigated possible relationships of thyroid hormones (THs), specifically free (F) and total (T) thyroxine (FT4; TT4) and triiodothyronine (FT3; TT3), and the expression of an immune-related microRNA biomarker (i.e., miR-155), with the concentrations of 11 PFAAs in nestling peregrine falcons (*Falco peregrinus*). Nestling peregrines (*n* = 56; usually two chicks of each sex per nest) were blood sampled when 23 $\pm$ 4 days old in urban and rural regions of the Laurentian Great Lakes Basin (Ontario, Canada) in 2016 and 2018.The circulating concentrations of several PFAAs were significantly associated with THs and estimated thyroid gland activity (TT3:TT4; FT3:FT4), including PFHxS (FT3; FT3:FT4), PFDS (TT3; TT3:TT4), PFOA (TT4; FT3:FT4), PFTeDA (TT4; FT3:FT4), PFHxDA (TT4; TT3:TT4) and ΣPFCAs (TT4). Our novel evaluation of miR-155 in peregrine nestlings identified significantly negative relationships of plasma miR-155 counts with PFHxS and PFOA concentrations, indicating potential down-regulation of miR-155 expression and impaired immunity. Several PFAA homologues significantly predicted the variation in THs and miR-155 in conjunction with year (e.g., inter-annual differences in weather, ambient temperature, rainfall), region (urban/rural), nestling age, and/or diet (trophic position; δ^{15}N), which suggests that multiple environmental and biological stressors, including PFAA-exposure, influenced thyroid activity and immune function in these nestlings. Further research is warranted to identify the mechanisms and additional impacts of PFAA-related thyroid and immune disruption on the growth, development, and health risks in developing birds.

1. Introduction

Appropriate thyroid function, including the thyroid hormones (THs) thyroxine (T4) and triiodothyronine (T3), are crucial for growth, development, reproduction, metabolism and thermoregulation (McNabb, 2007), and consequently, is highly conserved across vertebrates. Given their endocrine-disrupting potential, organohalogen compounds are likely to alter thyroid function, including circulating T4 and T3 (Brouwer et al., 1998; McNabb, 2007). In birds, polychlorinated biphenyls (PCBs), and polybrominated diphenyl ethers (PBDEs) and other flame retardants, have reportedly disrupted thyroid function (e.g., Fernie et al., 2005; Ucán-Marín et al., 2008; Guigueno and Fernie, 2017). Similarly, studies have observed associations between exposure to perfluoroalkyl acids (PFAAs), specifically perfluorinated carboxylic and sulfonic acids, and disrupted thyroid function and/or thyroid hormones (TH) in rodents (Thibodeaux et al., 2003; Yu et al., 2009), birds (Nøst et al., 2012; Løseth et al., 2019), and humans (Wen et al., 2013; Li et al., 2017; Preston et al., 2020). The role of THs differs in developing and adult individuals and is especially important during the developmental stage, when perturbance in thyroid function can have long-lasting effects (Bernal and Nunez, 1995; Zoeller et al., 2002).

MicroRNAs (miRNAs) are short non-coding RNA sequences (22–23 nucleotides) controlling post-transcriptional gene regulation (Rodriguez et al., 2004), and are involved in a range of physiological and pathophysiological processes (Bushati and Cohen, 2007; Ha and Kim, 2014). For example, the miRNA, miR-155, has been identified as a key regulator in the homeostasis and function of the immune system (Rodriguez et al., 2007). There is increasing evidence that exposure to environmental contaminants induces alterations in miRNA expression (Avissar-Whiting et al., 2010; Wang et al., 2012; Wang et al., 2015; Waugh et al., 2018; Badry et al., 2020). By extension, miRNAs have been suggested as promising potential biomarkers of environmental exposure (Vrijens et al., 2015), although to date, studies have predominantly focused on disease-related miRNA signatures (Etheridge et al., 2011; Vrijens et al., 2015). Moreover, it has been suggested that THs are involved in the regulation of miRNAs relevant to diseases and oxidative stress (Forini et al., 2019; Huang et al., 2019). It is thus timely to investigate the suitability of miRNAs as biomarkers for environmental exposure, as well as the relationships among exposure to environmental contaminants, miRNAs, immune function and thyroid activity in wildlife.

PFAAs and their precursors are widely used in industrial, commercial and consumer products, and some have been shown to be ubiquitous and persistent in the environment. For example, the highly bioaccumulative perfluorooctane sulfonic acid (PFOS; Houde et al., 2006) was listed under Annex B in 2009 of the U.N. Stockholm Convention on Persistent Organic Pollutants (SC-POPs), along

with its salts and related substances (Wang et al., 2009). Another prevalent PFAA, perfluorooctanoic acid (PFOA), its salts and perfluorooctane sulfonyl fluoride (PFOSF), were also listed under the SC-POPs in 2019 (Annex A; UNEP, 2019), with on-going assessments of other PFAAs, such as perfluorohexane sulfonic acid (PFHxS) for listing under the SC-POPs (POPRC, 2019). Since PFAAs tend to have higher bioaccumulative potential with longer fluorinated carbon chains (e.g., Conder et al., 2008), there has been increasing production and use of the short-chain PFAAs as replacements (Ritter, 2010), yet little is known about the toxicity of these replacement PFAAs to wildlife to date.

The peregrine falcon (*Falco peregrinus*) is an apex avian predator of the terrestrial food web and a well-established sentinel species for characterizing environmental contaminants and potential adverse effects on birds and potentially other wildlife (Fernie and Letcher, 2010; Smits and Fernie, 2013). The species is considered endangered under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Previously, the exposure of nestling peregrine falcons to various environmental contaminants in the Laurentian Great Lakes Basin of Canada was associated with altered circulating THs, retinol, hepatic function, bone growth and associated biochemistry measures (Smits and Fernie, 2013; Fernie et al., 2017).

In the present study, we examined regional differences and possible changes in circulating THs and miR-155 in association with PFAA exposure of nestling peregrine falcons from rural and urban regions across the Laurentian Great Lakes Basin (Ontario, Canada). The goal of the present study was to provide a novel evaluation of the relationships of PFAA exposure, thyroid activity and immune-related miRNA expression (i.e., miR-155). We present an integrated approach to assess such relationships incorporating the spatiotemporal variations in these measures, diet (inferred from the stable isotopes of carbon, nitrogen and sulfur), and biological factors (age, sex and body condition) of this apex predator of the terrestrial food web.

2. Material and Methods

2.1. Study species and sampling

Peregrine falcon nestlings (23 $\pm$ 4 days old) were banded, blood sampled, and measured in compliance with the guidelines of the Canadian Council of Animal Care and with all required scientific permits. Blood samples were collected from sibling nestlings (usually one male and one female; n = 56) from 25 active nests across the Laurentian Great Lakes Basin in 2016 and 2018. The sampling protocols and locations are described in detail elsewhere (Fernie et al., 2017; Sun et al., 2020). Briefly, blood samples (≤ 1.1 mL per chick) were collected, centrifuged, and immediately stored in liquid nitrogen until transferred and stored at –80 °C at the National Wildlife Research Centre (NWRC;

Ottawa, ON, Canada) or the Norwegian University of Science and Technology (miR-155 analysis only) (CITES permit: 16NO-052-IM).

2.2. Thyroid hormone analysis

Free (F) and total (T) thyroxine (T4) and triiodothyronine (T3) were analyzed in the plasma of each nestling at NWRC. Sample preparation has been described previously (Fernie et al., 2017). The hormone concentrations were determined using commercially available enzyme immunoassay (EIA) kits following the manufacturer's instructions (Diagnostics Biochem Canada Inc.). The concentrations were quantified using standard curves constructed from serial dilutions of the calibration standard. The method detection limits for the 2016 samples were 0.30 pg/mL, 0.08 ng/mL, 0.50 pg/mL and 3.00 ng/mL for FT3, TT3, FT4 and TT4, respectively, while for the 2018 samples, the limits were 0.15 pg/mL, 0.08 ng/mL, 0.50 pg/mL and 3.00 ng/mL, respectively. Results with a high coefficient of variability (%CV > 20) were excluded. The method accuracy and analytical precision were assessed by the analysis of standard reference material (SRM; human serum-based matrix samples provided by Diagnostics Biochem Canada Inc.) and duplicated samples. Recoveries of SRMs ranged from 85.4% to 114%, and relative percent differences (RPD) of duplicated samples were 9.8%, 7.9%, 5.3% and 15% for FT3, TT3, FT4 and TT4, respectively. Concentrations are expressed in ng/mL (TT3 and TT4) and pg/mL (FT3 and FT4).

2.3. MicroRNA-155 analysis

The analysis of miR-155 in nestling plasma was performed at the Norwegian University of Science and Technology using previously established methods (Matz et al., 2013; Waugh et al., 2018), and is described briefly here and reported in detail in the Supporting Information (SI). Sufficient plasma for the miR-155 analysis was only available for nestlings that were sampled in 2016 (n = 25) and not those sampled in 2018. Following manufacturer protocols, miRNA was extracted using a miRNeasy Mini Kit (Qiagen, Oslo, Norway). RNA concentrations (ng/µL) were quantified using a nandrop spectrophotometer. Reverse transcription (RT), for synthesis of cDNA from the RNA samples, was performed using the miScript II RT kit (Qiagen, Oslo, Norway). qPCR was conducted using a miScript SYBR Green PCR kit (Qiagen, Oslo, Norway) together with a gga-mir-155miScript custom assay (MSC0003997, Qiagen). qPCR was run in a LightCycler® 96 Instrument with the following: 15 min at 95 °C, three-step cycling at 15 s at 94 °C, 30 s at 55 °C and 30 s at 70 °C for 45 cycles. Analysis of data was performed using R 3.4.3 (R Core Team, 2017). We used an analysis that transformed raw Cq values from qPCR into molecule counts, a method explained in Matz et al., 2013.

2.4. PFAA analysis

PFAA analysis in plasma was conducted in Letcher's Organic Contaminants Research Laboratory (OCRL; NWRC), and the analytical and quality assurance/quality control (QA/QC) details are reported in Sun et al., 2020. Briefly, target compounds (mainly PFAAs) including five perfluoroalkane sulfonic acids (PFSAs): PFBS, PFHxS, PFEtCHxS, PFOS and PFDS, and 13 perfluoroalkyl carboxylic acids (PFCAs; C_4–C_{14}, C_{16} and C_{18}): PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUdA, PFDoA, PFTrDA, PFTeDA, PFHxDA and PFODA (full names are provided in Table S1), were quantified using UPLC-MS/MS operated in negative electrospray ionization (ESI) mode. Recoveries for internal standards ranged 73%–100% and RPDs of duplicates ranged 4%–17% for PFOS and C_9–C_{14} PFCAs. Concentrations are expressed in ng/g (*ww*; wet weight).

2.5. Stable isotope analysis

The analysis of stable carbon (^{13}C and ^{12}C), nitrogen (^{15}N and ^{14}N) and sulfur (^{34}S and ^{32}S) isotopes as proxies of trophic position (N) and food source (marine/freshwater-terrestrial gradient; C and S; Hobson, 1999) was conducted at the Ján Veizer Stable Isotope Laboratory (Ottawa, ON, Canada). Detailed analytical procedures and QA/QC results are reported elsewhere (Fernie et al., 2017; Sun et al., 2020). Briefly, nestling red blood cells were lipid-extracted and freeze-dried, and the stable isotopes were determined using an isotope ratio mass spectrometer coupled to an elemental analyzer. The ratios for C, N and S are expressed as δ values (‰) relative to their respective international standards Vienna Pee Dee Belemnite, atmospheric N_2 and Vienna Cañon Diablo Troilite, and normalized to calibrated internal standards. The analyses of internal standards revealed an imprecision of ≤ 0.2‰ for $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$.

2.6. Data analysis

All statistical analyses and plotting of the results were performed using R 4.0.2 (R Core Team, 2020). Analysis of variance (ANOVA) and Tukey HSD tests were used to determine the temporal (2016 vs. 2018) and regional (rural vs. urban) differences in TH concentrations. Shapiro-Wilk test was used to examine the normality of THs, and log transformation was performed to achieve the ANOVA assumption of normality when necessary. The non-parametric Kruskal-Wallis test was used to evaluate the regional and sex differences in miR-155 counts. Only PFAAs detected in more than 90% of the samples, i.e., three PFSAs (PFHxS, PFOS and PFDS) and eight PFCAs (C_8–C_{14} and C_{16}; Table S1), were included in further analysis. Non-detects were replaced with half of the detection limits, except when calculating sums at which time non-detects were set to zero. Pearson product moment

155 correlations were performed prior to modeling in order to assess the correlations among individual
156 PFAAs.

157 To investigate the relationships between PFAA exposure and THs, we fitted linear mixed-
158 effect models (LMMs; Bates et al., 2015) for each of the circulating THs and TH ratios (i.e., TT3:TT4
159 and FT3:FT4), the latter used to estimate thyroid gland activity (McNabb, 2007). Because of the strong
160 correlations among PFAA homologues and groups, each PFAA, Σ_3PFSAs and Σ_8PFCAs were included
161 individually in the LMMs. Covariates included year (potential inter-annual differences in, e.g., weather,
162 ambient temperatures, rainfall), region (rural and urban), nestling age, sex and dietary factors (δ^{13}C,
163 δ^{15}N, δ^{34}S) were incorporated into the models to adjust for potential confounding effects. Nest site
164 (i.e., the identity of each individual nest) was included as a random effect to control for possible
165 correlations among siblings. Normality of the response variables was identified using the Shapiro-
166 Wilks test and log-transformation of the data was performed when necessary. We compared the
167 regression coefficients and 95% confidence intervals among PFAAs to determine possible associations
168 with THs and TH ratios.

169 Potential relationships between PFAA exposure and miR-155 were evaluated using
170 generalized linear mixed-effect models (GLMMs) for the negative binomial family (Bates et al., 2015).
171 Negative binomial distribution was selected due to the over-dispersion of miR-155 count data. PFAAs
172 were added individually to the models, with region (rural and urban), nestling age, and δ^{15}N as
173 covariates, and individual nest site included as a random effect. Possibly because sampling occurred
174 in one year only (2016), δ^{13}C and δ^{34}S were highly correlated with each other and with δ^{15}N, and
175 therefore were not included as covariates in the GLMMs to avoid multicollinearity. Because of the
176 smaller sample size (n = 25) and the lack of a statistically significant difference in the miR-155 counts
177 between the sexes (P = 0.83), we did not include sex in the GLMMs to avoid over-fitting. Regression
178 coefficients and 95% confidence intervals were calculated and compared.

179 In addition, we evaluated the predictive abilities of PFAAs and covariates including year and/or
180 region, sex and/or age, and dietary tracers, for circulating THs and miR-155 employing a backward
181 elimination model selection procedure. The final models and predictors were selected based on AIC*c*
182 – Akaike information criterion corrected for small sample size values. Furthermore, the potential
183 relationships between body condition index (calculated as body weight:tarsus length) and THs or miR-
184 155, as well as between THs and miR-155, were investigated by fitting LMMs with estimated body
185 condition indices and THs as outcomes, and THs and miR-155 as primary predictors, respectively, while
186 including potential confounding covariates as mentioned above. Finally, we checked the variance
187 inflation factors (VIFs, high VIFs indicate multicollinearity), potential outliers (using the Bonferroni
188 outlier test of studentized residuals), as well as the residual diagnostics for all models. The resulting

VIFs were within an acceptable range (< 5; Gareth et al., 2013), no outliers were detected in the tested observations (all Bonferroni adjusted *P*-values ≥ 0.17), and model assumptions were validated.

3. Results and Discussion

3.1. PFAA exposure and circulating THs

Circulating concentrations of THs are closely regulated because of their importance to physiology, growth and survival. The thyroid gland produces and stores T4 and some T3, that then with appropriate stimulation, is released into circulation, with T4 deiodinated to T3, the more biologically active TH (Yen, 2001). Total measures of circulating T3 and T4 include the free and bound forms of each hormone, while the free or unbound forms, FT4 and especially FT3, are more biologically meaningful measures. Circulating FT3 is notably important physiologically since it enters cells and initiates related responses and actions (Abdalla and Bianco, 2014). When histological assessments are not possible, the ratios of circulating THs provide an approximation of the activity of the thyroid gland (McNabb, 2007). In the present study, the circulating TH concentrations in rural (TT4: 7–27 ng/mL; TT3: 1–3 ng/mL) and urban nestlings (TT4: 4–25 ng/mL; TT3: 1–3 ng/mL) were comparable to concentrations previously reported in peregrine nestlings from the same populations (Smits and Fernie, 2013; Fernie et al., 2017). The present study also measured circulating FT4 and the biologically active, FT3, in the same rural (6–14 and 2–10 pg/mL) and urban nestlings (4–12 and 2–7 pg/mL; Table S2), a first for peregrine falcons to the best of our knowledge.

Organohalogen compounds are known to induce changes in circulating THs through multiple mechanisms, including directly disrupting the activity of the thyroid gland and/or deiodinases and subsequent activation and/or inactivation of THs, interference of the synthesis and metabolism of transport proteins, and/or competitive binding to TH receptors (Brouwer et al., 1998; Gould et al., 1999; Ucán-Marín et al., 2008; Miller et al., 2009; Long et al., 2013; Ren et al., 2015; Fernie and Marteinson, 2016). Previous studies have reported significant correlations between circulating THs and various legacy contaminants such as PCBs, PBDEs, and alternative flame retardants such as octabromotrimethylphenyllindane (OBIND), in nestling peregrine falcons from the same populations (Smits and Fernie, 2013; Fernie et al., 2017).

In peregrine nestlings of the present study, we found significant associations of PFAA exposure with all of the measured circulating THs, except FT4, and estimated thyroid gland activity (TT3:TT4 and FT3:FT4; all $P \leq 0.04$). In addition, all significant relationships were positive, i.e., TT4 with PFOA, PFTeDA, PFHxDA and ΣPFCAs; FT3 with PFHxS; TT3 with PFDS; TT3:TT4 with PFDS; and FT3:FT4 with PFHxS, PFOA and PFTeDA, with one exception, the negative relationship of PFHxDA with TT3:TT4 (Fig.

1). Several studies have found significant relationships between THs and various PFAAs in nestlings of predatory birds: circulating T4 was significantly associated with ΣPFAAs in nestling white-tailed eagles from Norway (Løseth et al., 2019), and in seabirds, circulating T4 was significantly associated with PFHpS, PFOS and PFNA in nestling black-legged kittiwakes (*Rissa tridactyla*) and northern fulmars (*Fulmarus glacialis*) from Svalbard (Nøst et al., 2012).

In the present study, five of the 18 measured PFAA homologues, PFHxS, PFDS, PFOA, PFTeDA and PFHxDA, were repeatedly and positively related with measurements of thyroid activity in the nestling peregrines, that suggests probable disruption of the thyroid system of the peregrine falcon nestlings. In the peregrine nestlings, estimated thyroid gland activity (T3:T4) was related to circulating PFHxS, PFDS, PFOA, PFTeDA, and PFHxDA, and there was an observed increase in circulating T4 (TT4) in relation to three of these same PFAA homologues, specifically PFOA, PFTeDA, and PFHxDA. Since the thyroid gland produces and releases mostly T4 into circulation, we suggest that PFOA, PFTeDA and PFHxDA may alter T4-related activity in the thyroid gland thereby contributing to changes in circulating T4 concentrations. The positive relationships of PFHxS with FT3, and PFDS with TT3, suggest an increase in circulating T3 concentrations in association with the exposure of the peregrine falcons to these particular PFAAs, perhaps in relation to increasing circulating T4 and/or altered T4 deiodination to T3. If sufficiently disrupted, increased T3 concentrations may interfere with the negative feedback mechanisms of the hypothalamic-pituitary-thyroid axis including thyroid gland activity, disruption of TH homeostasis, and/or TH dependent processes (Abdalla and Bianco, 2014), potentially leading to fitness consequences for developing nestlings (see Section 3.4). Certainly in these same peregrine nestlings, there were significant associations among PFAAs, THs and/or body condition (see also Sun et al., 2020) that warrant further research, particularly to identify the various mechanisms involved in these changes.

In comparison with the more prevalent and/or well-studied PFAAs, such as PFOS and PFOA, our findings highlight the potential thyroid disruptive effects of shorter-chain PFSA (PFHxS) and long-chain PFAAs (PFDS, PFTeDA and PFHxDA) in peregrine falcon nestlings. Although epidemiological studies have reported associations of the comparatively less studied PFAAs such as PFHxS with altered circulating THs in adult and infant humans (Wen et al., 2013; Preston et al., 2020), such potential effects have received less attention in research with wildlife. Nevertheless, mechanistic studies have suggested several potential disruptive pathways of PFHxS and PFHxDA. For example, exposure to PFHxS (and other short-chain PFAAs) can induce downregulation of mRNA expression of transthyretin (a major TH binding protein in the bloodstream of birds) in herring gull embryonic neuronal cells (Vongphachan et al., 2011). In addition, exposure to high concentrations of PFHxS were found to increase rat pituitary T3-dependent cell growth, likely due to the similar modes of action shared by T3

and PFHxS (Long et al., 2013), while PFHxDA was found to significantly stimulate TH-responsive cell growth by activating the TH receptor-mediated pathway (Ren et al., 2015). Here, the observed significant associations of PFHxDA with TT4 and/or apparent decreased glandular activity (TT3:TT4), as well as of PFHxS with the biologically active FT3 and/or apparent increased glandular activity (FT3:FT4), are supportive of potential disruptive effects of these compounds *in vivo* on thyroid activity in developing birds. Further investigations are needed in order to elucidate the mechanisms involved in these relationships. It is also interesting that we did not observe a comparable association of PFOS and alterations in THs as was reported for humans (Wen et al., 2013; Preston et al., 2020), rat pups (Yu et al., 2009), and seabirds (Nøst et al., 2012), which may indicate possible species-specific effects of PFOS exposure that warrant further investigation.

Shorter-chain PFAAs such as PFHxS may have greater bioavailability compared to longer-chain PFAAs, as the stronger binding-potential to extracellular proteins of long-chain PFAAs may reduce their uptake into neuronal cells (Vongphachan et al., 2011). We also observed significant associations with PFHxS and THs, highlighting the need for further PFAA research with these birds and other wildlife, especially since global manufacturers have been replacing long-chain PFAAs with short-chain PFAAs in the past decade (Wang et al., 2014). Indeed, there appears to be a widespread presence of PFHxS in Canadian waters, given that its precursor perfluorohexane sulphonamide (FHxSA) has been detected in all urban sites and sites impacted by aqueous film forming foams (D'Agostino and Mabury, 2017). The results of the current study contribute to the growing evidence that PFAAs, here PFHxS, can modulate circulating THs, particularly the biologically active FT3, in growing birds. We further hypothesize that this occurs through PFAAs altering thyroid function including thyroid gland activity, and recommend future research investigate this hypothesis including possible modifications of the deiodination of T4 to T3 and changes to transport proteins.

3.2. PFAA exposure and plasma miR-155 count

In the (avian) immune system, miR-155 regulates cytokine production, T-cell differentiation, T-cell antibody responses, and B-cell proliferation (Rodriguez et al., 2007; Thai et al., 2007), and is likely a sensitive pathway for chemically-induced immunomodulation (Badry et al., 2020). To our knowledge, this is the first study that investigated potential relationships between miR-155 and PFAA exposure in a free-ranging raptor species. We observed significantly negative relationships of plasma miR-155 counts and concentrations of PFHxS ($P = 0.01$) and PFOA ($P < 0.001$; Fig. 2), suggesting that as concentrations of PFHxS and PFOA increased, there was an associated decline in miR-155 counts in the nestling peregrines.

Previous studies have reported potential adverse effects of various miRNAs from PFAA exposure, such as the significant positive associations of circulating PFOA with miR-26b and miR-199a-3p in humans (Wang et al., 2012), and significant alterations induced by PFOS on the expression of multiple miRNAs in livers of developing rats (Wang et al., 2015). Broadly consistent with these previous findings, our results suggest the potential inhibition and deregulation of PFHxS and PFOA exposure on miR-155 expression in nestling peregrine falcons. Our results, therefore, suggest that miR-155 may function as a marker for PFAA exposure in raptors and as an indicator of possible immunomodulation induced by exposure to environmental contaminants. The lack of a significant association between PFOS and miR-155 in the present study may nonetheless warrant further assessment in this and other species. miR-155 is essential for maintaining normal immune function, as it is involved in multiple core processes, such as the regulation of dendritic cells and T and B lymphocytes, which are required for protective immunity (Rodriguez et al., 2007; O'Connell et al., 2010). miR-155 deficiency may lead to profound disruption of the immune system and consequently impairment in antibody responses to disease and infection (Thai et al., 2007; Dudda et al., 2013). Increased susceptibility to disease through exposure to environmental contaminants has also been suggested, as significant downregulation of miR-155 was found in primary chicken fibroblasts after exposure to a commercial PCB mixture (Waugh et al., 2018). Consequently, we recommend that future studies investigating PFAA-related changes in avian immune function should include concurrent assessments of miR-155 and additional *in vivo* immune markers (e.g., immune-related blood cell counts). Furthermore, epigenetic mechanisms, including miRNAs, are linked to the regulation of synthesis and/or action of multiple hormones (Zhang and Ho, 2011). Thus, the significant negative relationships of miR-155 with PFHxS and PFOA observed in the present peregrine falcons, may have important implications for immune and potentially thyroid function of peregrine falcon nestlings in this study.

3.3. Optimal predictors for THs and miR-155

In the present study, we examined a suite of variables including PFAAs, biological factors (i.e., year and/or region, age, sex) and dietary tracers (i.e., δ^{15}N (trophic position), δ^{13}C and δ^{34}S (foraging location)), in predicting circulating THs, estimated glandular activity (T3:T4 ratios), and possible immunomodulation (miR-155). Consistent with our results (Figs. 1 and 2), several PFAAs were included as significant predictors in the most parsimonious model for all outcomes except FT4 (Table 1 and Fig. 3). In particular, PFHxDA significantly predicted circulating TT4 and TT3:TT4 ($P < 0.001$ and $P = 0.002$, respectively). In terms of T3, PFHxS significantly predicted circulating FT3 and FT3:FT4 ($P = 0.017$ and $P = 0.007$, respectively), and PFDS significantly predicted circulating TT3 ($P = 0.004$). Moreover, in terms of immune-related miRNA expression, PFOA significantly predicted miR-155 counts ($P = 0.001$).

Our results clearly demonstrate strong associations between the exposure of the peregrine nestlings to environmental concentrations of several PFAAs and variations in markers of thyroid and immune function in the nestlings, thereby providing novel insights in the endocrine and immune disruptive potential of these PFAAs. Future studies on the toxicity thresholds of these particular PFAAs are recommended.

Among various cues, environmental factors (e.g., weather, ambient temperature, rainfall) mediate avian thyroid gland activity and circulating THs (e.g., Fernie et al., 2019), and likely (partially) explain the between-year patterns in thyroid parameters observed in the present peregrine nestlings (Table 1). The year in which the birds were sampled significantly explained the variation in the concentrations of the circulating THs and thyroid gland activity (TT3:TT4) (all $P < 0.001$), consistent with the significant differences observed in circulating THs between 2016 and 2018 in both rural and urban nestlings (Fig. 4*A*–*D*). In addition, region was also a significant predictor of circulating TT4 and approximate glandular activity (TT3:TT4) in the most parsimonious models (Table 1), identifying thyroidal differences between urban and rural nestlings. We observed significantly higher concentrations of FT3 ($P = 0.03$) and TT4 ($P = 0.01$) in the rural nestlings compared to the urban chicks in 2016 (Fig. 4*A* and *D*), reflecting similar findings from the same peregrine populations sampled in the mid-2000s and 2010 (Smits and Fernie, 2013; Fernie et al., 2017). Furthermore, we found significantly different plasma miR-155 counts ($P = 0.005$) between rural (mean = 14; $n = 12$) and urban (mean = 67; $n = 13$) nestling peregrines in 2016 in the present study (Fig. 4*E*). Accordingly, region significantly predicted miR-155 counts ($P < 0.001$; Table 1).

To further elucidate any potential regional differences in the influence of PFAA exposure on THs and miR-155 expression, we fitted regression lines of the PFAAs included in the final models (Table 1) separately for rural and urban nestlings (Figs. S2 and S3). The pattern of circulating THs and ratios, as well as miR-155 counts, in relation to PFAAs appeared to be largely homogeneous in nestlings between the two regions, suggesting the likely consistent influence of PFAA exposure on thyroid activity and immune function in the present peregrine falcon nestlings irrespective of the regional populations. Nevertheless, potential divergent effects may exist between regions, as the relationships of FT3 and FT3:FT4 with PFHxS were more evident in urban nestlings compared to rural nestlings, while for the latter nestlings, the relationship of TT3 and PFDS was more evident. It may therefore be beneficial to further assess the regionally-specific effects of individual PFAAs (e.g., PFHxS, PFOA) on thyroid activity, immune function, and other physiological responses, especially in the context of multiple stressors to which birds are often exposed, in particular in urban environments (Suri et al., 2017; Isaksson, 2018; Marteinson and Verreault, 2020).

Consistent with the role of the thyroid system in regulating growth and development of young animals, we observed significantly positive relationships with nestling age, circulating TT3 and estimated thyroid gland activity (TT3:TT4) (both $P < 0.001$) in the present peregrine nestlings (Table 1). Likewise, significant associations with THs and nestling age have been observed in other raptor species (Fernie and Marteinson, 2016; Løseth et al., 2019). However, we did not observe an association between age and miR-155 in the studied nestlings.

In the present study, we also observed a significant relationship of trophic position, i.e., proxied by δ^{15}N, and estimated thyroid gland activity (FT3:FT4) ($\beta = -0.20$, $P = 0.04$). Such associations suggest a possible suppression of thyroid gland activity in peregrine falcon nestlings feeding on higher trophic level prey, and the contribution of other ecological factors, e.g., weather, should be investigated in further studies. Neither δ^{13}C nor δ^{34}S was included in the most parsimonious models for any of the circulating THs or estimated measures of thyroid gland activity, suggesting that trophic position rather than foraging location is likely a more suitable dietary predictor of thyroid gland activity in peregrine falcon nestlings. This is perhaps not surprising since nestlings, especially those at 17–26 days of age such as those in this study, are fed avian prey from within the breeding territory, and some breeding territories will have a greater selection of prey from a broader range of trophic positions (e.g., rural, northern birds) compared to urban birds that may predominantly consume rock doves (*Columba livia*).

3.4. Inter-relationships of PFAAs, THs, miR-155 and body condition

Alterations in thyroid function have been shown to affect avian metabolism and growth (McNabb, 2007). In the present study, the body condition of nestling peregrine falcons was best predicted by δ^{34}S, FT3, TT3 and sex (Fig. S4). Although epigenetic mechanisms such as miRNAs may be closely linked with endocrine function, including TH regulation, through the activation or repression of the expression of nuclear receptors (Zhang and Ho, 2011), in the present study we did not observe any relationship between miR-155 and THs, or between body condition and miR-155 (all $P > 0.12$).

We previously reported that a higher body condition index (i.e., better body condition) and significantly depleted δ^{34}S occurred in the urban nestlings compared to rural nestlings (Sun et al., 2020). This, in conjunction with the significant associations of body condition, δ^{34}S and T3 observed here, may suggest potential dietary mediation of body condition through modulation of circulating TH concentrations, in addition to the influence of other factors such as trophic level, food availability, and weather. Furthermore, we also found that nestling body condition was significantly and negatively associated with ΣPFCA burden in peregrine falcon nestlings (Sun et al., 2020). Thus, the significant relationships of estimated thyroid gland activity (FT3:FT4) with PFOA and PFTeDA that we observed

here, may imply that such associations could be through the mechanism of PFCA-related thyroid disruption. In addition, several PFAAs have been observed to be associated with telomere length or survival rates in glaucous gulls (*Larus hyperboreus*), further suggesting the effect of PFAA exposure on epigenetic mechanisms and ultimately and potentially, demographic responses (Sebastiano et al., 2020).

In summary, the present study characterized the relationships of a suite of biomarkers of thyroid activity (circulating THs, estimated thyroid gland activity) and immune-related factors (i.e., miR-155), with exposure to individual PFAAs in nestlings of an apex avian predator – the peregrine falcon. The significant relationships we identified among the birds' exposure to several PFAAs with most of these biomarkers, highlight the role of PFAAs in a complex network encompassing epigenetics, physiology and the environment. Our results thus provide novel evidence and insight into the effects on wildlife associated with their exposure to PFAAs, and we recommend further studies to elucidate the related mechanisms and to assess ultimate fitness consequences. Further research that concurrently assess miR-155 and other *in vivo* immune endpoints (e.g., immune-related blood cell counts) would be beneficial to further characterize PFAA-related changes in avian immune function. Additional associations among these biomarkers with other ecological and biological factors including year, region (rural/urban), age and/or diet were also observed. Such an integrated approach is thus encouraged for future studies to assess the toxicological impacts of PFAA exposure and accumulation in peregrine falcons and other wildlife, and that concurrently assess the potential influence of other ecological variables, e.g., weather, and environmental contaminants in mediating thyroid function and activity.

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Table legends

Table 1 Model output for plasma free and total thyroxine (FT4 and TT4) and triiodothyronine (FT3 and TT3), ratios of TT3:TT4 and FT3:FT4 (2016 and 2018) and miR-155 (2016) in peregrine falcon nestlings from the Laurentian Great Lakes Basin, Canada. Results are from the most parsimonious models (lowest AIC*c* value, see Table S3 and S4 for full models). FT3, TT3, TT3:TT4 and FT3:FT4 were log-normalized to meet model assumptions. Nest identity was included as a random effect in all models. The categorical variables of year and region represent 2018 and urban, respectively. Significant *P* values are bolded. R^2_m: marginal pseudo R^2.

Figure legends

Figure 1 Associations between PFAA exposure and circulating free and total thyroxine (FT4 and TT4) and triiodothyronine (FT3 and TT3) and ratios of TT3:TT4 and FT3:FT4 in nestling peregrine falcons sampled in 2016 and 2018 from the Laurentian Great Lakes Basin, Canada. Regression coefficients/estimates (β) and 95% confidence intervals are obtained from linear mixed-effect models with year, region (rural/urban), age, sex, dietary factors (δ^{13}C, δ^{15}N and δ^{34}S) and nest identity (random factor) adjusted. FT3, TT3, TT3:TT4 and FT3:FT4 were log-transformed to meet model assumptions. Asterisks indicate significant associations (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) between PFAA exposure and THs. *P* values for all PFAAs are given in the figure, model output details are given in Table S3.

Figure 2 Associations between PFAA exposure and plasma miRNA-155 counts in nestling peregrine falcons sampled in 2016 from the Laurentian Great Lakes Basin, Canada. Regression coefficients/estimates (β) and 95% confidence intervals are obtained from generalized linear mixed-effect models for the negative binomial family. Covariates including region (rural/urban), age, dietary factor (δ^{15}N) and nest identity (random factor) were adjusted. Asterisks indicate significant associations. *P* values for all PFAAs are given in the figure, model output details are given in Table S4. PFTeDA is not shown due to failed model convergence, nevertheless, the model included only region and PFTeDA showed an insignificant relationship between PFTeDA and miR-155 ($P = 0.969$).

Figure 3 Relationships of PFAAs with total thyroxine (TT4), free and total triiodothyronines (FT3 and TT3), ratios of TT3:TT4 and FT3:FT4 (2016 and 2018), and miR-155 (2016) in nestling peregrine falcons from the Laurentian Great Lakes Basin. Regression lines were fitted using model effect output and adjusted for covariates, e.g., year, region, and/or age (see Table 1 for further details). For better visualization, three data points with the highest concentration of PFHxDA (3 ng/g; TT4 effect dataset), PFDS (8 ng/g; TT3 effect dataset) and PFHxDA (0.9 ng/g;

588 TT3:TT4 effect dataset) are not shown (i.e., there are no statistical outliers: Bonferroni *P* values for studentized
589 residuals are 0.17, 0.28 and 0.45, respectively), and plots with the highest values are presented in Figure S1.
590
591 **Figure 4** Comparisons of plasma thyroid hormone concentrations (sampled in 2016 and 2018; *A*–*D*) and miR-155
592 counts (sampled in 2016; *E*) between rural and urban nestling peregrine falcons from the Laurentian Great Lakes
593 Basin. Significant differences in thyroid hormones were identified using ANOVA and Tukey tests, FT3 and TT3
594 were log-normalized and here are shown in original scales back-transformed using "emmeans" package. For
595 miR-155 counts we used the Kruskal-Wallis test (χ^2 = 8.03, P = 0.005). Significant differences are shown as: * P
596 < 0.05, ** P < 0.01, *** P < 0.001.
597

598 **Table 1**

	n	fixed effect	intercept	*estimate*, *P*-value	R^2_m
FT4	55	Year	10.5	*–3.03*, **<0.001**	0.47
TT4	55	Year + Region + PFHxDA	14.7	*5.21*, **<0.001**; *–3.19*, **0.002**; *4.44*, <**0.001**	0.56
FT3	55	Year + PFHxS	1.45	*–0.60*, **<0.001**; *0.12*, **0.017**	0.51
TT3	52	Year + Age + PFDS	–0.41	*–0.46*, **<0.001**; *0.05*, <**0.001**; *0.08*, **0.004**	0.67
TT3:TT4	51	Year + Region + Age + PFHxDA	–3.04	*–0.89*, **<0.001**; *0.40*, <**0.001**; *0.05*, **0.001**; *–0.94*, **0.002**	0.78
FT3:FT4	54	δ^{15}N + PFHxS	0.61	*–0.20*, **0.036**; *0.17*, **0.007**	0.17
miR-155	25	Region + PFOA	3.35	*2.54*, <**0.001**; *–1.73*, **0.001**	0.50

599

600

Figure 1

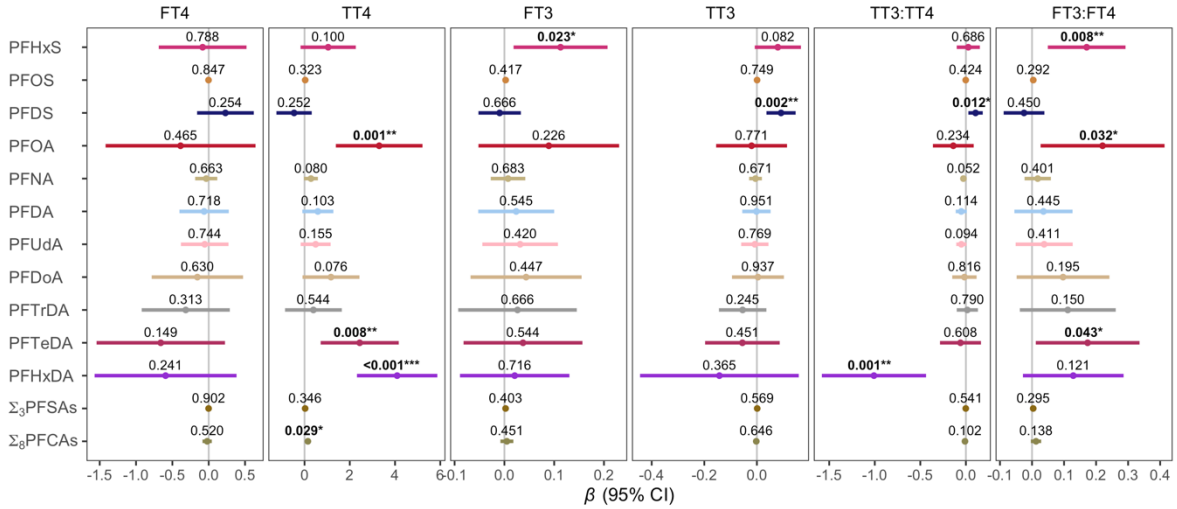


Figure 2

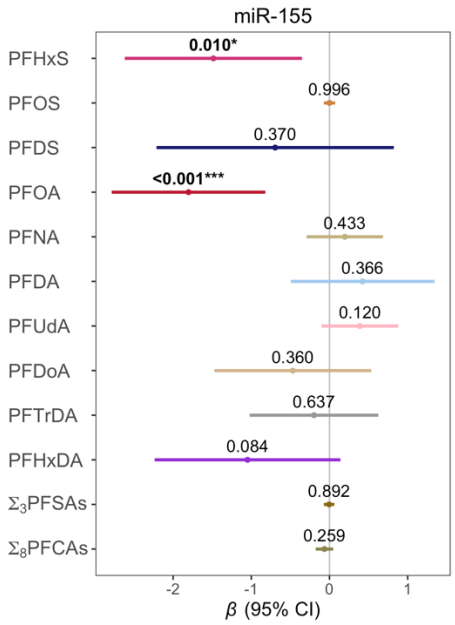
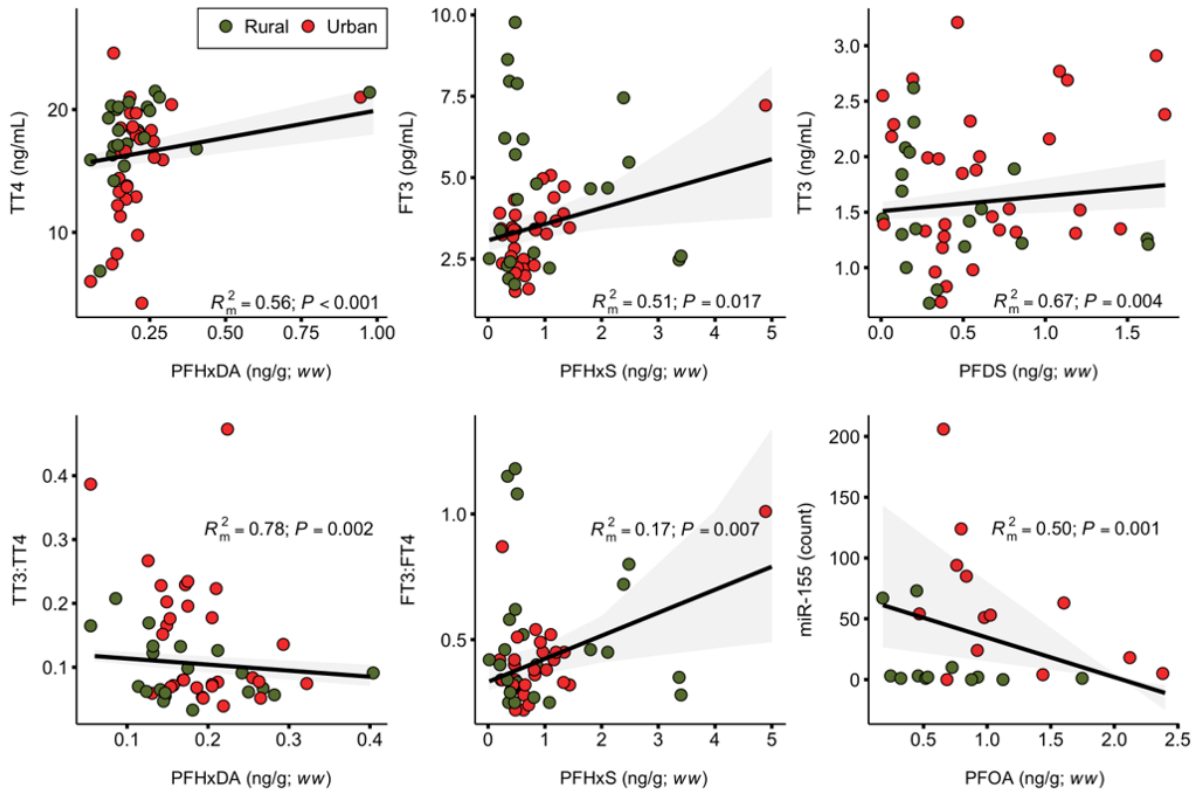


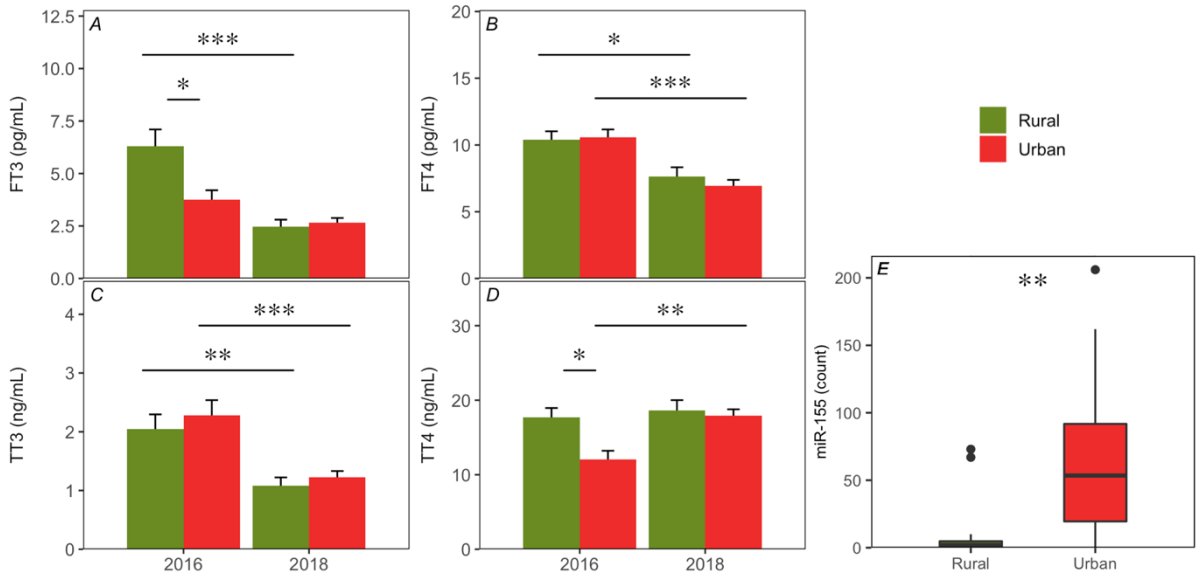
Figure 3


Rural
Urban
TT4 (ng/mL)
PFHxDA (ng/g; ww)
$R^2_m = 0.56$; $P < 0.001$
FT3 (pg/mL)
PFHxS (ng/g; ww)
$R^2_m = 0.51$; $P = 0.017$
TT3 (ng/mL)
PFDS (ng/g; ww)
$R^2_m = 0.67$; $P = 0.004$
TT3:TT4
PFHxDA (ng/g; ww)
$R^2_m = 0.78$; $P = 0.002$
FT3:FT4
PFHxS (ng/g; ww)
$R^2_m = 0.17$; $P = 0.007$
miR-155 (count)
PFOA (ng/g; ww)
$R^2_m = 0.50$; $P = 0.001$

626

627 **Figure 4**

628



629

630