Exploring the Impact of a Perfluoroalkyl Substance (PFAS) and Interspecific Variation in Passerine Metabolism

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Dedication

For the birds, and those that love and steward them.

Abstract

A comprehensive understanding of physiology is essential for improving risk assessment and management as organisms face compounding stressors, such as environmental contaminants and climate change. Passerines are a diverse and abundant group of birds that are sometimes vulnerable to environmental stressors, making it important to study their physiological responses in greater detail. I approached this challenge by using field flow-through respirometry, a novel method in this context, to investigate the effects of a perfluoroalkyl substance (PFAS), perfluorooctane sulfonate (PFOS), on the metabolic regulation and thyroid function of Tree Swallows (*Tachycineta bicolor*). Additionally, interspecific differences were examined in metabolic rates and liver weights among three passerine species in northern Minnesota: Tree Swallows, Northern House Wrens (*Troglodytes aedon*), and Black-capped Chickadees (*Poecile atricapillus*). It was found that PFOS exposure impacts thyroid hormone concentrations in Tree Swallows but does not significantly impact metabolic rate or growth. Furthermore, interspecific differences in metabolic rates and liver weights were significant, and liver weight may be better at explaining differences in metabolic rate than whole animal mass alone. This integrative study contributes to both an understanding of ecotoxicological impacts of PFAS and a foundation for future comparative physiology studies in passerines.

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v. Strategic Environmental Research and Development Program (SERDP)
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Chapter 1:

Investigating the impact of a Perfluoroalkyl substance (PFAS) on metabolic regulation and thyroid function in Tree Swallows (*Tachycineta bicolor*)

Introduction

Poly- and perfluoroalkyl substances (PFAS) are a large group of anthropogenic chemicals, some of which are highly persistent (OECD 2018). PFAS can be resistant to degradation due to their extremely strong carbon-fluorine bonds (Wang et al. 2017), a functionality developed for industrial and consumer applications since the 1940s (Kotthoff et al. 2015). The industrial use of PFAS includes aqueous film-forming foams (AFFF) (Cheng et al. 2010) commonly used in firefighting and military training (Moody et al. 2003). The chemical structure of many PFAS results in hydrophobic qualities, high stability, and mobility in water that is ultimately challenging to remove and treat (Kancharla et al. 2022).

PFAS contamination is concerning relative to effects across ecosystems and taxa, emphasizing the need to understand physiological responses in vulnerable species (Ankley et al. 2021). Detected globally (Dimitrakopoulou et al. 2024, Giesy and Kannan 2002), some PFAS are known to bioaccumulate and biomagnify (Ahren and Bundschuh 2014, Kelly et al. 2009), although their distribution and movement across systems remains poorly understood. Aquatic systems and biota tend to have the highest concentrations of PFAS (Dimitrakopoulou et al. 2024) and present greater exposure risk (Ahren and Bundschuh 2014). As of 2021, there were comparatively few (39) field-based studies on the effects of PFAS and fewer (19) on avian species (Ankley et al. 2021). Of particular interest to this study are insectivorous birds, which are often on the periphery of aquatic systems when feeding on emerging aquatic insects (Manning and Sullivan 2021) (Figure 1) and have been found to have high PFAS exposure (Dauwe et al. 2007).

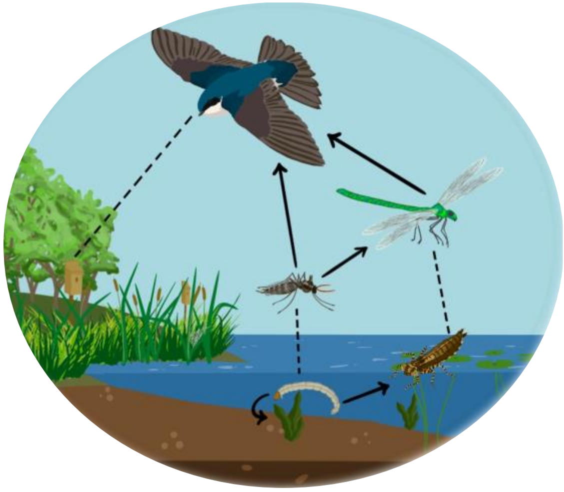

Figure 1. Periphery of aquatic systems impacted by PFAS. Aerial insectivores, like Tree Swallows, are exposed to PFAS through contaminated aquatic insects, potentially leading to adverse effects. Source: Abigail Odegard, unpublished 2024.

Tree Swallows (*Tachycineta bicolor,* swallows) are a common model species for studying environmental contaminants (Custer 2021). They readily use nest boxes, are tolerant of observer disturbance and handling, and they feed on aerial life stages of aquatic invertebrates exposed to sediment contaminants (Custer 2021, Jones 2003). Previous studies on swallows in the upper Midwest have reported contradictory findings regarding the effects of PFAS on reproduction (Custer et al. 2019, Custer et al. 2014). In addition to reproduction, inconsistent effects of PFAS have been shown in regulation of other endocrine systems including thyroid hormone (Ask et al. 2021) and metabolism (Blévin et al. 2017) in birds. It is critical to better understand the effects of these widely distributed bioaccumulative contaminants in vulnerable species of high exposure risk.

PFAS has been linked to disruptions in endocrine mechanisms that regulate metabolism (Sunderland et al. 2019), but contradictory findings suggest complex interactions. Specifically, some PFAS impact the hypothalamus-pituitary-thyroid (HPT) axis (Figure 2; Ishihara et al 2003). Thyroid hormones (TH) most relevant to metabolic activity, triiodothyronine (T3) and its precursor thyroxine (T4; Decuypere et al. 2005), are regulated by negative feedback loops of the HPT axis and are rarely free in serum as they are usually bound to transport proteins – mainly transthyretin (TTR) and albumin

(ALB) (McNabb 2007). PFAS can bind to ALB, potentially competing with THs and increasing plasma levels of free TH (Forsthuber et al. 2020). The disruption of the HPT axis by PFAS is not fully understood: PFAS were positively correlated with TH levels and female metabolism in adult Black-legged Kittiwakes (*Rissa tridactyla*) (Ask et al. 2021, Blévin et al. 2017), but TH levels were lower in chicken embryos with PFAS exposure (Cassone et al. 2012). Furthermore, changes in human metabolism due to PFAS exposure may be more associated with lipid levels than TH (Khaliq et al. 2014). These studies suggest the effects that PFAS may have on the HPT axis are complex and warrant further study.

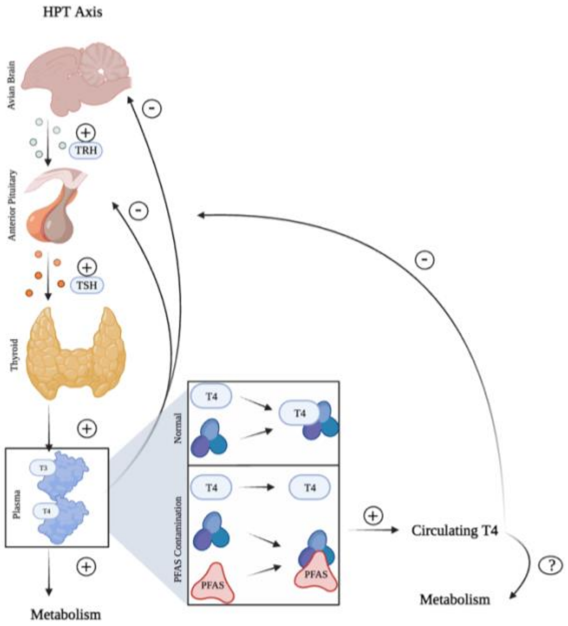

Figure 2. PFAS and the hypothalamus-pituitary-thyroid (HPT) axis. PFAS can bind to transport proteins and may compete with THs (T3 and T4) to alter TH levels through feedback mechanisms. Adapted from Hiller-Sturmhöfel et al. 1998 with use of BioRender.

If PFAS affects the HPT axis, it may impair individuals' ability to cope with temperature outside of the thermal neutral zone (TNZ), a concern in the context of global change. TNZ describes the range of ambient temperatures wherein physiological and metabolic processes that produce heat are balanced against heat loss, minimizing the energy an organism needs to expend to maintain body temperature. This is typically quantified through measurement of metabolic rate at rest, or basal metabolic rate (BMR) (Figure 3; Ritchison 2023). Previous research has determined TNZs for over 250 avian species (Khaliq et al. 2014), but not for swallows (Woodruff et al. 2023), though it may be roughly estimated using data from species of similar size (approximately 20-30 degrees C) (Khaliq et al. 2014, Hudson and Kimzey 1966). Metabolic data such as this can be applicable to energetics modeling that would inform future work involving risk assessment and further ecotoxicological work.

Outside the TNZ, metabolic rate increases to support thermoregulation, (Ritchison 2023; Figure 3), and may approach the summit metabolic rate (SMR) – a maximum output level typically associated with shivering that reaches values 4–8 times higher than BMR. The difference between the SMR and BMR is described as the aerobic scope and is indicative of individuals' ability to move from rest to maximal expenditure, mitigate effects of multiple stressors, and adapt to or tolerate stress (Bishop 1999, Sokolova et al. 2012). Therefore, a disruption in metabolic processes may hinder an organism's ability to deal with temperature-induced stress. While BMR provides insights into fundamental physiology, field metabolic rates (FMR) may offer a more realistic measure of energy demands in wild organisms. FMR reflects the elevated energy use observed in natural conditions (Ritchison 2023) and can inform broader ecological questions by linking individual energy balance to potential population-level responses (Treberg et al. 2016). This is particularly important in climate models for assessing resilience of vulnerability to thermal extremes.

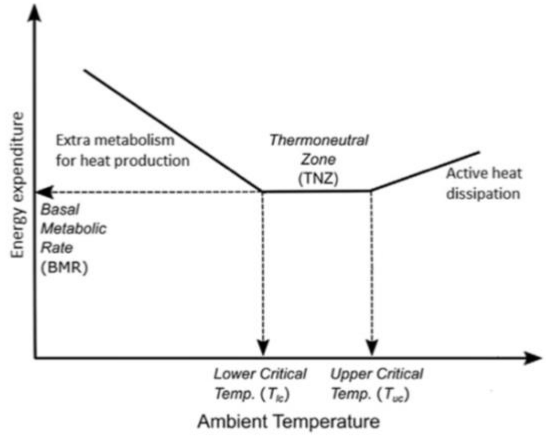

Figure 3. Theoretical illustration of thermoneutral zones (TNZ). Metabolism increases at temperatures below and above the TNZ. Source: Ritchison 2023.

PFAS have been detected at several sites in the Duluth, Minnesota, USA area (Aetna Group 2011) following the use of AFFF and prolonged transport in the water cycle (Fujii et al. 2007). AFFF was released at the Duluth Air National Guard Base (DANGB) during bimonthly fire training exercises from 1960 until 1987 (HydroGeoLogic Inc.). In 2019, PFAS was designated an emerging contaminant at the site (Air National Guard Readiness Center 2021). In 2020, the Strategic Environmental Research and Development Program (SERDP) began funding a study by the U.S. Environmental Protection Agency's Great Lakes Toxicology and Ecology Division (USEPA–GLTED) to investigate food-web exposure and the effects of PFAS on birds at DANGB and surrounding areas (SERDP & ESTCP 2023). This study aims, in part, to contribute to the SERDP study in better understanding the effects of PFAS within the context of swallow nestling physiology.

Given the potential for PFAS to disrupt metabolic regulation and reduce thermal tolerance by altering thyroid function, it is critical to understand how PFAS affect physiological performance in wild birds. This study combines controlled dosing and field respirometry to examine the effects of PFAS, specifically perfluorooctane sulfonate (PFOS), on swallow nestling physiology and the HPT axis. The controlled dosing study, though field-based, incorporates an experimental design typical of laboratory experiments that limits mixture interactions and administers known doses of PFOS—factors that often complicate field studies (Custer 2021). In the stress study, temperature-induced stress is used to measure summit metabolic rates and assess whether PFAS exposure near DANBG and surrounding areas may alter swallow nestling thermal tolerance. For both components (doing and stress studies) of this work, field metabolic rates were estimated from field flow-through respirometry, a relatively novel method in ecotoxicology that provides more ecologically relevant estimates of metabolic function that incorporates variation in characteristics like behavior, thermoregulation, and growth.

The objectives of this integrated study were to (1) apply field respirometry as a novel approach for understanding the effects of PFAS exposure in free-living nestlings,

(2) evaluate the impact of PFOS exposure on the HPT axis with a thyroid hormone concentration end point, (3) measure effects of PFOS exposure on FMR, (4) determine if PFOS impacts nestling development, as measured by body mass, (5) assess the differential ability of PFAS-exposed nestlings to cope with stress as measured by SMR, and (6) provide foundational data for energetics modeling applicable to future work such as risk assessment and ecotoxicology.

Methods

Site descriptions

Experimental site (dosing study, objectives 1- 4 and 6) nest boxes were established to understand the impact of PFOS on nestling metabolism, thyroid function, and mass. These sites included Lester Park Golf Course (LP) and the UMD Land Lab (UM), and they were established at least two years prior to this study (Figure 4) to ensure reliable occupancy (Jones 2003). These sites were selected based on prior environmental sampling conducted under the SERDP project, which indicated negligible PFAS contamination. LP is a public golf course with several small ponds that closed operations in 2020 but continued to receive minimal maintenance through 2023. UM is transitional farmland with temporary seasonal open water areas. Of the 63 total nest boxes (26 at LP, 37 at UM), 24 (14 at LP, 10 at UM) produced successful nests used in the 2023 dosing study conducted from 13 June to 1 July 2023.

Observational site (stress study, objectives 5 and 6) nest boxes were established along a gradient of PFAS contamination at least two years prior to this study (Figure 4) to understand the impact of PFAS exposure on summit metabolic rates. Previous environmental sampling established relative PFAS contamination levels at the following sites: DANGB and Martin Road (MR) (high sites); Rice Lake (RL) (medium site); Boulder Lake (BL), UM, and LP (low sites). DANGB (due to AFFF usage) is known to have elevated surface and groundwater PFAS contamination, and other downstream sites (MR, RL) are potentially affected by surface/groundwater transport (Custer 2011) resulting in a high and medium site designation. BL, UM, and LP were designated low PFAS contamination sites based on distance from DANGB. DANGB is a highly disturbed military site with constructed retention ponds and a dammed stream. MR, located just north of DANGB, features a roadside stream, culvert, and a beaver-dammed pond. Both RL and BL are large areas of open water and wetlands, with some residential development nearby. Of the 202 total nest boxes across sites, 21 nests provided nestlings used in the 2024 stress study.

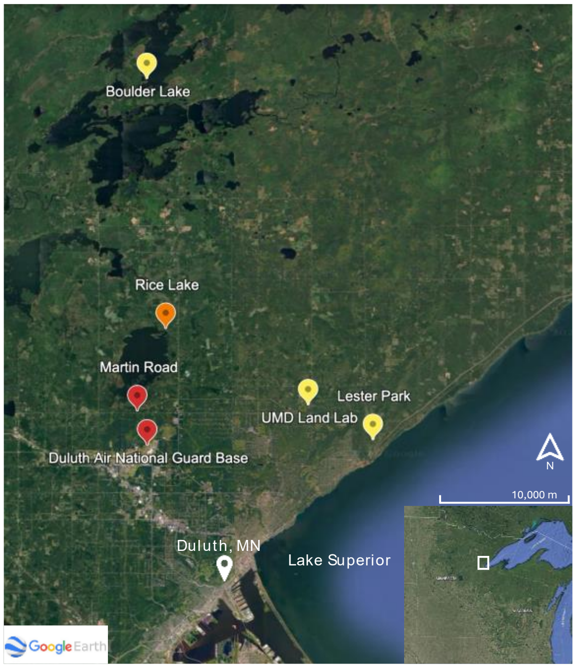

Figure 4. Field study sites in Duluth, MN, USA. Sites along a PFAS gradient include Duluth Air National Guard Base (DANGB) and Martin Road (MR) (red, high), Rice Lake (RL) (orange, medium), and Boulder Lake (BL), UMD land lab, and Lester Park Golf Course (LP) (yellow, low). Adapted from Google Earth.

Whole body residues of the dominant PFAS show PFOS as the highest concentrations at each site (Table 1). Detection limits were addressed by taking half the detection limit as the concentration value. All work was conducted under valid Minnesota Department of Natural Resources (35342) and United States Fish and Wildlife Service (MB830840-0) permits, as well as Animal Care and Use Protocol (Eco26-03-001 and Eco26-05-001) to M. Etterson.

Table 1. PFAS concentrations across study sites. Sites include Duluth Air National Guard Base (DANGB) and Martin Road (MR), Rice Lake (RL), and Boulder Lake (BL). Detection limits were addressed by taking half the detection limit as the concentration value. The three PFAS with highest concentrations at each site are shown. Means are presented ± SD.

Site	Analyte	Concentration (ng/g WW)
DANGB	**PFOS**	**36.36 ± 9.80**
	7:3 FTCA	11.52 ± 5.59
	PFHxS	7.77 ± 3.22
MR	**PFOS**	**13.11 ± 10.6**
	7:3 FTCA	2.28 ± 3.17
	5:3 FTCA	1.70 ± 0.01
RL	**PFOS**	**34.36 ± 17.77**
	PFHxS	2.48 ± 1.47
	7:3 FTCA	1.83 ± 1.29
BL	**PFOS**	**1.84 ± 1.42**
	5:3 FTCA	1.68 ± 0.01
	7:3 FTCA	0.91 ± 0.01

Study species

Tree Swallows are cavity nesters and primarily insectivorous during breeding season, with diet conent mainly consisting of emerging aquatic insects (Winkler et al. 2020). The nestlings are altricial, and adults range in mass from 16-25 g (Winkler et al. 2020). Swallow incubation periods range between 11 to 20 days, but more typically 13 to 14 days (Winkler et al. 2020). Nestlings remain in the nest from 15 to 25 days post-hatch (dph) (Wrinkler et al. 2020).

Respirometry and estimates of metabolism

Nest boxes were monitored twice weekly to estimate clutch initiation, hatch dates, and nestling ages for both studies. For the 2023 dosing study, nests were visited daily from nestling age 5 to 12 dph. For the 2024 stress study, nests were only visited during nest monitoring and for collection on day 12 or 13 post hatch. Field respirometry was conducted to estimate metabolic rates using a Qubit Q-box RP2LP field respirometer and Logger Pro 3 software, with few measurements taken with altered Q-Box RP1LP due to temporary unavailability of the RP2LP unit. This method was used to measure the effects of PFOS dosing on FMR (objectives 1 and 3) and the ability to cope with stress as measured by SMR (objectives 1 and 5). Additionally, the resulting data set can be applied to energetics modeling for future work (objective 6).

The respirometer was calibrated daily and at each site according to Qubit protocol (Qubit Systems Inc. 2020). To obtain comparable baseline readings, the respirometer collected data for approximately one minute before a nestling was placed in the chamber and continued for about one minute after its removal. Incurrent and excurrent CO2 and O2 data was manually extracted from Logger Pro by selecting values representing roughly 30 seconds of the most stable respiration periods, typically near the end of the run. FMR (2023 dosing study) and SMR were estimated by (2024 stress study) by multiplying VO2 (Equation 1) – a measure of oxygen consumption per unit time – by 0.00686 (Equation 2), using values derived from Bennett & Harvey (1987) and Vleck and Vleck (1980). This was then standardized to body weight by dividing kcal/day by g, to give kcal/g/day (Nichols et al. 1995). Resting metabolic rate (RMR), typically measured during fasting and low energy demands, was estimated using published activity coefficients (Nichols et al. 1995); FMR was divided by 1.0 for 5 dph, 1.214, for 8 dph and 1.5 for 12 dph.

$$Equation\ 1\!: VO2 = FR\,\frac{((FiO2 - FeO2) - FeO2)(FeCO2 - FiCO2)}{1 - FeO2}$$

$$Equation\ 2\!: \frac{1\ ul\ O2}{min}\,\frac{60\ min}{hour}\,\frac{24\ hour}{day}\,\frac{1\ ml\ O2}{1{,}000\ ul\ O2}\,\frac{1\ cal}{0.21\ ml\ O2}\,\frac{1\ kcal}{1{,}000\ cal} = \frac{0.00686 kcal}{day}$$

Nestlings were euthanized after respirometry measurements in both studies (12 dph for dosing and 12-13 dph for stress) to collect the following samples: plasma, thyroid, and whole carcass.

PFOS dosing and FMR, TH, and development

Each nest was randomly assigned dosing treatments and nestlings were individually marked using temporary oil-based Sharpie on the tarsus and claw. In addition to a handling control, the following dosing treatments were administered daily via oral gavage from day 5 to 12: Vehicle (ultra-pure water, 8 μL/g/day), low dose (0.32-0.47 mg/kg/day PFOS), high dose (3.37-4.80 mg/kg/day PFOS), and methimazole (~0.5

μL/kg/day), a positive control for inhibition of TH synthesis (Sirsat et al. 2022). Exact doses were calculated based on the mean nestling weight of each clutch, with a scaled dose administered to each nestling.

For the 2023 dosing study (objectives 2-4), two nestlings from each clutch were selected on days 5 (first day of dosing), 8 (midpoint), and 12 (final day of dosing) post-hatch. Prior to treatment, these nestlings were individually weighed before being placed in covered respirometer chambers for approximately 8 minutes. The body mass measurement was used to determine if PFOS impacts nestling development (objective 4) and use in estimations of FMR. A methanol solution was used on equipment to remove any shed PFOS between each clutch.

To evaluate the impact of PFOS dosing on thyroid hormone concentrations (objective 2), Total plasma T3 and T4 concentrations were analyzed using acid hydrolysis, solid-phase extraction, and liquid chromatography-mass spectrometry (Van Dingenen et al. 2023). PFOS concentrations were processed and measured via Batelle, a contracted third-party laboratory.

Statistical analyses were conducted using R Studio Version 2023.06.2+561 and R version 4.3.1 (2023-06-16). The packages packages readr, dplyr, ggplot2, Rcolorbrewer, ggpubr, nlme, MuMIn, and tidyr were utilized. were utilized. Welch's T-tests, one-way ANOVAs, and linear modelling with AICc were conducted. Welch's T-tests were conducted to determine if control and methimazole treatments were different from each other across ages (day 5, 8, and 12). Assumption of normality was tested with the Shapiro-Wilk test. Due to insignificant differences between control treatments, handling and vehicle treatments were combined into a single control treatment and methimazole was not included in further analyses. This was done to emphasize the effect of PFOS on TH, FMR, and mass (objectives 2 - 4).

To evaluate the impact of PFOS exposure on the HPT axis with a thyroid hormone concentration end point (objective 2), a one-way ANOVA was conducted to test the null hypothesis that PFOS treatment does not affect TH. This tested the effect of a single explanatory variable, treatment (controls, low, and high), on continuous response variables (T3 and T4 concentrations). TH, as concentrations were measured only on day

12 post hatch. Data was visually assessed for normality and homogeneity of variance using boxplots, residual plots, and QQ plots. The independence assumption was met by the study design. A post-hoc Tukey HSD test was used to identify significant differences between treatments.

To measure effects of PFOS exposure on FMR (objective 3) and determine if PFOS impacts nestling development with body mass as a metric (objective 4), linear mixed-effect modeling and AICc was performed. Repeated measures were used for dosing study analysis of controls, low, and high treatments to account for FMR and mass measurements taken through development (5, 8, and 12 dph). The mixed-effects models included treatment and age as fixed effects and nestling ID as a random effect. To model FMR, age was used as the covariate instead of mass due to high collinearity between mass and age (Pearson's $r = 0.89$). For modeling mass, treatment and age were included as predictors. AICc was used to compare models and the model with the lowest AICc was selected.

Cold-induced stress and SMR

In the 2024 stress study, two 11-13 dph nestlings (or one if the clutch contained only three nestlings) were weighed and individually placed each in a cooled respirometer chamber inside a cooler for approximately 15-20 minutes and temperatures ranging from 5 C – 22 C. The respirometry measurements were used to estimate SMR in PFAS-exposed nestlings and assess the differential ability to cope with stress (objectives 1 and 6).

Statistical analyses were conducted using R Studio Version 2023.06.2+561 and R version 4.3.1 (2023-06-16). The packages readr, dplyr, ggplot2, RColorBrewer, tidyr, and ggpubr were utilized. To assess the differential ability of PFAS-exposed nestlings to cope with stress as measured by SMR (objective 5), a Wilcoxon test and one-way ANOVA were conducted. A Shapiro-Wilk test indicated deviation from normality of metabolic rates measured at PFAS contaminated sites ($W = 0.60$, $p < 0.001$) and log-transformation did not normalize the data ($W = 0.89$, $p = 0.02$), so Wilcoxon test was selected to test the null hypothesis that control and stress metabolic measurements were the same. A one-way ANOVA was conducted to test the effect of a single categorical factor (site) on a continuous response variable (SMR) and the null hypothesis that PFAS exposure does not affect SMR (objective 5). Data was visually assessed for normality and homogeneity of variance using boxplots, residual plots, and QQ plots. The independence assumption was met by the study design.

Results

Respirometry

Respirometry measurements were conducted on 48 nestlings during the dosing study field season at experimental sites from 13 June to 1 July 2023 to measure the impact of PFOS exposure on FMR (objective 1, 3, and 6). Nests produced clutch sizes ranging from 2–6 nestlings, with respirometry measurements limited to two nestlings per clutch. Each treatment group consisted of five clutches, except for methimazole, which had four. A total of 144 observations were analyzed. Resting metabolic rates measured in this study were comparable to those previously reported for swallows (Nichols et al. 1995; Figure 5). During this period, air temperatures ranged from 17.1 °C to 31.7 °C, with a mean of 24.6 ± 3.4 °C, which was considered average (<0.5 °C deviation from normal) (NOAA 2023). Precipitation was significantly below average (<60% of normal), leading to drought conditions (NOAA 2023).

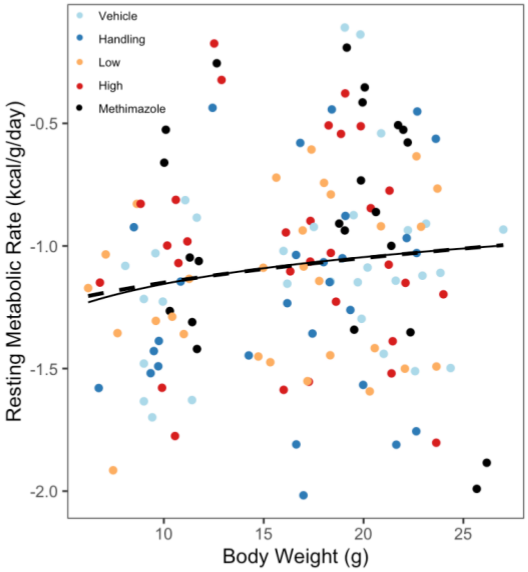

Figure 5. Comparison of Tree Swallow nestling resting metabolic rates and literature equivalents. Nichols et al. 1995 provides an allometric equation for resting metabolism across nestling passerine birds shown as a dashed line. An allometric equation for the respirometry data conducted during the 2023 dosing study is shown as a solid line. Treatments include controls (handling and vehicle of 8 µL/g/day ultra-pure water), low dose (0.32–0.47 mg/kg/day PFOS), high dose (3.37–4.80 mg/kg/day PFOS), and methimazole (~0.5 µL/kg/day).

For the 2024 stress study at observational sites, respirometry was conducted for 22 nestlings from the observational field sites between 25 June and 22 July to assess the differential ability of PFAS-exposed nestlings to cope with stress as measured by SMR (objective 1, 5, and 6). Clutch sample sizes were 10, 5, and 7 at high, medium, and low PFAS sites, respectively, resulting in 22 observations. Nestlings were measured once at 11–13 dph. July 2024 had above-average temperatures (1–2 °C increase from normal), with air temperatures ranging from 14.0 °C to 27.8 °C (mean: 21.1 ± 3.7 °C) (NOAA 2024). Precipitation was also below average (25–50% of normal) (NOAA 2024).

PFOS dosing and FMR, TH, and development

PFOS residues in nestlings were detected at expected relative levels across dosing treatments (Figure 6). Whole body residues in the high-dose and low-dose groups exceeded the residues observed in birds nesting at the AFFF PFAS contamination sites. Nestlings from vehicle and handling controls showed negligible PFOS residues.

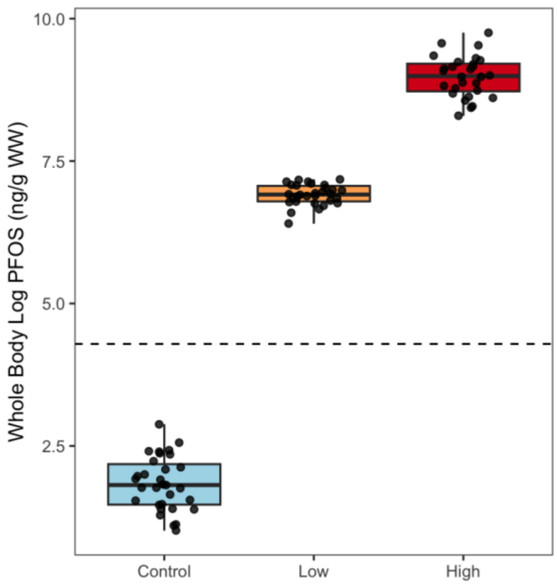

Figure 6. Whole body PFOS residues from Tree Swallow nestlings by treatment. Measured concentrations are shown relative to a dashed line that depicts environmentally relevant residues measured in Tree swallow tissues at observational sites proximal to AFFF PFAS contamination. Treatments include controls of handling and vehicle (ultra-pure water, 8 µL/g/day), low dose (0.32–0.47 mg/kg/day PFOS), and high dose (3.37–4.80 mg/kg/day PFOS).

Negative controls consisted of handling and vehicle (ultra-pure water), with methimazole as a positive control (~0.5 µL/kg/day). The two negative controls did not significantly differ from one another in T3, T4, FMR, or mass (Table 2). Methimazole treatments were not significantly different from negative controls in T3, FMR, or mass (Table 2). Methimazole treatment only showed a significant difference from controls in RMR (Table 2). Handling and vehicle controls were not different from one another and combined into a single control treatment for further statistical comparisons, and methimazole was excluded from further analysis as it did not result in the expected measurable thyroid inhibition.

Table 2. Welch’s T-test results for Tree Swallow nestlings. Negative control treatments include handling and vehicle (ultra-pure water, 8 µL/g/day), and a positive control treatment methimazole (~0.5 µL/kg/day). Respirometry was conducted on 5, 8, and 12 dph nestlings.

Treatments were administered daily and via oral gavage. Significant differences among treatments denoted by asterisks: * = 0.05, ** = 0.01, *** = 0.001

	Negative Controls			**Methimazole and Negative Controls**		
	t-value	p-value	df	t-value	p-value	df
T3 (ng/mL)	-0.25	0.81	17.91	-1.0	0.34	10.29
T4 (ng/mL)	-1.55	0.14	17.87	-2.03	0.07	11.70
Resting metabolic rate (kcal/g/day)	-0.51	0.61	55.44	-2.08	0.05*	34.23
Field metabolic rate (kcal/g/day)	-0.70	0.49	55.98	-1.85	0.07	35.46
Mass (g)	-0.52	0.61	55.70	-0.73	0.47	41.23

Results from one-way ANOVA indicated that dosing significantly affected both T3 (df = 2,36, F stat = 4.52, p-value = 0.018) and T4 (df = 2,36, F stat= 10.43, p-value < 0.001) concentrations (Figure 7). Control treatments did not have significantly different T3 levels compared to low and high-dose treatments (p-value = 0.55 and 0.06, respectively), but T3 levels in high-dose treatments were significantly decreased compared to low-dose (p-value = 0.02) (Tukey's HSD) (Figure 7a). Control treatments did not have significant T4 differences from low-dose treatments (p = 0.24) but were significantly elevated compared to high-dose treatments (p = 0.003). T4 levels of low-dose treatments were also significantly elevated compared to high-dose treatments (p < 0.001). (Figure 7b).

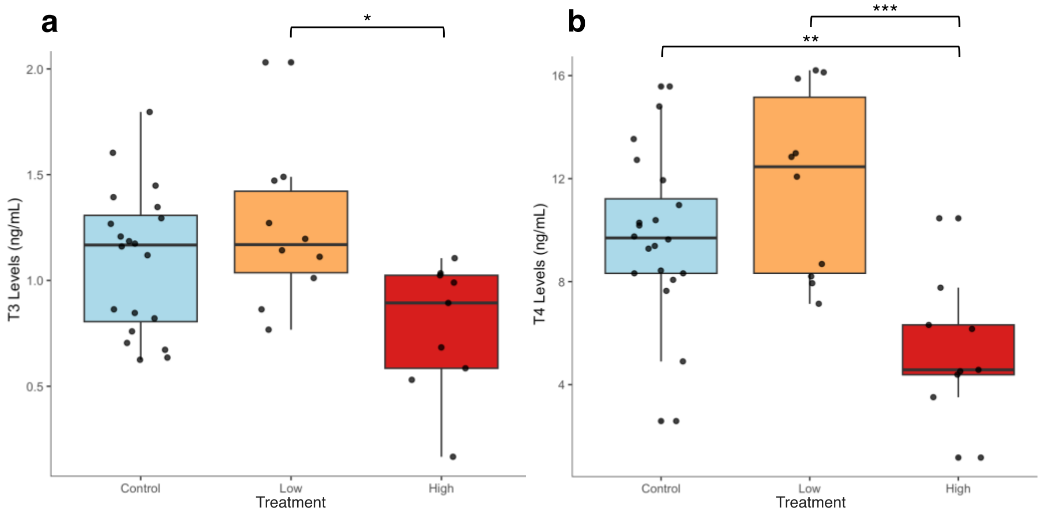

Figure 7. Tree Swallow nestling plasma thyroid hormones (**a**) T3 and (**b**) T4. ANOVA indicates treatment significantly affected both T3 (one-way ANOVA, df = 2, p-value = 0.02)

and T4 (one-way ANOVA, df = 2, p-value < 0.001) concentrations. Respirometry was conducted on 5, 8, and 12d ph nestlings. Plasma for thyroid hormone analysis was collected on 12 dph. Treatments were administered daily and via oral gavage (low dose 0.32–0.47 mg/kg/day PFOS, high dose 3.37–4.80 mg/kg/day PFOS). Significant differences among treatments denoted by asterisks: * = 0.05, ** = 0.01, *** = 0.001 (Tukey's HSD).

FMR, mass, and age were compared across dosing treatments with repeated measures to evaluate potential impacts of PFOS exposure during nestling development (Table 3). Based on AICc results, PFOS treatment and age were the most informative predictors of FMR (ΔAICc = 21.89). Linear mixed-effect modeling shows no significant differences between treatments for FMR (Low p = 0.61 and High p = 0.35) (Figure 8) or mass (p = 0.23 for low, p = 0.60 for high) (Figure 9).

Table 3. Tree Swallow nestling average mass and field metabolic rates by dosing treatment. Age is reported in days post-hatch (dph). Treatments include handling, vehicle (ultra-pure water, 8 µL/g/day), low dose (0.32–0.47 mg/kg/day PFOS), high dose (3.37–4.80 mg/kg/day PFOS), and methimazole (~0.5 µL/kg/day). Means are presented ± SD.

	Handling			**Vehicle**			**Low**			**High**			**Methimazole**		
Age (dph)	5	8	12	5	8	12	5	8	12	5	8	12	5	8	12
Average field metabolic rate (kcal/g/day)	3.8 ±1.7	11.2 ±4.8	13.3 ±4.0	2.8 ±0.9	9.1 ±2.6	18.8 ±3.1	2.4 ±0.9	8.1 ±1.7	16.1 ±1.9	2.6 ±1.3	8.5 ±3.0	12.8 ±4.1	3.0 ±1.1	9.7 ±2.3	18.7 ±4.5
Average mass (g)	11.1 ±3.2	17.8 ±1.2	20.4 ±2.2	9.8± 1.2	18.7 ±1.7	23.2 ±2.0	9.0 ±1.9	16.6 ±1.4	21.2 ±2.2	10.4 ±1.7	17.8 ±1.6	21.1 ±2.0	11.0 ±1.0	19.8 ±0.6	22.8 ±2.7

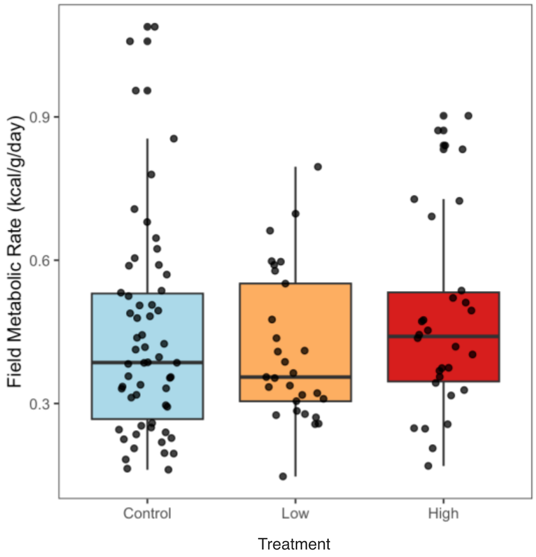

Figure 8. Tree Swallow nestling field metabolic rates at across PFOS dosing treatments. Treatments include controls (handling and vehicle 8 µL/g/day ultra-pure water), low dose (0.32–0.47 mg/kg/day PFOS), and high dose (3.37–4.80 mg/kg/day PFOS). Treatments were administered daily from 5-12 dph and via oral gavage. Respirometry was conducted at 5, 8, and 12 dph.

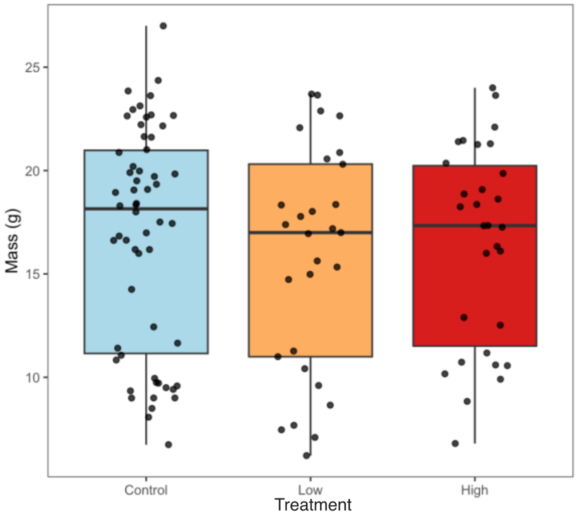

Figure 9. Tree Swallow nestling mass at across PFOS dosing treatments. Treatments include controls (handling and vehicle 8 µL/g/day ultra-pure water), low dose (0.32–0.47 mg/kg/day PFOS), and high dose (3.37–4.80 mg/kg/day PFOS).

Cold-induced stress and SMR

Whole body PFOS concentrations in nestlings of observational sites (2024) deviated from expected levels based on environmental gradients (Figure 10), but ANOVA indicated impacted sites (medium and high) were different from reference/low sites (df = 2,35, F stat = 23.3, p-value < 0.001). Medium and high site nestling PFOS concentrations were not different from one another (p-value = 0.77), but both were higher than the low sites (p-value < 0.001, Tukey's HSD).

Measured metabolic rates of the cold-stressed nestlings were significantly higher than that of 2023 control nestlings (Wilcoxon rank sum test, W = 58, p-value < 0.001) (Figure 11). Estimated SMR of cold-stressed nestlings were not significantly different across sites (ANOVA, df = 2,19, F stat = 0.60, p-value = 0.56) (Figure 12).

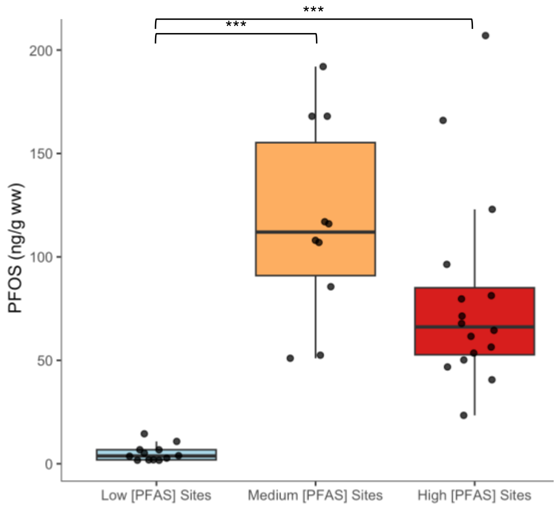

Figure 10. Whole body PFOS residues from Tree Swallow nestlings by site. These 12-13 dph nestlings were collected from observational sites along a PFAS gradient including Duluth Air National Guard Base (DANGB) and Martin Road (MR) (red, high), Rice Lake (RL) (orange, medium), and Boulder Lake (BL), UMD land lab, and Lester Park Golf Course (LP) (blue, low). Significant differences among treatments denoted by asterisks: * = 0.05, ** = 0.01, *** = 0.001 (Tukey's HSD).

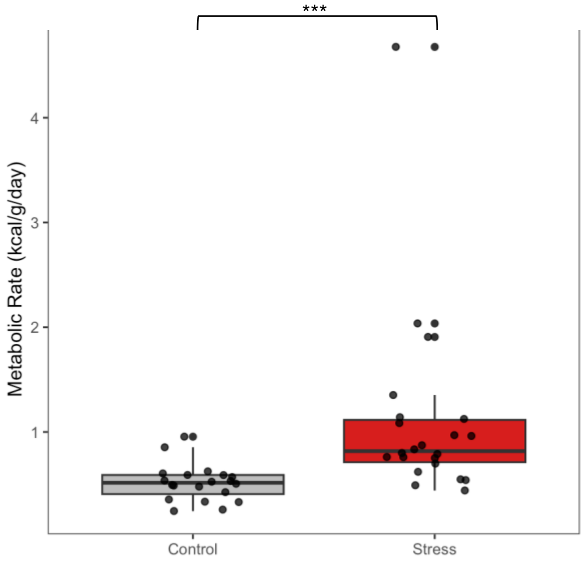

Figure 11. Comparison of Tree Swallow nestling metabolic rates between control and cold-stressed nestlings. 2024 nestlings were exposed to a cold treatment for 15-20 minutes, and their summit metabolic rates were measured. 2023 nestlings' field metabolic rates were

measured as a part of the dosing study. Welch's T-test indicates metabolism is affected by cold-induced stress (Wilcoxon rank sum test, W = 58, p-value < 0.001). Significant differences among treatments denoted by asterisks: * = 0.05, ** = 0.01, *** = 0.001.

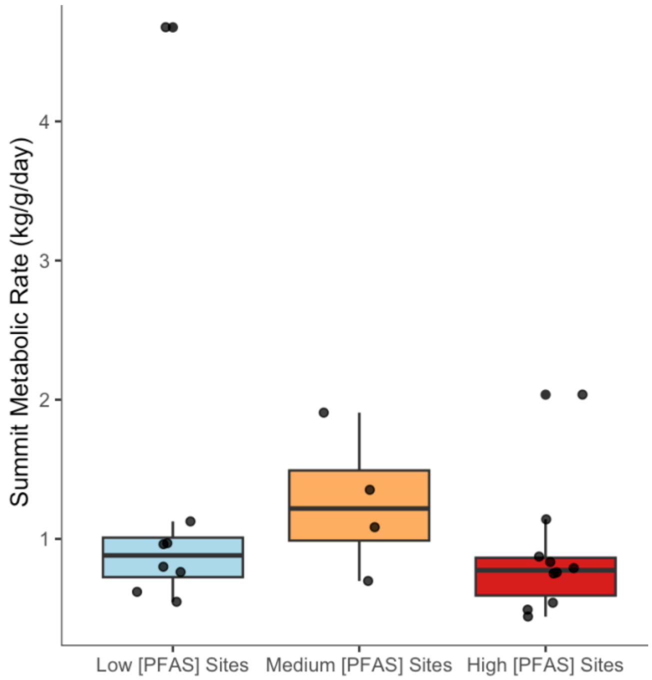

Figure 12. Comparison of Tree Swallow nestlings' summit metabolic rates across a PFAS concentration ([PFAS]) environmental gradient. Nestlings were exposed to a cold treatment for approximately 15-20 minutes, and their summit metabolic rates were measured. These nestlings were collected from observational sites along a PFAS gradient including Duluth Air National Guard Base (DANGB) and Martin Road (MR) (red, high), Rice Lake (RL) (orange, medium), and Boulder Lake (BL), UMD land lab, and Lester Park Golf Course (LP) (blue, low).

Discussion

This study finds field respirometry a valuable method in ecotoxicology and provides insight into the effects of PFOS exposure on the HPT axis. More specifically, there is partial support for the hypothesis that thyroid hormone (T3 and T4) levels differ with PFAS exposure. Our results indicate that T4 is affected while T3 is not (though it follows a similar trend as T4). Despite alterations in TH status, the results of this study failed to support the hypotheses that field metabolic rates, mass, and summit metabolic rates differ as a result of PFAS exposure. Additionally, the dataset provided can be applied to future work involving risk assessment and ecotoxicology.

The PFAS concentrations and metabolic rates measured in this study are similar to those found in previous work, therefore providing a valuable baseline for future studies investigating PFAS impacts on passerines or other avian species. In the 2023 controlled dosing study, nestlings incorporated PFOS into tissues as expected according to their treatment (Figure 6). Additionally, both the high and low doses, though exceeding the highest nestling PFOS concentration found at DANGB, are environmentally relevant. Mean PFOS concentrations in nestlings of the high dose are at the upper end of measured PFOS in field studies and comparable to egg concentrations of PFOS in Great Tits (*Parus major*) near fluorochemical plants in Belgium (10, 380 ng/g ww) (Groffen et al 2019), while mean PFOS concentrations of the low dose fall within the range of swallow plasma sampled along a concentration gradient in southern Minnesota (17.3 – 295 ng/g ww)(Custer 2013). The controls from the dosing study and testing of cold-induced stress provide estimates for establishing metabolic rates and TH concentrations, as well as the lower end of TNZs for developing swallow nestlings. This is potentially valuable for assessing physiological impacts of other stressors in swallows, and in energetics modeling.

Field flow-through respirometry appears to be an efficient and valid method for estimating metabolic rates in nestlings, as our measured values align with established literature across sites and treatments. However, field-based studies introduce additional variables like weather and food availability that may influence measurements. Both years of this study had above average temperatures and below average precipitation, which may affect food availability and thermoregulatory processes. It is important to note that field respirometry measurements were used to estimate FMRs that take these variables into account, rather than assuming we measured RMR which is typically measured during fasting and low energetic demands.

Thyroid hormone concentrations

Methimazole did not induce changes in circulating THs in this study, with the only significant difference from controls being in resting metabolic rate. This is contradictory to a study of Red-winged Blackbirds (*Agelaius phoenicius*), where T3 levels and mass were lower in methimazole-treated nestlings (Sirsat et al. 2022). However, that study used differing dose schedule (only day 2, 3, and 5-dph) and higher cumulative methimazole dose (10 mg methimazole/kg compared to our ~ 4.4 mg methimazole/kg) (Sirsat et al. 2022). These contradictory findings may suggest a species-specific response, or that there is an interaction of PFOS and methimazole, as they are both potentially impacting thyroid function. Published studies of simultaneous methimazole and PFAS dosing are not currently known. Additional research in possible interactions of methimazole with PFAS and the effects of methimazole in wild bird studies would be informative.

PFOS affected T4 more than T3 in our study (Figure 7). T3 levels, while not different in the control nestlings, were different between low and high doses. This follows a similar pattern that is found to be significant in T4 across treatments, where low dose nestlings had elevated circulating TH concentrations compared to high dose nestlings. A study on adult Black-legged Kittiwakes (*Rissa tridactyla*) showed a sex-dependent positive correlation of PFAS with male total T4 and female total T3 in plasma (Ask et al. 2021), whereas a study on Kittiwake chicks found no such correlation (Blévin et al. 2017).

Elevated concentrations of plasma TH in PFOS-exposed nestlings align with expectations that PFAS binds with albumin and increases concentrations of free TH in plasma (Forsthuber et al. 2020), but it is unclear as to why the low dose of PFOS results in elevated TH concentrations and high dose results in decreased TH. T4 may be less strictly regulated by the HPT axis, as it is the precursor to T3 (Decuypere et al. 2005). Furthermore, this suggests that feedback mechanisms within the HPT axis may be overwhelmed in high PFOS concentrations wherein TH concentrations are unable to maintain homeostasis. While thyroid adverse outcomes due to contaminant exposure has been studied in avian reproductive success contexts (Noyes et al. 2019, Chen et al 2008) there are still unknowns regarding PFAS adverse outcomes within wild birds (Ankley et al. 2009). Evidence that T4 is impacted by PFOS exposure is present, but further mechanistic research into adverse outcome pathways would strengthen understanding of which feedback mechanisms may have been affected within the HPT axis.

Field metabolic rates & mass

PFOS does not seem to affect field metabolic rates of developing swallow nestlings despite disruptions in circulating T4 (Figure 8). This suggests there may be another mechanism more tightly regulating FMR in nestlings so that other physiological processes may take priority for development. BMR was positively associated with T3 in adult Kittiwakes, but FMR had no relationship (Welcker et al. 2013). Contradictory findings in adult Glaucous Gulls (*Larus hyperboreus*) suggest TH concentrations were not associated with BMR (Verreault et al. 2007). The present study by design cannot assume BMR, but additional work in understanding thyroid disruptions and effects on FMR specifically would strengthen the connection of PFOS, HPT axis impacts, and changes in metabolism. Additionally, the highest TH levels may occur as altricial nestlings develop endothermy (Ritchison 2023). Therefore, it would be valuable to continue monitoring post-fledge or repeat this study on adults to see if findings are life-stage dependent.

Mass, or growth in the case of developing nestlings, was also unaffected by PFOS exposure (Figure 9). Despite this, these results may be comparable to a study done on Red-winged Blackbirds, where supplemental T3 ultimately resulted in a decrease of mass and length of femur and tarsus (Sirsat et al. 2022). These results show that even with disrupted TH concentrations due to PFO exposure, mass was not affected. This further corroborates with speculation that there may be mitigation of the HPT response to maintain development despite PFOS exposure. Future work would benefit from using more specific growth metrics to capture potentially more subtle effects of PFAS on development in nestlings.

Summit metabolic rates

In the 2024 study, whole body residues of PFAS deviated from original expectations of environmental gradients but were still higher in impacted sites (medium = 116.5±47.7 ng/g ww and high = 80.6±47.9 ng/g ww) than low (5.1±4.0 ng/g ww) sites

(Figure 10). While medium and high sites were not different as expected, the significance between these impacted sites and the low/reference sites still allows speculation on the possible effects of PFAS on SMR. The PFAS concentrations found in the present study are comparable to the previously reported concentrations at sites in Minnesota (17.3 – 295 ng/g ww) (Custer et al. 2013). SMR did not differ between sites (Figure 12), despite the varying PFAS concentration in nestlings. It was anecdotally noted that there were differing nestling responses that did not correlate to site, as some responded by shivering and others by reducing activity that may be related to a torpor (Ruf and Geiser 2015). With Red-winged Blackbird nestlings, increased T3 did not influence VO2 and ventilation in response to cooling (Sirsat et al. 2022). While it was found that PFAS may influence TH (Figure 7), these findings align with previous findings that respiration is not affected by PFAS-impacted TH concentrations (Verreault et al. 2007). Additional research analyzing temporal response differences would improve understanding of stress response impacts from contaminant exposure.

Implications & conclusions

This work contributes to a better understanding of the effects of PFAS on TH and basic metabolism in birds. It provides baseline thyroid, metabolism, and thermoregulation data for future passerine studies that are valuable in assessing effects of contaminants and risk to populations, as well as applications in climate and energetics modeling. Additionally, it contributes to the growing work investigating interactions of compounding stressors (i.e., food availability, stress) and contaminants.

Using novel approaches of controlled-dosing field-based study and field respirometry, the effects of PFAS (especially PFOS) on Tree Swallow physiology and metabolism were investigated. It was found that PFOS may affect TH concentrations, but that further impacts to development and metabolism are not apparent . The HPT axis regulation of metabolism may be dependent on the level of PFAS exposure, and the PFAS-HPT axis-metabolism connection may not be as direct as originally hypothesized due to other regulatory mechanisms that may prioritize development in nestlings. Further research should investigate PFAS effects on the HPT axis and its mechanisms for regulating THs, metabolism, and thermoregulation while using multiple life stages and measuring more specific metrics (BMR, growth, etc.) to capture more subtle effects of PFAS. Ultimately, a complete understanding of PFAS effects on metabolism and thermoregulation has implications for assessing risk to vulnerable species.

Chapter 2:

Investigating interspecific variation in liver weight and energy metabolism in northern Minnesota passerines

Introduction

Understanding physiological differences among species is increasingly important in the context of environmental stressors. A fundamental goal of ecotoxicological risk assessment is to predict potential adverse effects on individuals and species (Ankley et al. 2009) that may be exposed. Physiological research (including aspects of energy metabolism) with wild bird studies can provide valuable insight into potential vulnerabilities (Daan et al. 1990, Garcia-Reyero and Perkins 2011). While basal metabolic rate (BMR) is useful in fundamental physiological research, estimates of field metabolic rates (FMR) may be more useful in studying stressor response in wild organisms. FMR, a measure of metabolism that assumes higher energy demands of organisms observed in field studies (Ritchison 2023), allows for an understanding of an individual's energy balance that may be extrapolated to population level questions (Treberg et al. 2016). This information is important in assessing population risk, especially within context of often-compounding (Semenza et al. 2022, Holmstrup et al. 2009) stressors related to energy balance such as climate change (IPCC 2021), disease (Sur et al. 2025), and environmental contaminants like per- and polyfluoroalkyl substances (PFAS) (Remili et al. 2025). In addition to applicability in energetics modeling, metabolic data can be used to address gaps in metabolic scaling in passerines.

In passerine birds, FMR varies with body mass (Hudson et al. 2013), but interspecific differences in metabolic rates cannot be explained by body mass alone. Factors like trophic status and organ weight may also contribute to differences (Williams et al. 2010). BMR is defined as the minimum energy required for living (Hulbert and Else 2004) and does not include additional energetic costs that contribute to FMR such as food digestion, growth, and reproduction. BMR has been thought to scale allometrically with body mass due to constraints of volume to surface ratio, at approximately 0.67-0.75 (Walsberg and King 1978), but further work in modelling of allometric scaling provides evidence for other explanations such as hypotheses that incorporate the fractal nature of nutrient delivery (West et al. 1997). There is residual variation around the allometric scaling of mass and metabolism that is not accounted for in earlier work, as animals of the same body mass have wide ranges in BMR (Mueller and Diamond 2001, McNab 2009). Some factors that may explain metabolic variation with body mass in passerines include phylogenetic history, feeding strategy, trophic level, latitude, migratory status, habitat, and organ weight (Ritchison 2023). Comparisons of these factors between passerine species would provide insight into ecological and evolutionary differences in addition to providing baseline and ecologically relevant data.

Tree Swallows (*Tachycineta bicolor*, swallows), Northern House Wrens (*Troglodytes aedon*, wrens), and Black-capped Chickadees (*Poecile atricapillus*, chickadees) are three northern Minnesota species that differ across these factors that may drive interspecific variation in metabolic rates. These species have a range of body sizes, varying diets during breeding season, and different migratory status (Cornell Lab of Ornithology 2019). Ecological factors such as mentioned here, while valuable for context, are more difficult to quantify. Trophic level may be best quantified with isotope analyses but requires extensive sampling and instrumental analyses. Quantifying migration has complexities including the large temporal and spatial scales involved, in the context of which species may experience different environmental conditions, food availability, and energetic demands throughout migration. Organ weight in nestlings could offer a more simple, direct, and quantifiable comparison to better understand drivers of interspecific differences in metabolism.

There are contradictory findings regarding to what degree the various factors mentioned above may explain the residual differences in metabolism, specifically in the correlation of organ weight and metabolism. Organ weight was found to be correlated to habitat but not BMR in Tieleman et al. 2003, whereas Hammond et al. 2000 showed a sex-dependent correlation of BMR and some internal organs.The liver has important functions in metabolism (Vikramjit and Metcalf 2009). It also provides a mechanistic lens to explore metabolic differences and contributes to a broader understanding of how internal organ investment may drive or reflect energy use strategies in developing birds. A previous comparative study proposed liver weight as an explanatory factor for differences in metabolic rate between tropical and temperate birds (Jimenez et al 2014). There has been work done in swallows and wren liver weights in ecotoxicological contexts (Dods et al. 2005, Custer et al 2002), and in chickadees regarding phenotypic plasticity (Liknes and Swanson 2011), but baseline liver weight data has yet to be established. There has been work on measuring adult field metabolic rates of swallows (Williams 1988), wrens (Tieleman et al. 2006), and chickadees (Olson et al. 2010), but studies have used different methodologies and reported values that are difficult to directly compare. Therefore, it would be extremely valuable to measure the interspecific differences of nestlings with a consistent methodology to more definitively relate organ weight as a factor that influences metabolism. Furthermore, this information would offer insight into the possible co-evolution of organ development and metabolism and contribute to the understanding of the evolution of physiological diversity in birds.

For this comparative study of field metabolic rates in three passerines in northern Minnesota, we used field respirometry to better understand the relationship between interspecific differences in metabolism and liver weight. Field metabolic rates were estimated from field flow-through respirometry and compared among nestlings of three passerine species. Liver weights were measured to determine if interspecific physiological differences, such as organ size, help explain variation in metabolic rates among these species. By comparing three species that differ in life history, developmental rate, and habitat use, this study offers insights into how evolutionary and ecological strategies shape physiological traits like metabolism and organ development.

The objectives of this integrated study were to (1) apply field respirometry as a novel approach for understanding drivers of interspecific differences in free-living nestlings, (2) evaluate differences liver weight and FMR of three passerine species, (3) assess the relationship between liver weight and FMR in passerines, (4) use comparisons of three passerine species to provide insight into ecological and evolutionary differences, and (5) provide foundational data to address gaps in avian developmental physiology.

Methods

Site descriptions

Nest boxes were established at the following sites (Figure 1) at least two years prior to this study. Lester Park Golf Course (LP) is a public golf course with several small ponds, that closed operations in 2020 but continued to receive minimal maintenance through 2023. University of Minnesota – Duluth Land Lab (UM) is transitional farmland with temporary seasonal open water areas. W.R. Bagley Nature Area (BG) is a highly recreated area that includes a small pond. Boulder Lake (BL) is a large area of open water and wetlands, with some residential development nearby. Boy Scout Landing (BS) is a highly disturbed public water access point to the St. Louis River. Of the nest boxes at these sites, 11 (1 at LP, 1 at UM, 2 at BG, 2 at BL, 5 at BS) produced successful wren and chickadee nests that were used in the 2024 season. Swallow nestling measurements were conducted in 2023 and obtained from 10 nests across the UM and LP sites.

All work was conducted under valid Minnesota Department of Natural Resources (35342) and United States Fish and Wildlife Service (MB830840-0) permits, as well as Animal Care and Use Protocol (Eco26-03-001 and Eco26-05-001) to M. Etterson.

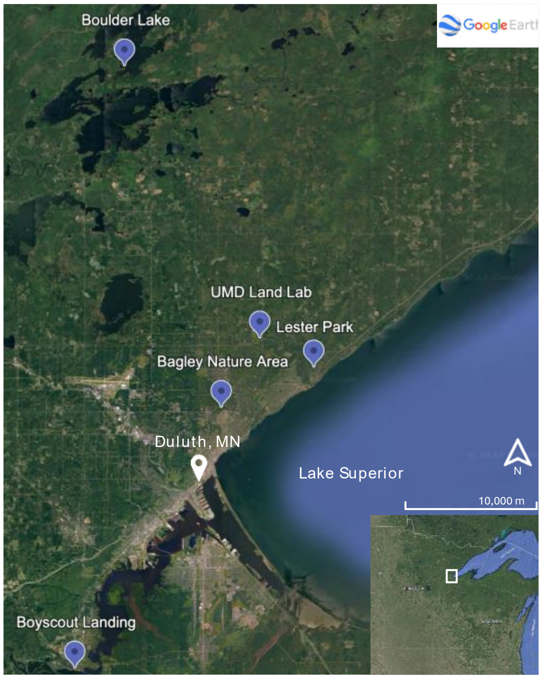

Figure 1. Field study sites in Duluth, MN, USA. Data were collected in the summers of 2023 and 2024.

Study species

To understand drivers of interspecific differences in free-living nestlings, three passerine species –swallows, wrens, and chickadees – were utlizied. They are all cavity nesters and primarily insectivourous during breeding season, but they vary in mass (Adult swallows range from 16-25g; wrens 10-12g; chickadees 9-14g, Cornell Lab of Ornithology 2019) and diet content. These species' trophic level and feeding strategies differ; swallows feed on emerging aquatic insects (Winkler et al. 2020), wrens glean invertebrates from ground and shrubs with a high proportion of spiders (Johnson 2020), and chickadees feed on herbivorous terrestrial insects like caterpillars (Foote et al. 2020). Additionally, as adults, chickadees are considered year-round residents of northern Minnesota (Foote et al. 2020) whereas swallows and wrens migrate to the southern U.S. and Central America (Winkler et al. 2020, Johnson 2020).

These species vary in development as well, with incubation and fledge time being different between species. Temperate swallows typically incubate for 13-14 days and fledge between 18 and 22 days post-hatch (dph) (Winkler et al. 2020) whereas wren and chickadees in similar latitudes have shorter development times. Wrens incubate for approximately 12 days and typically fledge between 16 and 18 dph (Johnson et al. 2004). Chickadees typically incubate for 12-13 days and fledge between 12 and 16 dph (Foote et al. 2020).

Percent of development at which nestlings were measured is based on established literature of average fledging age (Birds of the World 2025). This was calculated by dividing the age (since onset of incubation) at measurement by the species' average development time: 33.5 for swallows (incubation of 13.5 days, fledge 20 dph), 26.5 for chickadees (incubation of 12.5 days, fledge 14 dph), and 28.5 for wrens (incubation of 12.5 days, fledge 16 dph). Using R Studio Version 2023.06.2+561 and R version 4.3.1 (2023-06-16) with packages readr, dplyr, ggplot2, RColorBrewer, tidyr, ggpubr, and MuMIN being utilized, a one-way ANOVA was conducted to determine if development at time of FMR measurement varied between species. This tested the effect of a single categorical factor (species) on a continuous response variable (development %) to test the null hypothesis that species were measured at the same development stage. Data was visually assessed for normality and homogeneity of variance using boxplots, residual plots, and QQ plots. The independence assumption was met by the study design.

Respirometry & FMR

Nest boxes were monitored twice weekly to estimate clutch initiation, hatch dates, and nestling ages for both studies. Field respirometry was conducted using a Qubit Q-box RP2LP field respirometer and Logger Pro 3 software. This method was used to measure the FMR of three passerine species (objectives 1-3). Additionally, this dataset can provide foundational data to address gaps in avian development physiology (objective 5). The respirometer was calibrated daily and at each site according to Qubit protocols (Qubit Systems Inc. 2020). Two nestlings (or one if the clutch contained only three nestlings) were weighed and individually placed in the covered respirometry chamber. To avoid force-fledging for chickadees and wrens, 9-11 dph nestlings were used while for swallows, 11-13 dph nestlings were examined. Measurements were still conducted at relatively the same stage of development. To obtain comparable baseline readings, the respirometer run began approximately one minute before a nestling was placed in the chamber and continued for about one minute after its removal. Incurrent and excurrent CO2 and O2 data were manually extracted from Logger Pro by selecting values representing roughly 30 seconds of the most stable respiration periods, typically near the end of the run.

Field metabolic rate was estimated by multiplying VO2 (Equation 1) (Bennet and Harvey 1987)—a measure of oxygen consumption per unit time—by 0.00686 (Equation 2) (Vleck and Vleck 1980), using values derived from Bennett & Harvey (1987) and Vleck et al. (1980) to give kcal/day. FMR was then standardized to body weight by dividing kcal/day by g, to give kcal/g/day (Nichols et al. 1995).

$$Equation\ 1{:}\ VO2 = FR\,\frac{((FiO2 - FeO2) - FeO2)(FeCO2 - FiCO2)}{1 - FeO2}$$

$$Equation\ 2{:}\ \frac{1\,ul\,O2}{min}\,\frac{60\,min}{hour}\,\frac{24\,hour}{day}\,\frac{1\,ml\,O2}{1{,}000\,ul\,O2}\,\frac{1\,cal}{0.21\,ml\,O2}\,\frac{1\,kcal}{1{,}000\,cal} = \frac{0.00686 kcal}{day}$$

Using R Studio Version 2023.06.2+561 and R version 4.3.1 (2023-06-16) with packages readr, dplyr, ggplot2, RColorBrewer, tidyr, ggpubr, and MuMIN being utilized, a one-way ANOVA was performed to test the effect of a single categorical factor (species) on continuous response variables (FMR). This tested the null hypothesis that FMR was the same between species (objectives 1 and 2). Boxplots, residual plots, and QQ plots were used to visually assess data for normality and homogeneity of variance. The independence assumption was met by the study design. Tukey HSD test was conducted to identify significant differences between species.

Liver weights

Following respirometry, nestlings were euthanized to collect liver samples. Small, negligible liver subsamples were taken for a separate transcriptomic study before livers were weighed. Liver weight was calculated as a percentage of body weight (hepatosomatic index, HSI) by dividing remaining liver mass by total nestling mass at time of respirometry measurement (HSI = 100 * Liver Weight/Body Weight). HSI is often used in toxicology as a measure of metabolic activity (Verma and Prakash 2020). This was done to evaluate differences liver weight of three passerine species (objective 2).

Using R Studio Version 2023.06.2+561 and R version 4.3.1 (2023-06-16) with packages readr, dplyr, ggplot2, RColorBrewer, tidyr, ggpubr, and MuMIN being utilized, a one-way ANOVA was performed to test the effect of a single categorical factor (species) on continuous response variables (HSI). This tested the null hypothesis that HSI was the same between species (objectives 1 and 2). Boxplots, residual plots, and QQ plots were visually assessed data for normality and homogeneity of variance. The independence assumption was met by the study design. Tukey HSD test was conducted to identify significant differences between species.

FMR modeling

Using R Studio Version 2023.06.2+561 and R version 4.3.1 (2023-06-16) with packages readr, dplyr, ggplot2, RColorBrewer, tidyr, ggpubr, and MuMIN being utilized, modelling of FMR was used to assess the relationship between liver weight and metabolic rate and to provide insight for differences between species (objectives 3 and 4).

Univariate models with mass, liver, HSI, and species were conducted and AICc was used to compare models. A global linear model was conducted with log-transformed metabolic rate (MR) as the response variable and predictors being log-transformed body mass, log-transformed liver mass, species identity, and the interaction between log body mass and log liver mass. Nested models were conducted, and AICc was used to compare models.

Results

Respirometry measurements were conducted on 33 nestlings (11 wrens, 12 chickadees) during the field season from 7 June to 16 July 2024 (Figure 2) and 18 swallow nestlings from 13 June to 1 July 2023. Nests produced clutch sizes ranging from 2–7 nestlings, with measurements taken from one nestling in two-nestling clutches and two nestlings in clutches of three or more. A total of 51 measurements were analyzed.

July 2024 had above-average temperatures (1–2 °C increase from normal) with air temperatures ranging from 14.0 °C to 27.8 °C (mean: 21.1 ± 3.7 °C) (NOAA 2024). Precipitation was below average (25–50% of normal). During the 2023 study air temperatures ranged from 17.1 °C to 31.7 °C, with a mean of 24.6 ± 3.4 °C, which was considered average (<0.5 °C deviation from normal) (NOAA 2023). However, precipitation was significantly below average (<60% of normal), leading to drought conditions (NOAA 2023).

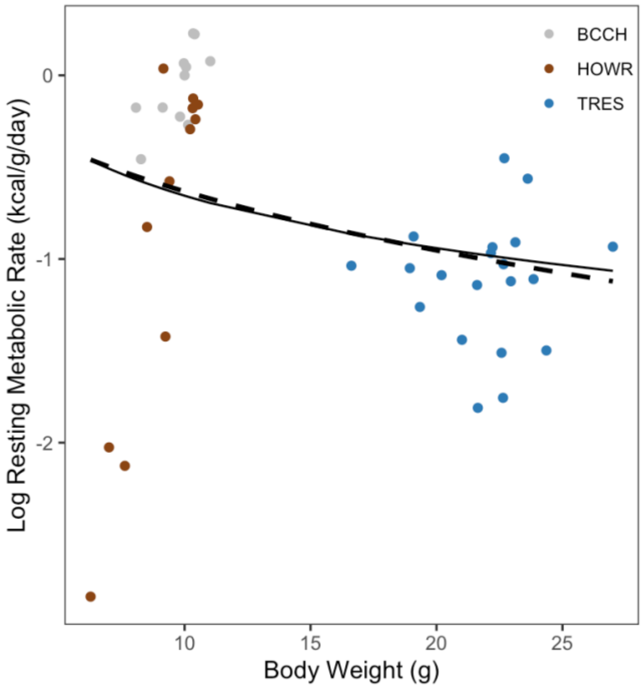

Figure 2. Comparison of three Passerine species nestling resting metabolic rates and literature equivalents. Nichols et al. 1995 provides the allometric equation for resting metabolism across nestling passerine birds shown as a dashed line. An allometric equation for the respirometry data conducted during for this study is shown as a solid line. BCCH: Black-capped Chickadees; HOWR: Northern House Wrens; TRES: Tree Swallows.

Study species

Chickadees and wrens were measured on either day 9 (n = 4 both species), 10 (n = 2 both species), 11 (n = 5 and 4, respectively), or 12 (n = 2 HOWR) post hatch, whereas swallows were measured on either day 11 (n = 6), 12 (n = 12), or 13 (n = 3) post hatch. There was not a significant difference of age as a percentage of development between species (ANOVA, df = 2,40, F stat = 2.49, p-value = 0.10). Relative developmental stage at which respirometry measurements were taken did not differ among species.

Interspecific differences in FMR & HSI

Metabolic rates of nestlings differed significantly among species (Figure 3) (ANOVA, df = 2,40, F stat =20.44, p < 0.001). Chickadees had significantly higher mass-specific metabolic rates than swallows (Tukey's HSD p < 0.001) and wrens (Tukey's HSD, p < 0.001). There was no significant difference between swallows and wrens (Tukey's HSD, p = 0.19).

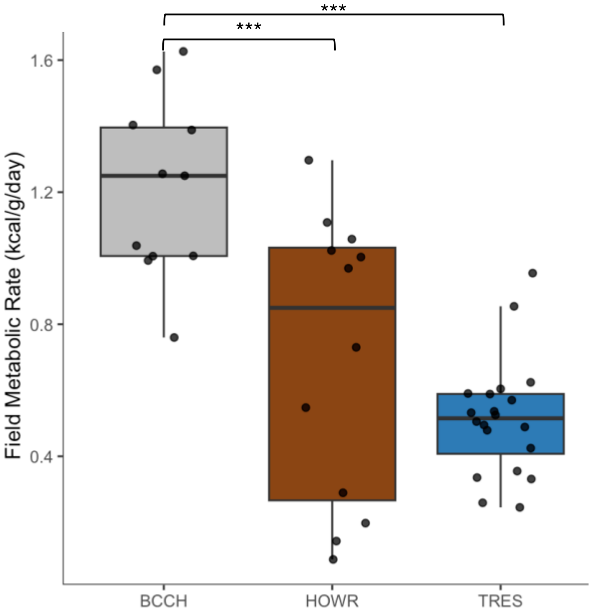

Figure 3. Nestling field metabolic rates of three Passerine species. Metabolic rates were significantly different across species (ANOVA, df = 38, p < 0.001). Tukey's HSD revealed significance for Black-capped chickadees (BCCH), but not for other species comparisons (Northern House Wren, HOWR and Tree Swallows, TRES). Significant differences among treatments denoted by asterisks: * = 0.05, ** = 0.01, *** = 0.001 (Tukey's HSD).

HSI also differed significantly across species (Figure 4) (ANOVA, df = 2,40, F stat =4.31, p-value = 0.02). Swallows had significantly higher HSI than chickadees (Tukey's HSD, p = 0.17), but were not different from wrens (Tukey's HSD, p = 0.27). There were no significant differences between wrens and chickadees (Tukey's HSD, p = 0.45). These results confirm species-specific variation in liver mass.

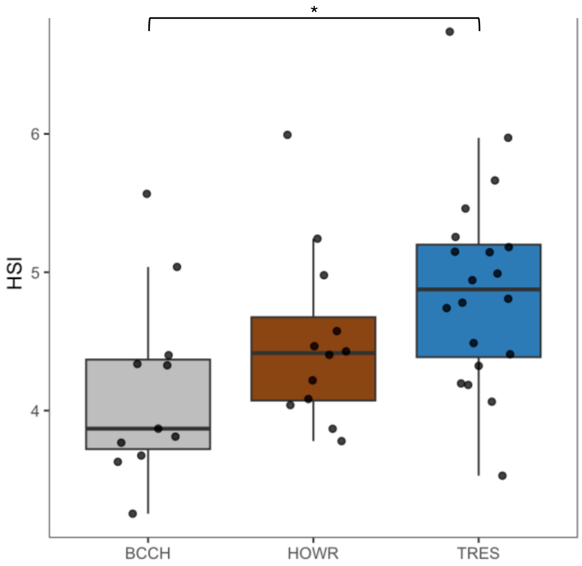

Figure 4. Nestling liver weights as percentage of body mass (HSI) for three Passerine species. HSI was significantly different across species (ANOVA, df = 38, p = 0.02). Tukey's HSD revealed significance for Black-capped Chickadees (BCCH) and Tree Swallows (TRES), but not for other comparisons of BCCH and House Wrens (HOWR) or HOWR and TRES. Significant differences among treatments denoted by asterisks: * = 0.05, ** = 0.01, *** = 0.001 (Tukey's HSD).

FMR and liver modeling

Linear regression was used to assess the relationship between total liver mass and FMR across species, with body mass included as a covariate. Liver mass was significantly negatively associated with metabolic rate ($p = 0.01$, adjusted $R^2 = 0.13$). This suggests that nestlings with larger livers tended to have lower metabolic rates (Figure 5).

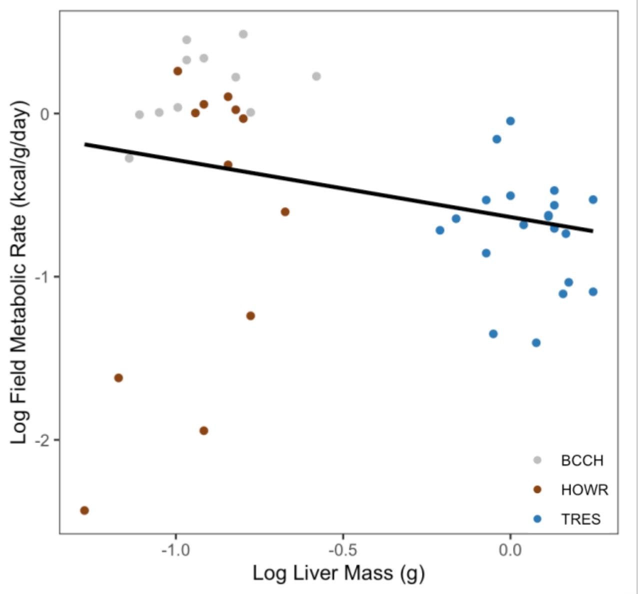

Figure 5. Nestling liver weights compared to field metabolic rates for three Passerine species. Liver mass was significantly negatively associated with metabolic rate ($p = 0.01$, adjusted $R^2 = 0.13$) when accounting for body mass. BCCH: Black-capped Chickadees; HOWR: Northern House Wrens; TRES: Tree Swallows.

Modelling and AICc was used to further assess the liver and FMR relationship between species, as well as provide insight into interspecific differences. Due to the fact mass and HSI are highly correlated (Pearon's correlation = 0.95), simple liver mass (i.e., not as a percentage of body weight) was used in univariate and multivariate models. The univariate models were allometrically fitted to variables and showed that liver mass ($p = 0.04$) and species identity ($p < 0.001$) are significant predictors of FMR while mass was not ($p = 0.13$). Liver mass had 2.17 ΔAICc units from null and species had 12.55 ΔAICc. While not significant, the coefficient of the mass model of - 0.25 is consistent ¾ scaling.

Log-transformed liver mass as used in multivariate models, and with using log(FMR) as a predictor variable, log(HSI) was implicitly tested. Moving forward with multivariate models showed that including liver and species as covariates improved the model fit (Table 1). It was found that the effect mass has on FMR is species dependent (Table 1, model 2). Including an interaction of mass and liver further improved model fit (Table 1, model 3) compared to a model without the interaction (Table 1, model 1). A model including both species and the interaction term was the best fitting model (Table 1, model 4; ΔAICc = 41.11).

Table 1. Models using Akaike Information Criterion corrected for small sample sizes (AICc) for allometrically fitted models that predict field metabolic rate (log(FMR)) of three passerine species. Nestlings' metabolic rates were measured with field respirometry for Black-capped Chickadees, Northern House Wrens, and Tree Swallows at 9-12dph.

Model	**df**	**AICc**	**ΔAICc**
Null	42	89.54	
Model 1	40	86.05	3.49
$\log(\mathrm{FMR}) = \beta 0 + \log(\mathrm{mass}) + \log(\mathrm{liver}) + \epsilon$			
Model 2	38	59.53	30.01
$\log(\mathrm{FMR}) = \beta 0 + \log(\mathrm{mass}) + \log(\mathrm{liver}) + \mathrm{species} + \epsilon$			
Model 3	39	66.28	23.26
$\log(\mathrm{FMR}) = \beta 0 + \log(\mathrm{mass}) + \log(\mathrm{liver}) + (\log(\mathrm{mass}) \times \log(\mathrm{liver}) + \epsilon$			
Model 4	37	48.43	41.11
$\log(\mathrm{FMR}) = \beta 0 + \log(\mathrm{mass}) + \log(\mathrm{liver}) + \mathrm{species} + (\log(\mathrm{mass}) \times \log(\mathrm{liver}) + \epsilon$			

Discussion

This study finds field respirometry a valid method in comparative physiology and provides insight into ecological and evolutionary differences of passerines. More specifically, this study provides support for the hypotheses that FMR and HSI differ among swallows, wrens, and chickadees. Furthermore, liver explains metabolic variation among swallow, wren, and chickadees beyond body mass alone.

Field metabolic rates

Field flow-through respirometry is a valid method for estimating metabolic rates in nestlings, as our measured values align with established literature (Figure 2). It is important to note that we used field respirometry measurements to estimate FMR that take these variables into account, rather than assuming we measured resting metabolism rate (RMR), which are typically measured during fasting and low energetic demands (Nichols et al. 1995). However, the close fit of the expected scaling and our data suggests that the estimations of RMR from field respirometry-measured FMR are valid.

Respirometry of swallows, wrens, and chickadees were measured at similar stages of development, and I found that FMR differed across these species (Figure 3) with swallows having the lowest mass-specific FMR followed by wrens and chickadees. Swallows and wrens had lower FMR than chickadees, which is expected based on the aforementioned scaling of body size and metabolism (McNab 2009). However, there was no significant difference found between swallow and wren FMR, which can be interpreted as evidence for factors that explain FMR beyond mass and aligns with previous work showing variation in mass-specific metabolic rates among organisms (Walsberg and King 1978, Mueller and Diamond 2001). Of the species measured, swallows are the most studied – largely due to their role as an ecological and ecotoxicological model species (Jones 2003) – and have nestling metabolism data with fairly similar methodology available for comparison to this study (Piepenburg 2016). Wren nestlings' FMR have been measured with a different but well-known method, doubly labelled water (Dykstra and Karasov 1993). The only published FMR studies for chickadees were measured on adults and often in a thermoregulatory context (Olson et al. 2010). This study provides valuable FMR measurements obtained with consistent methods that are more easily comparable to future research and applicable to energetics modeling that helps inform predictions about species resilience or vulnerability. Additionally, contributes to addressing gaps in the limited body of research comparing metabolic scaling across passerine species,

The large spread of FMR in wrens (Figure 3) may suggest a species-specific sensitivity disturbance. Wren nestlings are especially active during handling and are most likely to prematurely fledge when the nest box is opened. While there is anecdotal evidence of capture sensitivity of wrens in other studies (Lothery et al. 2014), it is common for wren adults to desert and destroy nests (Freed 1981, Belles-Isles and Picman 1986). There were technical issues with the respirometer for the 2024 season, which impacted sample size. An increased sample size in future work could either reduce the variability of data or provide further evidence for increased disturbance sensitivity in HOWR. Additionally, swallows were measured in 2023 while wrens and chickadees were measured in 2024. Future work could obtain interspecific respirometry measurements within the same field seasons.

Liver weight

Swallows, wrens, and chickadees had species-specific variation in HSI and that the differences seemed to be inversely related to MR (Figure 4) with swallows having the highest HSI followed by wrens and chickadees. Wrens, however, did not have a significant difference in HSI compared to either species. When considering the insignificant difference in wren and swallow FMR values, an insignificant difference in HSI as well may suggest that their FMR is similar because their HSI values are similar. AICc results indicate that models incorporating HSI do not distinguish between swallow and wren FMR, and chickadees differed in both metabolic rate and HSI. In other words, HSI may help to explain why swallow metabolic rates do not differ from those of wrens. Liver weight was found to be different between temperate and tropical birds of the same mass (Jimenez et al. 2014), suggesting there may be differences among species that reflect evolutionary history in this context as each of the study species here belong to different families (swallows to Hirundinidae, wrens Troglodytidae, and chickadees Paridae). This study did not further investigate if ecological characteristics like migratory status or trophic level explains variation, but future work in comparing additional species (perhaps within similar families) would improve understanding of how species-specific ecological and physiological differences shape differences in FMR.

Metabolism & liver weight

It was found that liver weight and FMR were correlated (Figure 5) and liver explains FMR better than body mass alone in models that do not include species identity. However, in additional models (Table 1) that included species identity, it was found that species may be incorporating much of the variation originally attributed to liver in the univariate model. The best-fitting model including an interaction of mass and liver, with species as an additional covariate (model 4, ΔAICc = 41.11), but including mass and liver alone still improved model fit (ΔAICc = 3.49). In model 4, the slope was very high (y =. 4.97), suggesting that FMR univariate model with mass alone showed a -0.25 coefficient, which while not significant, is as expected with a ¾ allometric scaling assumption.

Further, species is the best fitting univariate model with liver improving model fit better than mass alone, which suggests liver may be explaining a mechanistic driver of FMR that species identity is incorporating.

In a study of the changing body composition of Red Knot (*Calidris canutus islandica*) during migration, it was found that the mass of organs associated with nutrient extraction (including the liver) was correlated with mass gain (Piersma et al 1999). Body mass and composition are considered morphometric indices of body condition (Labocha and Hayes 2012), a metric for that relates to an individual's survival, reproduction, and behavior. These results show a relationship between FMR and Liver, and with organ weight being related to body composition, so FMR may be used as a measure of overall condition. The use of field respirometry for studies as a proxy of condition may therefore be a valuable, nondestructive alternative. Future studies directly measuring both FMR and body condition indices could further validate these findings.

Implications & conclusions

In providing evidence for interspecific variation in, and correlation of, FMR and liver weight among three passerine species (Tree Swallows, Northern House Wrens, and Black-capped Chickadees), this study contributes to the field of organismal and comparative physiology. Specifically, my study suggests that these species show deviations in FMR that is not explained by mass alone, and that liver weight may be a driving factor. Furthermore, FMR may be indirectly linked to body condition and therefore field respirometry could be a valuable, nondestructive alternative in physiological studies that assess the condition of individuals. Further work that includes more individuals within a single season may be beneficial to solidify conclusions made here, as well as investigating comparisons between more species to investigate whether other factors (ecological or physiological) may drive interspecific variation of FMR. This study and the purposed future work would be important for assessing risk to individuals and populations.

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