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Changes in thyroid function of nestling tree swallows (*Tachycineta bicolor*) in relation to polycyclic aromatic compounds and other environmental stressors in the Athabasca Oil Sands Region

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ABSTRACT

In the Canadian Athabasca Oil Sands Region (AOSR), nestling tree swallows (*Tachycineta bicolor*) raised near mining-related activities accumulated greater concentrations of polycyclic aromatic compounds (PACs) that contributed to their poorer condition, growth, and reproductive success. Here, we report changes in thyroid function of the same 14 day old (do) nestlings (N ≤ 68) at these mining-related sites (OS1, OS2) compared to reference nestlings (REF1), and in relation to multiple environmental stressors that influence avian thyroid function. Thyroid function was compromised for OS1 nestlings but generally comparable between OS2 and REF1 chicks. In 2012, circulating total triiodothyronine (TT3) and thyroxine (TT4) were similar among all nestlings. The OS1 chicks had more active thyroid glands based on histological endpoints. Hepatic T4 outer-ring deiodinase (T4-ORD) activity was suppressed in OS1 and OS2 chicks. Despite inter-annual differences, OS1 chicks continued experiencing compromised thyroid function with significantly higher circulating TT4 and more active thyroid glands in 2013. The OS2 chicks had less active thyroid glands, which conceivably contributed to their suppressed growth (previously reported) relative to the heavier OS1 nestlings with more active thyroid glands. Thyroid gland activity was more influenced by the chicks' accumulation of (muscle), than exposure (feces) to naphthalene, C2-naphthalenes, and C1-fluorenes. Of four major volatile organic contaminants, sulfur dioxide (SO_2) primarily influenced thyroid gland activity and structure, supporting previous findings with captive birds. When collectively considering environmental-thyroidal stressors, chicks had a greater thyroidal response when they experienced colder temperatures, accumulated more C2-naphthalenes, and consumed aquatic-emerging insects with higher PAC burdens than terrestrial insects (carbon ($\delta^{13}C$)). We hypothesize that the more active thyroid glands and higher circulating TT4 of the OS1 chicks supported their growth and survival despite having the highest PAC burdens, whereas the lack of thyroid response in the OS2 chicks combined with high PAC burdens, contributed to their smaller size, poorer condition and poorer survival.

1. Introduction

The Athabasca Oil Sands Region (AOSR) in western Canada, is the third largest known oil reserve in the world (Government of Alberta 2012) with nearly 4 million barrels of crude oil extracted daily in 2015 and expected to increase (CCAoP, 2016; Giesy et al., 2010; Parajulee and Wania, 2013). In the AOSR, polycyclic aromatic compounds (PACs) occur naturally and are further mobilized by mining activities from related emissions, aerial deposition, and seepage into local waterways (Kelly et al., 2009; Zhang et al., 2016) that have directly increased environmental concentrations of many PACs (2.5–23 fold) since industrial extraction began (Kurek et al., 2013). There is limited understanding of the exposure to and/or toxicity of these PACs to regional wildlife. The PAC profiles of regional mammals (Lundin et al., 2015)

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and birds (Fernie and Marteinson et al., 2018a) are dominated by alkylated polycyclic aromatic hydrocarbons (alkyl-PAHs) and dibenzothiophenes (DBTs), suggesting petrogenic origins associated with recent mining activity ((Schuster et al., 2015) and references therein). Nestling tree swallows (*Tachycineta bicolor*) were exposed to and accumulated higher concentrations of 42 PACs (31–106 ng/g wet weight (ww)) when raised near mining-related sites not directly receiving oil-sands processed water (OSPW) compared to reference nestlings (13–27 ng/g ww) (Fernie and Marteinson et al., 2018a).

Several studies have shown that tree swallows in the AOSR experience reproductive and developmental changes that vary annually (Smits et al., 2000; Gentes et al., 2006; Fernie and Marteinson et al., 2018b). Hatching and fledging success were inconsistently reduced across years for tree swallows that bred at one of several reclaimed wetlands in the AOSR where sediments and water were contaminated with naphthenic acids, parent- and alkyl-PAHs, and DBTs (Smits et al., 2000). The increased mortality rate of nestling tree swallows on some reclaimed wetlands suggested reduced fitness of the nestlings according to Gentes et al. (2006). Similarly, regional nestling tree swallows were lighter in weight and in poorer condition at a wetland near mining-related activity but not receiving OSPW (Fernie and Marteinson et al., 2018b). The reduced fledgling production and developmental changes observed in these birds were related to their exposure and accumulation of specific PACs (i.e., ΣDBTs, C1-naphthalene, C1-phenanthrenes, C2-fluorenes, and/or Σalkyl-PAHs), clutch initiation dates, the nestlings' diet and their exposure to heavy rainfall during growth (Fernie and Marteinson et al., 2018b). Collectively, these changes suggest that disrupted thyroid function may be an important adverse outcome pathway for AOSR-related contaminants.

Appropriate thyroid function regulates growth and reproduction as well as neurodevelopment, immune function, and metabolism in birds and other vertebrates. Many environmental pollutants disrupt thyroid function in biota, with evidence of thyroid disruption occurring in birds in the AOSR in relation to OSPW and air pollutants. In the AOSR, increased concentrations of triiodothyronine (T3) and thyroxine (T4) in the thyroid glands of nestling tree swallows raised on reclaimed wetlands, suggested that thyroid disruption in these nestlings was related to PAHs in the environment (Gentes et al., 2007). Adult male mallards (*Anas platyrhynchos domesticus*) exposed to OSPW experienced altered circulating T3:T4 ratios that suggested disruption of hormone production and/or release by the thyroid glands (Beck et al., 2014). When captive adult female American kestrels (*Falco sparverius*) inhaled a mixture of major air pollutants associated with industrial activities in the AOSR (i.e., benzene, toluene, nitrogen dioxide (NO2) and sulfur dioxide (SO4)), circulating T4 was suppressed and changes in their thyroid glands suggested sustained production and release of T4 (Fernie et al., 2016). Together, these studies provide additional support for the hypothesis that environmental pollutants in the AOSR have the potential to disrupt the thyroid axis of birds in the region.

Most research concerning the potential effects of PACs has focused on parent PAHs, not alkyl-PAHs that dominate the PAC profile of biota in the AOSR. The embryonic exposure of birds to parent PAHs elicits various physiological effects in embryos and nestlings, including reduced growth (reviewed in: Albers, 2006; Leighton, 1993) with some evidence for weak thyroid disrupting effects of PAHs in general (Fowles et al., 2016). The effects and risks of exposure to other types of PACs (e.g., alkyl-PAHs) is largely unknown although some alkyl-PAHs are thought to play a role in the toxic effects of environmental PAH mixtures (reviewed in: Baird et al., 2007). We have shown that tree swallow nestlings accumulate alkyl-PAHs which may contribute to their smaller size, poorer condition, and lower survival (Fernie and Marteinson et al., 2018b). We therefore hypothesize that changes in the thyroid function of these nestlings may also be occurring and predict that this may be related to the chicks' exposure to and/or accumulation of PAHs, airborne contaminants, diet, and weather variables during nestling development in the AOSR. Using tree swallows as an avian model, the objectives of the present study were to investigate and characterize changes in the thyroid function of nestling birds in relation to their exposure and accumulation of parent PAHs, alkyl-PAHs and DBTs in the AOSR (Fernie and Marteinson et al., 2018a). We characterized thyroid function in nestling tree swallows raised on sites near active OS mining areas, but which did not directly receive OSPW, and compared it to those of nestlings raised on a reference site within the AOSR. We sought to determine if any observed changes in nestling thyroid function were related to changes in the growth of the same nestlings, their diet, air quality measures, and weather variables because of an extreme weather event that broke 100-year rainfall regional records in 2013.

2. Materials and methods

2.1. Study sites and subjects

This study, including handling and all related methodologies with the tree swallows, was approved in accordance with the guidelines of the Canadian Council of Animal Care. Appropriate permits were obtained from the Canadian Wildlife Service and all provincial (Alberta) agencies. As previously described (Cruz-Martinez et al., 2015; Fernie and Marteinson et al., 2018a, 2018b), nest boxes (N = 15–34 per site) for tree swallows were established ~3 m apart within 10–20 m of on-site fresh water sources, with thyroid function of nestlings monitored for two years (2012, 2013) at two study sites (OS1, OS2) within 5 km of active mining (mine pits, processing plants), and at one of two reference sites (REF1) ~60 km south of Fort McMurray in the AOSR (Fig. 1). There was no direct source of OSPW to the freshwater bodies at any of our study sites.

Tree swallows, which began to occupy nest boxes between May 10 and 20 in 2012 and 2013, were monitored daily until clutch completion (no further eggs were laid for 3 d), and again after 9–10 d of incubation when nests were undisturbed. Hatch dates of each brood were recorded, with nestling sizes (i.e., body weight, ninth primary feather length) recorded at 9, 12 and 14 days post-hatch (dph). At 14 dph, when chicks were considered ready to fledge, two or three nestlings from each nest were randomly selected, a volunteered fecal sample collected individually (for chemical analysis), with each chick then anesthetized and euthanized by cervical dislocation, followed by dissection (Cruz-Martinez et al., 2015). Nestlings were examined for sex, deformities and disease, and samples of pectoral muscle (for chemical analysis) and dorsal feathers (for dietary analysis of $\delta^{13}C$ and $\delta^{15}N$) were collected (Fernie and Marteinson et al., 2018a). Reproductive success for each pair of adult tree swallows has been previously described by Fernie and Marteinson et al. (2018b).

2.2. Weather and volatile organic compounds

We calculated the means of weather (minimum ambient temperatures, total precipitation) and air quality variables specific to the 14 d growth period for each brood of tree swallow nestlings preceding assessment of thyroid function. Total precipitation (mm) experienced by each brood was calculated using data from the Environment and Climate Change Canada (ECCC) weather station in Fort McMurray (ID: 3062696; 56°39′04″ N, 111°12′48″ W), the closest weather station to all four study sites (https://weather.gc.ca/city/pages/ab-20_metric_e.html). Site-specific minimum ambient temperatures (°C), and mean air concentrations of sulfur dioxide (SO_2) and total hydrocarbons (THC), were obtained from Wood Buffalo Environmental Association (WBEA) (http://www.wbea.org/monitoring-stations-and-data/historical-monitoring-data) at the weather stations closest to OS1 (Muskeg River), OS2 (Buffalo Viewpoint, Lower Camp), and REF1 (Anzac). Air concentrations of nitrous oxide (NO), nitrogen dioxide (NO_2), nitrogen oxides (NO_X), total reduced sulfur (TRS), ozone (O_3), particulate matter 2.5 ($PM_{2.5}$), and hydrogen sulfide (H_2S)recorded at the same WBEA

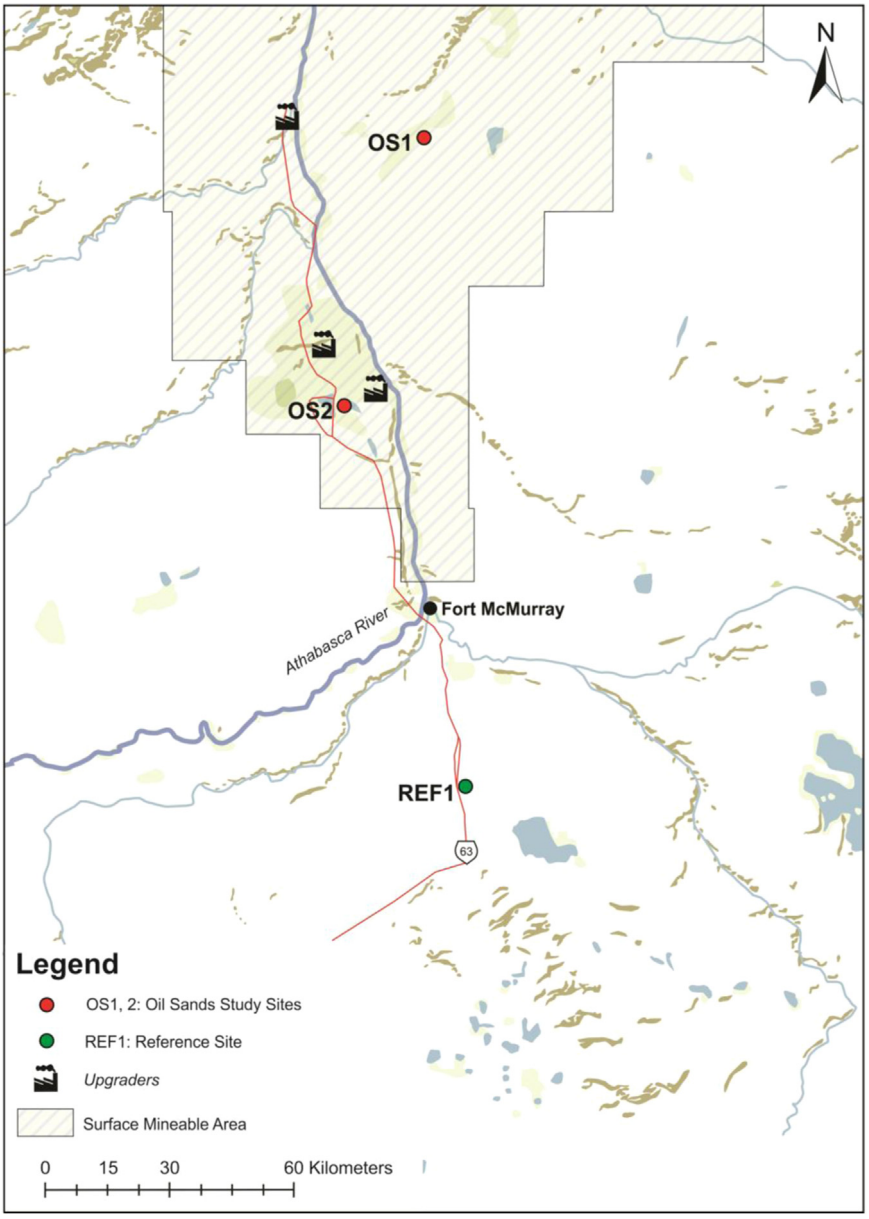

Fig. 1. The study sites where tree swallow chicks were raised and their thyroid function was assessed in the Athabasca Oil Sands Region. (Modified from Fernie and Marteinson et al. 2018a, 2018b).

weather stations, were also used for site-specific measures at OS1 and REF1.

2.3. Stable isotopes

The analysis of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotopes in the dorsal feathers of each nestling were analyzed at the G.G. Hatch Isotope Laboratories (Ottawa, ON, Canada), with methods previously described in detail (Fernie et al., 2017, Fernie and Marteinson et al., 2018a, 2018b). Briefly, feathers and standards were weighed, placed in an elemental analyzer interfaced to an isotope ratio mass spectrometer (IRMS) and flash-combusted at ~1800 °C (Dumas combustion). The resulting gas products were separated by a "purge-and-trap" adsorption column and sent to IRMS interface followed by IRMS. The data are reported in δ notation, and the unit of measurement, per mL (‰), defined as δX = [(Rsample - Rstandard)/Rstandard]*1000; with $\delta^{13}C$ or $\delta^{15}N$ values substituted for X, and R the corresponding ratio of $^{13}C/^{12}C$ or $^{15}N/^{14}N$. The δ15N values are reported as ‰ versus atmospheric nitrogen (AIR) and normalized to internal standards calibrated to international standards (i.e., IAEA-N1 (+0.4‰), IAEA-N2 (+20.3‰), USGS-40 (−4.52‰), USGS-41 (47.57‰)). The $\delta^{13}C$ values are reported as ‰ vs. V-PDB and similarly, normalized to internal standards calibrated to international standards (i.e., IAEA-CH-6 (−10.4‰), NBS-22 (−29.91‰), USGS-40 (−26.24‰), USGS-41 (37.76‰)). Measurement precision was estimated to be 0.08‰ and 0.04% for 13 °C and 15°N, respectively.

2.4. Analysis of thyroid function

Thyroid function was assessed by determining circulating TT3 and TT4 concentrations in the plasma, hepatic T4 outer ring deiodinase activity (T4-ORD), and histological assessments of the thyroid glands, using previously described methods (Fernie et al., 2015, 2016; Marteinson et al., 2011). Given the small size of these nestlings, the limited volume of plasma obtained precluded an assessment of circulating concentrations of free T3 or free T4. The concentrations of TT3

and TT4 in plasma were determined using commercially available radioimmunoassays (MP Biomedicals, Solon, OH) (TT3: 06B254215; TT4: 06B263676) following the manufacturer's instructions and based on linear responses versus concentration calibration curves (R^2: TT3 = 0.997, TT4 = 0.953) across the physiological range of hormone concentrations (TT3 = 0.5–8 ng/mL, TT4 = 20–200 ng/mL).

Hepatic T4-ORD activity was determined for each individual bird using an S9 liver homogenate prepared by homogenizing 100–200 mg of liver in 1 mL of 0.1 M sodium phosphate buffer (pH 7.4) that contained 1 mM ethylenediaminetetraacetic acid (EDTA) and 20 mM diothiothreitol (DTT), using a Precellys®24 ceramic bead tissue homogenizer (Bertin Technologies, Rockville MD). Homogenization buffer was prepared fresh daily and kept on ice. Homogenization was accomplished with two separate 30 s pulses in 2-mL microcentrifuge tubes. The homogenates were centrifuged at 9000 × *g* at 4 °C for 5 min and an aliquot of the supernatant (S9 fraction) was pipetted into a sterile microcentrifuge vial and frozen at −80 °C until analysis.

For the hepatic deiodinase enzyme assays, 3000 ng of T4 in 5 μL acetone was spiked into 15 mL glass culture tubes containing 950 μL of the homogenization buffer. To begin the reactions, 10 μL of S9 supernatant (or buffer for blanks) was added and the reaction was incubated in a water bath at 37 °C for 90 min. Reactions were terminated by the addition of 1 mL of ice-cold methanol to each tube, followed by thorough vortex mixing. The final reaction mixtures were then used to determine liberated T3 using coated tube radioimmunassay kits (MP Biomedical, Santa Ana CA, USA) as described above for the analysis of the plasma thyroid hormones. Reactions were performed in triplicate and corrections were made for T3 measured in assays where buffer had been substituted for the S9 fraction volume. Protein was determined in S9 fractions by the method of Bradford (1976) and deiodinase activity was expressed as pg of T3 liberated per minute per mg of protein in the assay.

Formalin-fixed tissues were processed and embedded in paraffin using standard procedures (Luna, 1968). Tissues were sectioned (~7 μm) and mounted on slides, then stained with hematoxylin and eosin (H & E). Each histological slide was examined using a Carl Zeiss EMS stereoscope with an APoLumar SI.2Y objective, and an AxioCam HRC digital camera at 26 ms (ms) exposure to capture images of the thyroid tissues for measurement. Digital images of two randomly selected fields of view for each tissue section were taken at 40x magnification. For each thyroid gland, ten complete follicles per image (total of 20 follicles) were selected and analyzed (Park et al., 2011). Briefly, epithelial cell heights (ECH) were measured (μm) at four locations per follicle, approximately 90 degrees from each other, and then averaged to calculate a mean ECH per follicle. Area (μm^2) of the colloid (CA) inside the same 20 thyroid follicles was also measured. The data were expressed as mean thyroid ECH and mean colloid area (CA) per individual bird. The mean ratio of ECH:colloid diameter (ECH:CD) was used as an indicator of thyroid gland activation and the potential for thyroid hormone production (Bocian-Sobkowska et al., 2007). All analyses were performed using Zen Lite 2012 (2012) software by the same individual (LP).

2.5. Analysis of PAC concentrations in nestling feces and muscle

PAC concentrations were assessed in nestling fecal (2012, 2013) and muscle (2013) samples. We consider the fecal concentrations of the nestlings to represent their exposure to and elimination of the measured PAHs and metabolites (henceforth referred to as “exposure”), and their muscle concentrations to represent their exposure, uptake and accumulation of the PAHs (henceforth “uptake” or “accumulation”) (Fernie and Marteinson et al., 2018a). The analytical and QA/QC methods for the 42 PAHs and DBTs (Chen Laboratory) have been previously described (Fernie and Marteinson et al., 2018a, 2018b). Briefly, the samples were homogenized and spiked with a surrogate standard mixture of deuterated PAHs (d-PAHs), then extracted by accelerated solvent (ASE 350, Dionex, Sunnyvale, CA, USA) with dichloromethane (100 °C, 1500 psi). Following lipid content determination, the remaining extract was loaded on a 2-g Isolute® silica solid phase extraction (SPE) column. The silica sorbent was pre-cleaned with DCM to remove potential contamination; the packed SPE column was conditioned with 10 mL hexane (HEX). After loading the sample followed by 3 mL HEX, the SPE cartridge was eluted with 11 mL of a 40:60 (v/v) mixture of DCM:HEX that contained the analytes of interest and which was concentrated and spiked with an internal standard d14-dibenzo(a,i) pyrene (Toronto Research Chemicals, Toronto, Canada). Target PACs were separated and quantified using an Agilent 7890 gas chromatograph (GC; Agilent Technologies, Palo Alto, CA) coupled with a single quadrupole mass analyzer (Agilent 5977A MS) in the electron impact (EI) and selected ion monitoring (SIM) mode. The 30 m HP-5MS column (0.25 mm i.d., 0.25 μm, J&W Scientific, Agilent Tech.) was used and the injector was operated in pulsed-splitless mode.

Our QA/QC measures for analyzing the swallow samples included analysis of Standard Reference Materials (SRMs), spiking experiments, examination of surrogate standard recoveries, and a process of procedural blanks as previously reported [10]. Recoveries (mean ± standard deviation) of individual PAC analytes ranged from 75 ± 5% to 94 ± 5% based on the spiking experiments. Concentrations of PACs ranged from 75 ± 4% to 107 ± 4% of the certified concentrations in the NIST SRM 1974c (standard based on mussel tissue; *Mytilus edulis*) that was included in every three batches of test samples. A sum (Σ) PAC concentration of 0.3–1.35 ng/g was determined in procedural blanks run with each set of 5 samples, and were subtracted from the concentrations measured in authentic samples. PAC concentrations in the fecal and muscle samples were corrected with surrogate standard recoveries (70 ± 4% to 91 ± 6%). The limit of detection for PACs ranged from 0.05 to 0.15 ng/g ww.

As previously described (Fernie and Marteinson et al., 2018a, 2018b), we calculated concentrations of Σalkyl-PAHs (C1,4-naphthalenes, C1–3-fluorenes, C1–4-phenanthrenes, C1–4-fluoranthenes/pyrenes, C1–4-benz(a)anthracenes/chrysenes), Σparent PAHs (naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, 1,2-benzofluorene, 2,3-benzofluorene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k,i)fluoranthene, benzo(e)pyrene, benzo(g,*h*,i)perylene), and Σdibenzothiophenes (ΣDBTs: dibenzothiophene, C1–4-dibenzothiophenes). We refer to six major PACs, specifically naphthalene, C1- and C2-naphthalenes, C1- and C2-fluorenes, and C1-phenanthrenes, that were measured in at least 60% of the nestlings (Fernie and Marteinson et al., 2018a).

2.6. Statistical methods

The statistical analysis was conducted with SAS 9.4©. We considered the significance level to be at $p \leq 0.05$, with those *p*-values between 0.06 and 0.07 considered as evidence of statistical trends and potential biological importance. Residuals were tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's Test), but could not be transformed to normality. Consequently, non-parametric statistical tests were used for all statistical comparisons. Highly significant differences were evident between years (*p*-values < 0.0001) but not between sexes for all thyroid-related measures, so data were combined by sex for each endpoint and analyzed separately by year. Kruskal-Wallis tests were used to identify overall significant differences in thyroid-related parameters (2012, 2013) and nestling body mass (14 do; 2013 only) across the three study sites, followed by pairwise site comparisons using Mann-Whitney *U* tests. Spearman's r correlations were used to identify significant correlations among the thyroid-related measures and nestling weight at 9, 12 and 14 dph, or with concentrations of the 6 major PACs, Σalkyl-PAHs, Σparent PAHs, and ΣDBTs, or with concentrations of the volatile SO_2, NO, NO_2, NO_X, TRS, THC, O_3, $PM_{2.5}$, and H_2S. Non-parametric multiple regressions (with identity link

function and Gaussian distribution) were conducted to identify significant relationships between hepatic T4-ORD activity or thyroid gland measures (ECH, CD, ECH:CD), and fecal (exposure) and muscle (accumulated) concentrations of the 6 major PACs, Σalkyl-PAHs, Σparent PAHs, and ΣDBTs, or concentrations of selected air pollutants (SO_2, NO, NO_2, NO_X) based on previous significant correlations (Spearman's r correlation analysis). The significant individual PACs and VOCs, plus the nestling diet ($\delta^{13}C$, $\delta^{15}N$ values), and weather variables (i.e., total rain, minimum ambient temperatures) for each brood, were subsequently used in a similar non-parametric multiple regression model to understand the influence of these factors on nestling thyroid function; this approach included only environmental factors known to or highly suspected of influencing avian thyroid function, reducing the number of factors in the model and improving the meaningfulness of the results.

3. Results and discussion

In this study, we investigated changes in the thyroid function of tree swallows in the AOSR, that had shown poorer reproduction, growth and condition, partly in relation to their greater PAC burdens, when raised within 5 km of active mining and processing activities (Fernie and Marteinson et al., 2018a, 2018b). In light of these reproductive and developmental changes and increased PAC burdens, we investigated potential changes in nestling thyroid function and sought to identify relationships with altered thyroid function, greater PAC burdens, developmental differences, and environmental stressors known to influence avian thyroid function.

3.1. Thyroid function: differences among sites

Previous studies in the AOSR have reported alterations in thyroid hormone concentrations in frogs (Hersikorn and Smits, 2011; Pollet and Bendell-Young, 2000) and birds (Gentes et al., 2007; Beck et al., 2014). Nestling tree swallows at OSPM sites had higher circulating T3 concentrations and elevated glandular T4 concentrations (Gentes et al., 2007), and juvenile mallard ducks experienced sex-specific alterations in the ratio of plasma T3:T4 when exposed to OSPW (Beck et al., 2014). Circulating levels of thyroid hormones are physiologically regulated by balancing production and release from the thyroid glands and conversion of T4 to T3 through deiodinases, predominantly occurring in the liver of birds (McNabb, 2007). In the present study in 2012, there were no significant differences in circulating concentrations of TT3 ($p = 0.37$) or TT4 ($p = 0.96$) in the nestling tree swallows (Table 1) (Fig. 2), yet their hepatic T4-ORD activity significantly differed among the sites ($p = 0.02$) (Table 1) (Fig. 3). Compared to the REF1 chicks, hepatic T4-ORD activity was significantly suppressed in the exposed chicks at OS1 ($Z = -2.55$ df $= 1$ $p = 0.01$) and OS2 ($Z = 2.66$ df $= 1$ $p = 0.008$), which had similar T4-ORD activity (Fig. 3).

Activity of thyroid glands in producing and releasing THs is governed by the production and release of thyroid stimulating hormone (TSH) from the pituitary gland in the brain. Within the thyroid gland, the ECH of thyroid follicles indicates thyroid gland activity, increased ECH indicates activated follicles to increase production of T4 (Park et al., 2011), and colloid volume indicates active TH production and the reserve of thyroglobulin, the TH precursor (Bocian-Sobkowska et al., 2007; Stathatos, 2012). The epithelium:colloid ratio reflects stimulation of the thyroid glands by TSH and provides an overall indication of the activation of the thyroid follicles and the capacity for producing TH (Bocian-Sobkowska et al., 2007). Birds with lower ECH:CD ratios have a greater amount of colloid and less TH production while a higher ratio indicates epithelial cells are actively producing TH and depleting colloid stores. In 2012, there were significant differences among the tree swallow nestlings in the activity ($p = 0.0002$) and structure of their thyroid glands (i.e., ECH: $p = 0.0008$; CA: $p = 0.0009$; CD: $p = 0.0008$) (Table 1): thyroid glandular activity was significantly greater for the chicks at OS1 than at REF1 ($p = 0.0008$) or OS2 ($p = 0.0002$) (Table 1) (Fig. 4a). Moreover, the chicks at OS1 had higher ECH ($p \leq 0.0005$) (Fig. 4b) and depleted colloid (p-values ≤ 0.0002) (Table 1) (Fig. 4c, d). These results for 2012 suggest that the OS1 chicks had active TH production that depleted colloid stores in response to TSH stimulation. TSH concentrations were not measured making this a logical speculation rather than confirmable. Previous research has shown that circulating TSH concentrations were negatively correlated with plasma concentrations of PAHs in heavily oiled guillemots (*Uria aalge*) (Troisi et al., 2016), and we know that the tree swallow chicks at OS1, significantly more so than those at OS2, were exposed to and accumulated significantly higher concentrations of PAHs than the reference nestlings (Fernie and Marteinson et al., 2018a). However, thyroid gland structure and activity were similar in the chicks at REF1 and OS2 (p-values ≥ 0.45) (Fig. 4) (Table 1) leaving only OS1 birds with measurable differences in thyroid structure and function, relative to their cohorts.

3.2. Inter-annual comparisons of thyroid function in nestling tree swallows

Similar changes in several measures of thyroid function were observed in the tree swallow chicks in 2013, but differences between years

Table 1
Measures of thyroid function in nestling tree swallows (14 dph) in the AOSR.

	Study Sites	Reference 1 (REF1)			Oil Sands 1 (OS1)			Oil Sands 2 (OS2)			Kruskal-Wallis			Wilcoxon (p-values)		
Year	Variable	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	X2	df	p-value	Ref1 vs OS1	Ref1 vs OS2	OS1 vs OS2
2012	Blood TT3 (ng/mL)	14	0.92	0.06	6	1.15	0.19	9	0.93	0.06	–	–	NS	NS	NS	NS
	Blood TT4 (ng/mL)	14	2.60	0.34	7	2.48	0.18	9	2.55	0.21	–	–	NS	NS	NS	NS
	Hepatic T4-ORD (pgT3/min/mg protein)	15	0.26	0.03	7	0.19	0.05	18	0.16	0.03	8.15	2	0.02	0.01	0.008	0.07
	TG activity (ECH:CD)	14	0.23	0.02	7	0.58	0.08	18	0.18	0.01	17.38	2	0.0002	0.0008	0.15	0.0002
	ECH (μm)	14	7.70	0.38	7	11.30	0.77	18	7.07	0.34	14.37	2	0.0008	0.004	> 0.45	0.0005
	Colloid area (μm^2)	14	1318	199	7	403	65	18	1375	84	14.02	2	0.0009	0.009	> 0.45	0.0002
	Colloid diameter (μm)	15	36.36	3.08	7	20.82	1.71	18	39.57	1.20	14.24	2	0.0008	0.008	> 0.45	0.0002
2013	TT3 (ng/mL)	21	1.44	0.12	22	1.25	0.15	12	1.40	0.17	–	–	NS	NS	NS	NS
	TT4 (ng/mL)	18	0.09	0.01	21	0.12	0.01	12	0.08	0.01	7.01	2	0.03	0.03	0.51	0.03
	Hepatic T4-ORD (pgT3/min/mg protein)	19	0.23	0.01	16	0.24	0.02	8	0.22	0.02	–	–	NS	NS	NS	NS
	TG activity (ECH:CD)	25	0.14	0.01	22	0.17	0.01	10	0.13	0.01	12.1	2	0.002	0.004	0.94	0.01
	ECH (μm)	25	6.23	0.13	22	7.26	0.20	10	5.89	0.18	20.79	2	< 0.0001	0.0001	NS	0.0002
	Colloid area (μm^2)	25	1592	108	22	1329	60	10	1592	241	–	–	NS	NS	NS	NS
	Colloid diameter (μm)	25	47.04	1.71	22	43.57	1.04	10	47.11	3.36	–	–	NS	NS	NS	NS

Hepatic T4-ORD: hepatic T4 outer ring deiodinase activity; TG activity: thyroid gland activity; ECH: epithelial cell height.

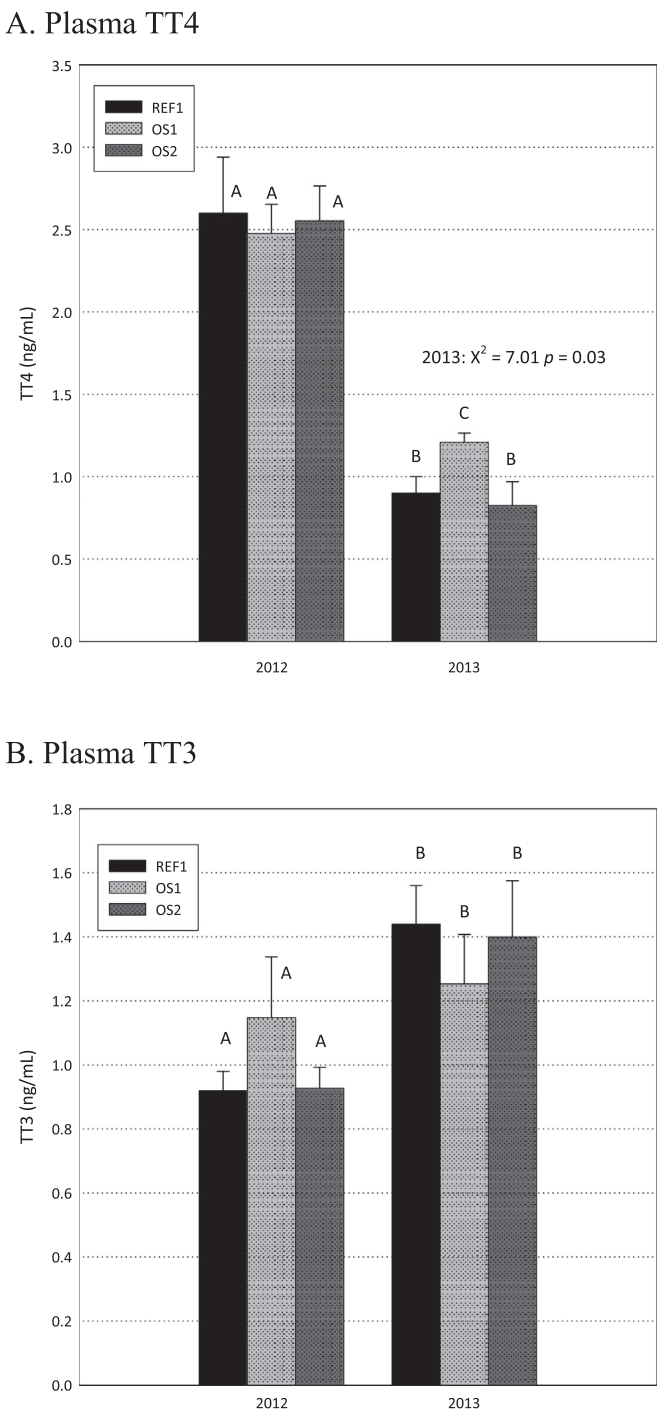

Fig. 2. Circulating TT4 ng/mL (A) and TT3 ng/mL (B) concentrations of tree swallow nestlings, 14 dph, in the Athabasca Oil Sands Region of western Canada.

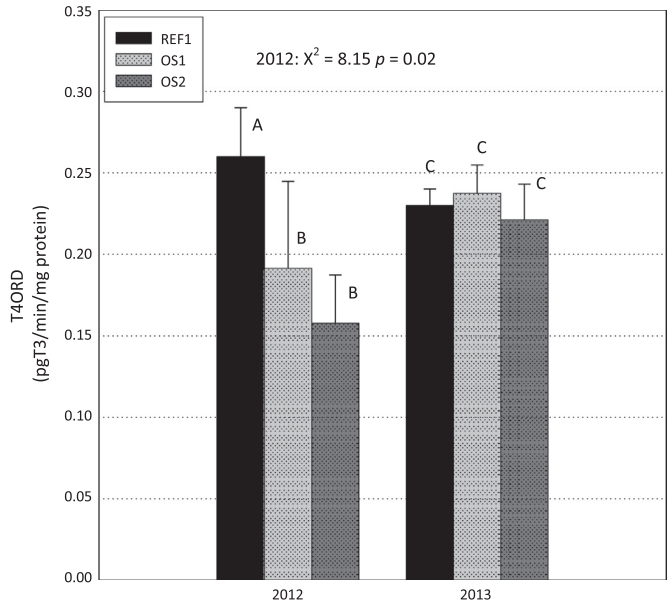

Fig. 3. Hepatic T4-ORD enzyme activity differed across the study sites in tree swallow chicks at 14 dph in the AOSR.

were also apparent likely because of annual differences in weather, food supply, and other factors that will be discussed shortly. As in 2012, there were no significant differences in circulating TT3 concentrations ($p = 0.65$) (Fig. 2a), but significant overall site differences in plasma TT4 concentrations appeared ($p = 0.03$) (Fig. 2b) (Table 1). Compared with the previous year, hepatic T4-ORD activity no longer significantly differed ($p = 0.88$) among the chicks in 2013 (Fig. 3) (Table 1). However, there were ongoing site differences in thyroid gland activity ($p = 0.002$) (Fig. 4a) and structure, but only for ECH ($p < 0.0001$) (Fig. 4b) and no longer colloid content (p-values ≥ 0.18) (Fig. 4c, d) (Table 1). Compared to the nestlings at REF1 and OS2, the chicks at OS1 had significantly higher plasma TT4 concentrations (p-values ≤ 0.03) (Fig. 2b), more active thyroid glands (ECH:CD ratio: $p \leq 0.004$) (Fig. 4a) and significantly higher follicular ECH ($p \leq 0.0002$) (Table 1) (Fig. 4b). Once again, all thyroid-related measures were similar for the chicks at REF1 and OS2 (p-values ≥ 0.19) (Table 1) (Figs. 2–4).

Our results demonstrate that the thyroid function of the 14 do tree swallow chicks differed greatly between years in the AOSR and was particularly altered in the OS1 chicks, and nominally so in the OS2 chicks, compared to the REF1 chicks. In 2012, the thyroid glands of the OS1 chicks were very active (ECH:CD), presumably to meet increased requirements to maintain circulating T4 (Park et al., 2011), as reflected in the higher ECH together with depleted colloid. That year, hepatic T4-ORD activity was suppressed in the OS1 and OS2 chicks, possibly suggesting these hepatic enzymes were being inhibited by exposure to higher concentrations of PACs and air pollutants (Fernie and Marteinson et al., 2018a). PACs induce hepatic enzymes in birds (Head et al., 2015), including those in petroleum coke in the AOSR that decreased EROD activity and altered CYP1A- and thyroid-pathways in vitro (Crump et al., 2017). Through the increased activation of the thyroid glands of the OS1 chicks, and the suppressed T4-ORD enzyme activity of the OS1 and OS2 chicks, circulating concentrations of TT3 and TT4 were maintained at appropriate levels since they were consistent with those of the reference chicks in 2012. A similar pattern was evident in 2013: OS1 chicks had more active thyroid glands plus elevated blood T4 levels but without increased circulating T3 levels and stable T4-ORD activity. The differences in nestling thyroid function between 2012 and 2013 may well reflect the extreme weather with much colder temperatures and record-breaking rainfalls that happened during brood rearing in 2013. In fact, the body mass of chicks at 14 dph was related to their exposure to highly inclement rains, their hatch dates and their sex (Fernie and Marteinson et al., 2018b). These and other factors (e.g., diet) are suspected of influencing avian thyroid function and will be examined shortly.

In relation to other studies examining thyroid endpoints in animals inhabiting the AOSR, our present results with the tree swallow nestlings suggest that industrial-related activities aside from OSPW are sufficient to elicit responsive changes in thyroid gland activity relating to T4 production and/or release between years, reflecting that previously reported for tree swallow chicks raised on OSPM wetlands (Gentes et al., 2007). In small mammals exposed to alkyl-PAHs on reclaimed sites in the AOSR, marked pathological changes were reported in the

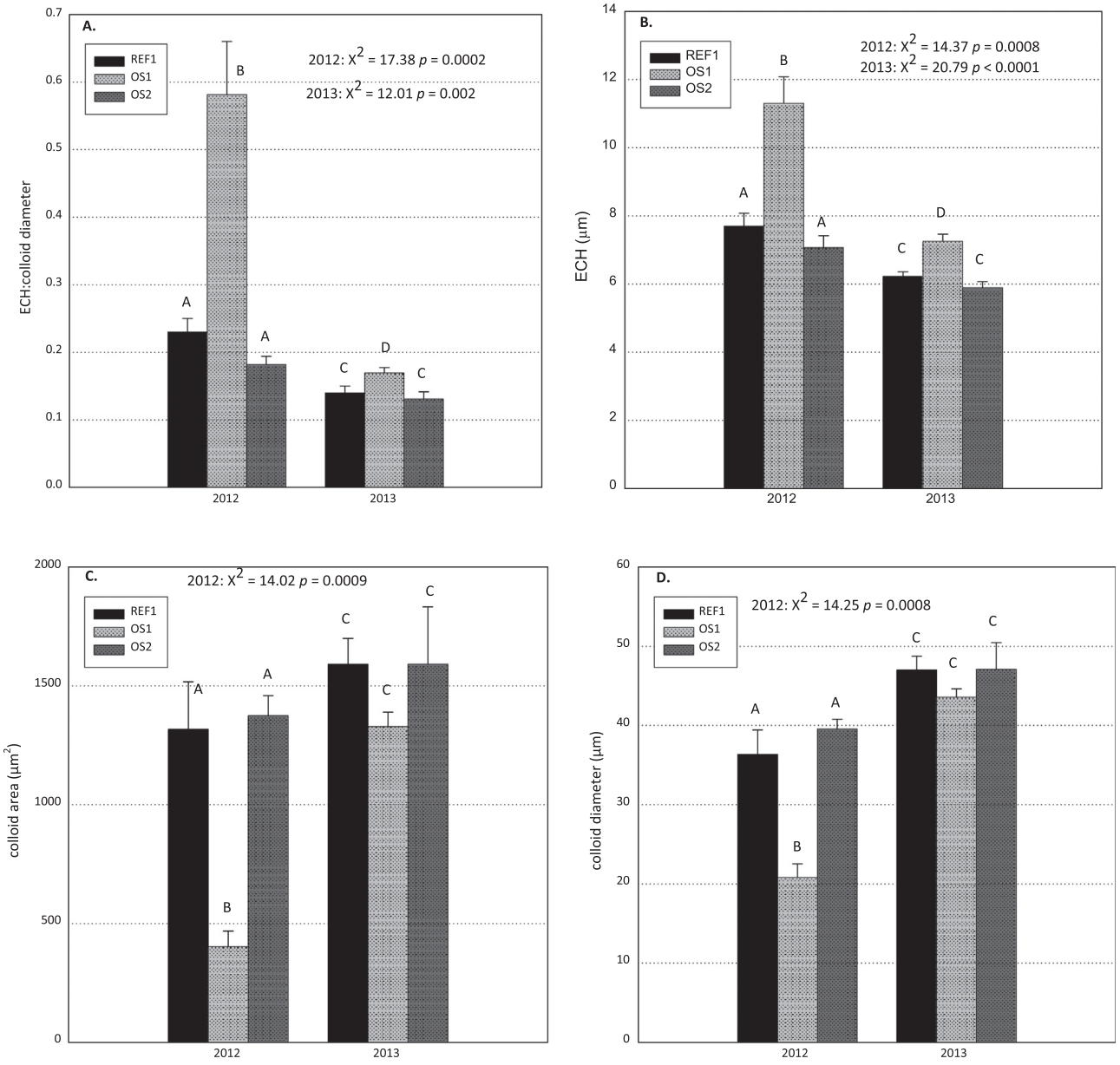

Fig. 4. Multiple changes were evident in thyroid measures of nestling tree swallows at 14 dph in the AOS:. A. Changes in thyroid gland activity (2012, 2013); B. follicular epithelial cell height (ECH) (2012, 2013); C, D. and follicular colloid stores (area: C; diameter: D) (2012 only).

thyroid glands along with changes in circulating thyroid hormones (Movasseghi et al., 2017). In the present nestling tree swallows, hepatic deiodinase enzymes as well as circulating hormones were inconsistently affected across years, likely reflecting the responsive nature of this complex and crucial endocrine network in meeting metabolic demands under variable natural conditions, and to best support growth, thermoregulation, immune function, and ultimately survival.

3.3. Correlations with thyroid endpoints and nestling size

As previously reported, the nestling tree swallows at OS2 were significantly lighter than the chicks at the other two sites (2012, 2013 combined) (Fernie and Marteinson et al., 2018b). The same pattern was evident in 2013 only, when the weight of the 14 do chicks significantly differed across all three study sites ($Z = 31.59$ df $= 2$ $p < 0.0001$). The OS2 chicks (21.1 ± 0.3 g) were significantly lighter than the reference chicks (22.6 ± 0.2 g), that in turn, were significantly lighter than the OS1 chicks (23.3 ± 0.2 g) (all p-values ≤ 0.009). In a previous study (Gentes et al., 2007) with nestling tree swallows at OSPM wetlands, plasma T3 concentrations and thyroidal gland weight increased with the body weight of the chicks. We found a similar pattern of association in 2013 with the present 14 do nestlings: their body weight was positively correlated with ECH (N = 57 Spearman's r = 0.26 $p = 0.05$) (Fig. 5a) and modestly with thyroid gland activity (ECH:CD: N = 57 Spearman's r = 0.25 $p = 0.07$) (Fig. 5b). Since increased ECH indicates increased T4 production, the positive association between body mass and ECH suggests that increased T4 production contributed to increased body weight in the tree swallow chicks, which was especially evident with the OS1 chicks (Fig. 5a). Consistent with this hypothesis is the marginal association with the ECH:CD ratio that reflects TSH stimulation of the thyroid gland, follicular activation and glandular capacity for producing THs. Together these results suggest that heavier tree swallow chicks (mostly OS1 chicks) had activated follicles for greater T4 production and marginally more active thyroid glands in contrast to lighter chicks (mostly OS2 chicks) (Fig. 5b). Thyroid function regulates growth and thermoregulation, and appropriate thyroid regulation of

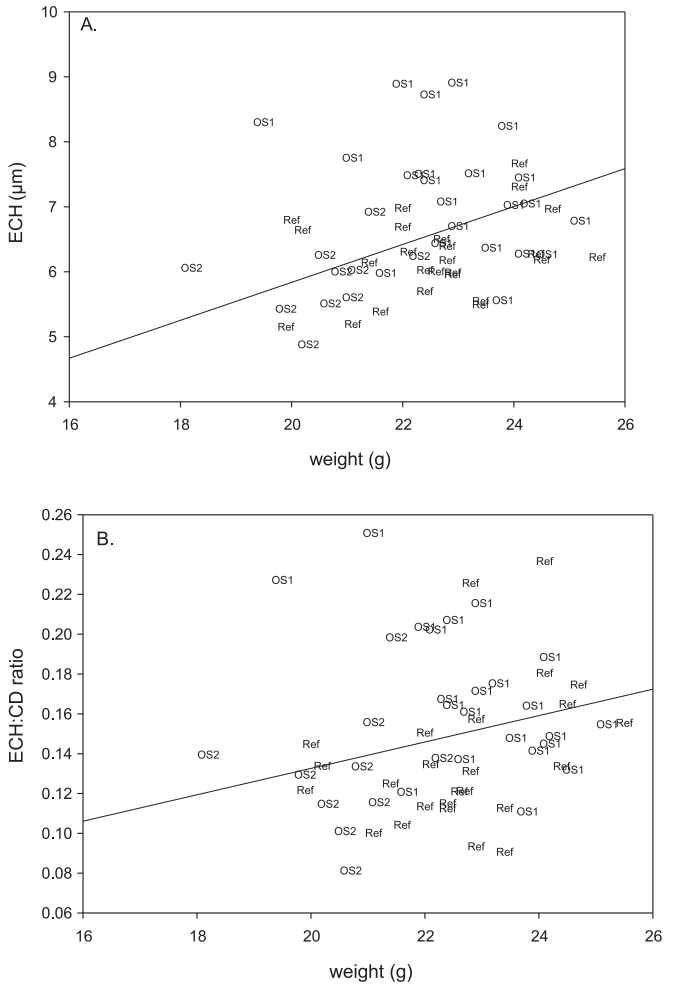

Fig. 5. Correlations were evident in 2013 only between A. thyroid gland structure (ECH) (Spearman's r = 0.26 p = 0.05), or B. modestly with glandular activity (ECH:CD) (Spearman's r = 0.25 p = 0.07), and the body weight of nestling tree swallows at 14 d of age.

these processes is essential to survival. We speculate that the lack of increased activation of the thyroid glands in OS2 nestlings, in combination with their accumulation of higher PAC burdens compared to the reference chicks (Fernie and Marteinson et al., 2018a), may have contributed to them being lighter, in poorer condition, with poorer survival (Fernie and Marteinson et al., 2018b), and likely reduced their ability to thermoregulate when experiencing the extreme rains and cold temperatures in 2013. In contrast, the greater activation of the thyroid glands of the OS1 chicks may have supported their survival during the challenging 2013 nestling period despite their accumulation of the highest PAC burdens, as their survival was similar to the reference chicks (Fernie and Marteinson et al., 2018b). Like Gentes et al. (2007) who found no relationships with plasma T4 or thyroidal hormone content and nestling body weight, we too found no correlations with the other thyroid measurements and nestling size at 9, 12 or 14 dph, and none were evident in 2012 (p-values ≥ 0.08).

3.4. Thyroid function and PACs

What contaminating factors may have contributed to the changes in the thyroid function of the tree swallow nestlings in this study? Although the effects of PAHs on birds has been widely studied (e.g., Bursian et al., 2017; Bianchini and Morrissey, 2018; Albers, 2006), to the best of our knowledge, only Gentes et al. (2007) appear to have examined the potential thyroidal effects in birds. Using Spearman's R correlation and then non-parametric multiple regression, we examined the potential influence on nestling thyroid function of the six major individual PAC congeners (i.e., those having the greatest concentrations in nestling feces and muscle), ΣPACs, Σparent PAHs, Σalkyl-PAHs, and ΣDBTs, that the tree swallow chicks were exposed to (fecal concentrations) and accumulated (muscle concentrations). The PAC burdens were highest in the OS1 chicks, followed by the OS2 chicks, and then the reference chicks in descending order (Fernie and Marteinson et al., 2018a). With both years combined, the chicks' exposure (fecal concentrations) to most but not all of these PAC measures was positively correlated with glandular activity and structure and negatively correlated with hepatic T4-ORD activity (p-values ≤ 0.03) (Table 2), while the chicks' accumulation of several PACs was positively correlated with glandular structure (i.e., ECH) and activity (p-values ≤ 0.05), but not with hepatic T4-ORD activity (Table 2).

Since these nestling swallows were exposed to and accumulated all of these PAC congeners collectively and not in isolation (Fernie and Marteinson et al., 2018a), we used non-parametric multiple regression to identify which of these PAC congeners were related to the chicks' thyroid function. Examining the PAC congeners collectively demonstrated that glandular ECH was significantly related to fecal concentrations of Σparent PAHs (p = 0.01), and colloid diameter marginally related to ΣDBTs (p = 0.06) and Σparent PAHs (p = 0.07) (Table 3), suggesting that activation of the epithelial cells to support TH production appeared to be influenced by the chicks' exposure and elimination of the Σparent PAHs and possibly ΣDBTs. Hepatic T4-ORD activity was significantly related to fecal concentrations of C1-phenanthrenes (p = 0.04) and Σparent PAHs (p = 0.03) (Table 3). In comparison, tissue residues of the same PACs more strongly influenced thyroid function (i.e., more relationships were evident): thyroid gland activation was related to accumulated naphthalene (p = 0.004), C2-naphthalene (p = 0.0005), and C1-fluorenes (p = 0.01), follicular colloid to accumulated C2-naphthalene (p = 0.007), and ECH to accumulated naphthalene (p = 0.01), C2-naphthalenes (p = 0.01), and C1-fluorenes (p = 0.01), with evidence that hepatic T4-ORD activity was marginally related to accumulated ΣDBTs (p = 0.07) (Table 3). There were no other significant relationships between the chicks' thyroid function and their exposure (feces) and accumulation (muscle) of the other PACs (all remaining p-values ≥ 0.10) (Table 3). Taken together, our results suggest it is the uptake and accumulated concentrations of PACs, more so than the exposure and elimination of PACs by birds, that has a greater influence on thyroid function during nestling development. These relationships likely help to explain the increased thyroidal activation of the OS1 birds only that also had the highest PAC burdens. These results also support our prediction that PACs, both individually and collectively (i.e., summed concentrations), affect thyroid function of nestling birds, and support the hypothesis of Gentes et al. (2007) that PAHs may have been involved in the thyroidal hormonal gland changes that they observed in nestling tree swallows at OSPM wetlands. Similarly, in small mammals exposed to PACs and other oil sands-related contaminants on reclaimed mine sites, dramatic changes were evident in the thyroid glands (Movasseghi et al., 2017).

3.5. Thyroid function and volatile organic contaminants

During their development, the tree swallow chicks at OS1 were generally exposed to higher air concentrations of SO_2 (2012 only), TRS, NO, NO_2, NO_x, and $PM_{2.5}$, than the chicks at REF1 (Fernie and Marteinson et al., 2018b), that may also explain some of the thyroidal differences observed between the OS1 and REF1 chicks. Some of the same air contaminants (i.e., SO_2, NO_2), combined with benzene and toluene that are also commonly measured in the AOSR, were reported to affect thyroid function when inhaled by captive birds (Fernie et al., 2016). American kestrels experienced suppressed circulating T4, depleted follicular colloid and increased ECH, with sustained T4 production but no changes in circulating T3 measures or hepatic T4-ORD

Table 2
Significant associations were identified for thyroid-related measures in nestling tree swallows and their exposure (fecal concentrations) and accumulation (muscle concentrations) of six major PAC congeners, ΣPACs, Σparent-PAHs, Σalkyl-PAHs, or ΣDBTs. The results of the Spearman's R correlations (R-value) are presented here. napthal = naphthalene; phenan = phenanthrene; N = sample size.

		Fecal Concentrations				Muscle Concentrations			
		ECH	CD	ECH:CD	T4-ORD	ECH	CD	ECH:CD	T4-ORD
C1-naphthal	R	0.10	0.01	0.04	−0.24	0.02	−0.10	0.17	−0.07
	p-value	0.36	0.90	0.74	**0.04**	0.89	0.46	0.22	0.66
	N	84	84	84	71	52	52	52	39
C2-naphthal	R	0.24	−0.13	0.19	−0.33	0.40	−0.28	0.45	0.02
	p-value	**0.03**	0.24	0.09	**0.00**	**0.00**	**0.05**	**0.00**	0.92
	N	84	84	84	71	52	52	52	39
C1-fluorenes	R	0.22	−0.03	0.13	−0.37	0.32	−0.14	0.30	0.07
	p-value	**0.05**	0.79	0.26	**0.00**	**0.02**	0.32	**0.03**	0.69
	N	84	84	84	71	52	52	52	39
C2-fluorenes	R	0.28	−0.29	0.30	−0.38	0.32	−0.21	0.35	−0.10
	p-value	**0.01**	**0.01**	**0.00**	**0.00**	**0.02**	0.13	**0.01**	0.55
	N	84	84	84	71	52	52	52	39
C1-phenan	R	0.11	−0.17	0.17	0.15	0.36	−0.12	0.31	0.12
	p-value	0.30	0.12	0.13	0.22	**0.01**	0.39	**0.02**	0.46
	N	84	84	84	71	52	52	52	39
ΣPACs	R	0.30	−0.18	0.24	−0.25	0.35	−0.06	0.24	0.08
	p-value	**0.01**	0.11	**0.03**	**0.04**	**0.01**	0.69	0.08	0.64
	N	84	84	84	71	52	52	52	39
Σparent-PAHs	R	0.36	−0.24	0.33	−0.09	−0.17	−0.14	0.03	−0.10
	p-value	**0.00**	**0.03**	**0.00**	0.46	0.24	0.31	0.85	0.55
	N	84	84	84	71	52	52	52	39
Σalkyl-PAHs	R	0.27	−0.20	0.24	−0.29	0.41	−0.10	0.31	0.08
	p-value	**0.01**	0.07	**0.03**	**0.02**	**0.00**	0.49	**0.02**	0.62
	N	84	84	84	71	52	52	52	39
ΣDBTs	R	0.28	−0.33	0.29	−0.25	0.35	0.00	0.17	−0.14
	p-value	**0.01**	**0.00**	**0.01**	**0.03**	**0.01**	0.98	0.22	0.38
	N	84	84	84	71	52	52	52	39

(Fernie et al., 2016). In the present study, air pollutants were correlated with thyroid function of the tree swallow chicks at OS1 and REF1: in 2012, thyroid gland activity (ECH:CD ratio) was positively correlated with air concentrations of NO (N = 21 Spearman's r = 0.53 $p = 0.01$), NO_2 (N = 21 Spearman's r = 0.49 $p = 0.03$), NO_x (N = 21 Spearman's r = 0.47 $p = 0.03$), and marginally with $PM_{2.5}$ (N = 21 Spearman's r = 0.42 $p = 0.06$) (Table 4), suggesting that exposure to higher concentrations of NO, NO_2, NO_x and possibly $PM_{2.5}$ was associated with more active glands producing T4. This pattern was repeated in 2013 when circulating T4 levels were elevated for the OS1 chicks: thyroid glands were more active in producing T4 (ECH:CD ratio) with increasing air concentrations of NO, NO_2, and NO_x again, as well as SO_2 and THC (all p-values ≤ 0.01) (Table 3), reflecting the findings with captive American kestrels after they inhaled benzene, toluene, NO_2 and SO_2 for 18 days (Fernie et al., 2016). Indeed, in the tree swallow chicks, circulating TT4 concentrations were positively correlated with increasing concentrations of SO_2 (Spearman's r = 0.30 $p = 0.03$) and NO_2 (Spearman's r = 0.36 $p = 0.02$) in 2013 (Table 4). Worthy of note is that the thyroid glands of the wild tree swallow chicks became less active in producing T4 with increasing air concentrations of TRS, O_3, and H_2S present in the air (all p-values ≤ 0.04). Hepatic T4-ORD was associated with air concentrations of SO_2 (N = 37 Spearman's r = -0.54 $p = 0.0005$) and H_2S (N = 15 Spearman's r = 0.68 $p = 0.005$) in 2012 but not in 2013, perhaps simply reflecting the suppression of T4-ORD in 2012 only when air PAC concentrations were higher (Fernie and Marteinson et al., 2018a). The difference between exposures in the wild and those in captive birds suggests that multiple contaminants in conjunction with SO_2 and H_2S may underlie the correlations with T4-ORD activity.

Since the tree swallow chicks were concurrently exposed to multiple air pollutants (Fernie and Marteinson et al., 2018a), we used non-parametric multiple regression to identify which of SO_2, NO, NO_2, and NO_X, may have resulted in the changes in nestling thyroid function. We identified that of these major air pollutants, only SO_2 levels were significantly related to thyroid gland activity (N = 68; ECH:CD, $p = 0.0001$) and ECH ($p < 0.0001$), while follicular colloid volume (CD) was related to both SO_2 ($p = 0.0006$) and NO_2 ($p = 0.01$) (Table 3). However, the air concentrations of SO_2, NO, NO_2, and NO_X, were not related to hepatic T4-ORD activity (N = 57 $p \geq 0.33$) (Table 3). The consistent relationships with SO_2 and thyroid activity of the tree swallow chicks help to substantiate the hypothesis of Fernie and colleagues (2017) that SO_2 in particular, may have explained the decrease in glandular colloid and (likely) thyroglobulin, and the increased activation of the thyroid glands of the captive kestrels, since sulfur inhibits iodide transport and incorporation into thyroglobulin during the production of T4 in the thyroid gland (Duntas and Doumas, 2009).

3.6. Inter-relationships: nestling thyroid function and their exposure, accumulation, and elimination of PAHs, diet, air contaminants, and weather variables during brood rearing

Birds are concurrently exposed to a multitude of stressors on a daily basis. Ambient temperatures and food availability have the greatest effects on avian thyroid function, with food sources (among other factors) also influencing thyroid parameters (McNabb, 2007). Warm and cold temperatures, and partial food restriction or ingestion, may alter circulating T3, and T4 to a lesser extent, through changes in deiodinase activity (McNabb, 2007). Consequently, we also examined the possible roles of multiple variables on thyroid gland function and hepatic T4-ORD enzyme activity in the 14 do nestling tree swallows. Multiple stressors that were included in the non-parametric multiple regression models were exposure (fecal concentrations) and accumulation (muscle concentrations) of Σalkyl-PAHs, C2-naphthalenes or C1-phenanthrenes (the two PACs with the highest concentration in the chicks (Fernie and Marteinson et al., 2018a, 2018b), $\delta^{13}C$ and $\delta^{15}N$, as well as air concentrations of SO_2, total rain and minimum ambient temperatures that occurred during nestling growth (14 d). The assessment of these

Table 3
Relationships among the thyroid function of nestling tree swallows and their exposure (feces; Model 1) or accumulation (muscle; Model 2) of PACs, or volatile organic compounds (Model 3), or in combination with environmental stressors known to influence avian thyroid function (Model 4), were identified using non-parametric multiple regression. (PE = parameter estimate; SEM = standard error; T = t-value; p = p-value; Sulfur dioxide (SO_2); Nitrous oxide (NO); Nitrogen dioxide (NO_2); Nitrogen oxides (NO_X); napthal = naphthalene; phenan = phenanthrene; M = muscle concentrations; F = fecal concentrations; M.A. Temp. = minimum ambient temperatures).

	Epithelial Cell Height (ECH)				Colloid Diameter (CD)				Glandular Activity (ECH:CD)				Hepatic T4-ORD activity			
	PE	SE	T	p	PE	SE	T	p	PE	SE	T	p	PE	SE	T	p
Model 1: PACs - Fecal concentrations **(N = 84)**																
Intercept	6.02	0.41	14.62	< 0.0001	43.28	2.49	17.36	< 0.0001	0.14	0.02	7.48	< 0.0001	0.27	0.03	10.67	< 0.0001
C1-naphthal	0.04	0.13	0.35	0.73	0.85	0.77	1.11	0.27	−0.01	0.01	−0.93	0.36	−0.01	0.01	−0.96	0.34
C2-naphthal	−0.03	0.07	−0.40	0.69	−0.34	0.42	−0.82	0.41	0.00	0.00	0.45	0.65	0.00	0.00	−1.28	0.21
C1-fluorenes	0.10	0.31	0.33	0.74	1.82	1.89	0.96	0.34	0.02	0.01	1.46	0.15	−0.03	0.02	−1.63	0.11
C2-fluorenes	−0.17	0.24	−0.70	0.49	−1.28	1.47	−0.87	0.39	0.02	0.01	1.65	0.11	−0.01	0.01	−0.87	0.39
C1-phenan	−0.12	0.08	−1.53	0.13	0.13	0.47	0.27	0.79	0.00	0.00	0.80	0.43	0.01	0.00	2.11	**0.04**
ΣPACs	−0.06	0.06	−0.97	0.33	0.40	0.38	1.06	0.29	0.00	0.00	−1.18	0.24	0.00	0.00	0.02	0.99
Σparent-PAHs	0.22	0.08	2.66	**0.01**	−0.92	0.50	−1.83	0.07	0.01	0.01	0.77	0.45	0.01	0.00	2.18	**0.03**
Σalkyl-PAHs	0.11	0.07	1.58	0.12	−0.34	0.44	−0.77	0.44	0.00	0.00	0.44	0.66	0.00	0.00	0.11	0.92
ΣDBTs	0.03	0.09	0.32	0.75	−1.03	0.53	−1.94	0.06	0.00	0.01	0.16	0.87	−0.01	0.01	−1.59	0.12
Model 2: PACs - Muscle concentrations																
Sample size	N = 52				N = 52				N = 52				N = 39			
Intercept	5.65	0.33	17.25	< 0.0001	49.37	3.08	16.05	< 0.0001	0.11	0.01	7.64	< 0.0001	0.24	0.03	7.14	< 0.0001
naphthal	−0.32	0.12	−2.67	**0.01**	2.16	1.12	1.92	0.06	−0.02	0.01	−3.02	**0.004**	0.00	0.01	0.06	0.95
C1-naphthal	−0.03	0.08	−0.42	0.68	0.09	0.74	0.12	0.91	0.00	0.00	−0.13	0.90	−0.01	0.01	−1.16	0.26
C2-naphthal	0.10	0.04	2.55	**0.01**	−1.06	0.37	−2.83	**0.007**	0.01	0.00	3.78	**0.0005**	0.00	0.00	−0.18	0.86
C1-fluorenes	0.44	0.15	2.91	**0.01**	−1.73	1.40	−1.23	0.23	0.02	0.01	2.56	**0.01**	−0.01	0.02	−0.77	0.45
C2-fluorenes	−0.03	0.13	−0.25	0.80	−1.54	1.19	−1.29	0.21	0.01	0.01	0.97	0.34	−0.02	0.01	−1.38	0.18
C1-phenan	−0.03	0.03	−0.83	0.41	−0.17	0.29	−0.59	0.56	0.00	0.00	0.10	0.92	0.00	0.00	−0.40	0.70
ΣPACs	0.01	0.02	0.31	0.76	0.39	0.23	1.66	0.10	0.00	0.00	−1.06	0.30	0.00	0.00	0.98	0.33
Σparent-PAHs	−0.02	0.06	−0.27	0.79	−0.85	0.57	−1.50	0.14	0.00	0.00	1.43	0.16	0.00	0.01	−0.24	0.81
Σalkyl-PAHs	−0.01	0.03	−0.20	0.84	−0.14	0.27	−0.52	0.61	0.00	0.00	0.12	0.91	0.00	0.00	−0.11	0.91
ΣDBTs	0.01	0.06	0.11	0.91	−0.32	0.58	−0.55	0.58	0.00	0.00	0.41	0.68	−0.01	0.01	−1.88	0.07
Model 3: Volatile Organic Compounds																
Intercept	7.73	0.39	19.77	< 0.0001	36.90	2.85	12.94	< 0.0001	0.27	0.04	7.58	< 0.0001	0.24	0.03	7.79	< 0.0001
SO_2	−3.98	1.06	−3.76	< **0.0001**	26.77	7.45	3.59	**0.0006**	−0.39	0.10	−4.04	< **0.0001**	0.01	0.08	0.09	0.93
NO	−11.89	24.49	−0.49	0.63	−5.72	7.34	−0.78	0.44	0.25	2.24	0.11	0.91	−1.89	1.91	−0.99	0.33
NO_2	−11.23	24.62	−0.46	0.65	−17.17	6.74	−2.55	**0.01**	0.35	2.25	0.16	0.88	−1.88	1.92	−0.98	0.33
NO_X	11.89	24.55	0.48	0.63	10.47	6.37	1.65	0.10	−0.28	2.24	−0.12	0.90	1.88	1.92	0.98	0.33
Model 4: Combined parameters																
Intercept	8.19	2.86	2.87	0.01	20.61	19.12	1.08	0.29	0.33	0.10	3.28	0.002	−0.06	0.23	−0.25	0.81
M: C2-naphthal	0.02	0.06	0.27	0.79	−1.45	0.38	−3.84	< **0.0001**	0.01	0.00	2.81	**0.008**	0.00	0.00	−0.05	0.96
M: C1-naphthal	−0.05	0.04	−1.25	0.22	0.02	0.28	0.06	0.95	0.00	0.00	−0.94	0.35	0.00	0.00	0.21	0.83
M: Σalkyl-PAHs	0.03	0.02	1.19	0.24	0.10	0.16	0.64	0.53	0.00	0.00	0.33	0.74	0.00	0.00	−0.44	0.67
F: C2-naphthal	−0.01	0.10	−0.08	0.94	1.88	0.66	2.84	**0.01**	−0.01	0.00	−1.64	0.11	0.00	0.01	0.14	0.89
F: C1-phenan	0.07	0.09	0.79	0.44	−0.62	0.61	−1.01	0.32	0.00	0.00	1.15	0.26	0.01	0.01	1.94	0.06
F: Σalkyl-PAHs	−0.03	0.05	−0.58	0.57	−0.11	0.35	−0.31	0.76	0.00	0.00	−0.29	0.78	0.00	0.00	−0.51	0.61
SO_2	0.32	0.74	0.44	0.66	2.45	4.95	0.50	0.62	0.00	0.03	0.19	0.85	0.05	0.05	1.13	0.27
M.A. Temp.	0.12	0.12	1.00	0.32	−2.45	0.79	−3.12	**0.003**	0.01	0.00	2.23	**0.03**	0.00	0.01	0.14	0.89
Carbon ($\delta^{13}C$)	0.13	0.09	1.42	0.16	−2.12	0.63	−3.36	**0.002**	0.01	0.00	3.48	**0.001**	−0.01	0.01	−1.25	0.22

multiple stressors on the thyroid function was restricted to 2013, the year of extremely adverse weather and the only year in which we assessed PAH residues in the chicks. At 14 dph, thyroid gland activity (ECH:CD) and follicular colloid (CD only) were significantly related to the chicks' accumulation of C2-naphthalenes (N = 50, $p \leq 0.008$), minimum ambient temperatures ($p \leq 0.03$) during growth, and the source of their diet ($\delta^{13}C$: $p \leq 0.001$) from local aquatic or terrestrial sources, but not the trophic position of their diet ($\delta^{15}N$) (Table 3). Aquatic emerging aerial insects, an important food source for tree swallows (Winkler et al., 2011) in the AOSR (Godwin et al., 2016), accumulate PACs (Custer et al., 2017). PAH concentrations were correlated between sediments and stomach contents of nestling tree swallows (Custer et al., 2017). Consequently, we hypothesize that thyroid gland activity increased when chicks were exposed to cold temperatures, had higher C2-naphthalene burdens, and ate aquatic emerging insects that had heavier PAC burdens from contaminated wetland sediments, than terrestrial insects with lower PAH burdens. Surprisingly, it appeared that the production of thyroglobulin, as reflected by ECH, was not related to the factors addressed in the present study, and hepatic T4-ORD activity was only marginally related to the chicks' exposure to C1-phenanthrenes. These latter findings contradict our predictions.

It was evident that multiple stressors were influencing thyroid function in the nestling tree swallows in the AOSR in 2013. These results are consistent with the knowledge that the thyroid system is responsible for regulating thermoregulation, metabolism, and physical growth of vertebrates. Collectively, it appears that the primary mode of action of these multiple stressors was on the activity of the thyroid gland in producing and releasing T4 into circulation. There may have been some influence, albeit relatively minor on the processes involved in converting T4 to T3 via hepatic T4-ORD, possibly reflecting the need to tightly regulate very low levels of blood T3. T3 is the most relevant and biologically active end product of deiodinase activity, regulating physiological parameters that support survival of the individual. With the myriad roles of TH and the responsive nature of the thyroid gland, it may not be reasonable to expect clear and predictable responses since we cannot know all of the stressors and demands challenging birds in the AOSR, or when best to sample them in order to capture transient changes. Endocrine systems in general are regulated, with compensatory changes that may be transient, and the timing of such changes may differ each year because of external stressors. Thus, it is conceivable that changes in circulating TH and T4-ORD activity may have occurred

Table 4
Thyroid function measures in nestling tree swallows were significantly associated with some but not all volatile organic contaminants in the AOSR based on Spearman's R correlations (R-value). (Sulfur dioxide (SO_2), total reduced sulfur (TRS), nitrous oxide (NO), nitrogen dioxide (NO_2), nitrogen oxides (NO_X), total hydrocarbons (THC), ozone (O_3), particulate matter 2.5 ($PM_{2.5}$), hydrogen sulfide (H_2S); N = sample size).

		2012				2013			
		ECH:CD	T4ORD	TT3	TT4	ECH:CD	T4ORD	TT3	TT4
SO_2	R-value	−0.18	−0.54	0.13	−0.01	0.34	0.04	−0.08	0.30
	p-value	0.28	**0.0005**	0.52	0.95	**0.01**	0.78	0.54	**0.03**
	N	36	37	28	29	57	43	55	51
TRS	R-value	−0.13	0.02	−0.50	0.32	−0.41	0.29	−0.22	−0.24
	p-value	0.67	0.95	0.07	0.26	**0.04**	0.23	0.34	0.33
	N	14	15	14	14	25	19	21	18
NO	R-value	0.53	−0.17	0.20	0.00	0.39	0.07	−0.17	0.25
	p-value	**0.01**	0.46	0.39	1.00	**0.01**	0.68	0.28	0.12
	N	21	22	20	21	47	35	43	39
NO_2	R-value	0.49	−0.18	0.37	−0.20	0.67	−0.15	−0.08	0.36
	p-value	**0.03**	0.43	0.10	0.39	< **0.0001**	0.40	0.62	**0.02**
	N	21	22	20	21	47	35	43	39
NO_X	R-value	0.47	−0.12	0.28	−0.06	0.47	0.06	−0.16	0.25
	p-value	**0.03**	0.60	0.24	0.79	**0.0009**	0.75	0.31	0.12
	N	21	22	20	21	47	35	43	39
THC	R-value	0.30	−0.29	0.14	−0.02	0.35	0.10	−0.19	0.24
	p-value	0.07	0.08	0.48	0.93	**0.01**	0.54	0.16	0.09
	N	36	37	28	29	57	43	55	51
O_3	R-value	−0.16	−0.09	−0.25	0.09	−0.44	0.18	−0.34	0.02
	p-value	0.59	0.75	0.38	0.76	**0.03**	0.46	0.14	0.92
	N	14	15	14	14	25	19	21	18
$PM_{2.5}$	R-value	0.42	−0.01	−0.25	−0.01	0.12	−0.06	−0.11	0.18
	p-value	0.06	0.98	0.29	0.95	0.42	0.72	0.48	0.27
	N	21	22	20	21	47	35	43	39
H_2S	R-value	0.22	0.68	−0.06	0.57	−0.70	0.08	−0.36	−0.01
	p-value	0.44	**0.01**	0.88	0.14	**0.02**	0.86	0.25	0.98
	N	15	15	8	8	10	8	12	12

before or after our sampling of the tree swallow chicks.

4. Conclusions

Previously, Gentes et al. (2006) demonstrated that thyroidal content was altered in nestling tree swallows on OSPW-treated wetlands in the AOSR, and hypothesized that this was the result of PAHs. Our results expand on the limited understanding of the influence of PAHs on avian thyroid function. Compared to nestling tree swallows on the reference site in the AOSR, thyroid function and hepatic T4-ORD enzyme activity differed between years in chicks on sites close to mining-related activity where they were exposed to and accumulated greater concentrations of PAHs (Fernie and Marteinson et al., 2018a). Changes in the production and/or release of T4 from the thyroid gland combined with changes in T4-ORD activity, generally resulted in the maintenance of appropriate concentrations of circulating T3 and T4, although plasma T4 was elevated in 2013. Other birds in the AOSR experienced alterations in circulating T3, T4 and/or T3:T4 in some but not all studies (e.g., Gentes et al., 2007). In the present study, heavier chicks (mostly OS1 chicks) had more active thyroid glands in contrast to the lighter chicks (mostly OS2 chicks), and this may have supported appropriate thermoregulation and survival of the OS1 chicks but not the OS2 chicks that were smaller, in poorer condition, and had compromised survival, during the adverse weather of 2013. With the chicks' exposure to a combination of multiple stressors, results suggest that the greatest influences on avian thyroid function in the AOSR, especially the thyroid gland, came from: 1., the chicks' accumulation more so than their exposure to PAHs, and particularly to C2-naphthalenes; 2., their consumption of aquatic-emerging insects with higher PAC burdens than terrestrial insects ($\delta^{13}C$); and 3., colder (minimum) temperatures during their 14 d of development as nestlings. Air pollutants, NO, NO_2, and NO_X, and especially SO_2, during the nestling period in the AOSR, also contributed to alterations in avian thyroid function. Since thyroid function regulates reproduction, and reproductive success of these tree swallows was also compromised (Fernie and Marteinson et al., 2018b), an assessment of potential changes in thyroid function of adult birds in the AOSR would be beneficial, as would future research further examining the connections between chemical pollutants (e.g., PACs), increased hepatic metabolism, and altered thyroid function.

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