Metagenomics Reveals Trophic Transfer of Hg in Songbirds in Acadia National Park

by

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ABSTRACT

Mercury is a toxic pollutant which is spread around the earth through both natural and anthropogenic dispersion. Further investigation into the flow of mercury through riparian ecosystems is needed. Studies show that adult breeding songbirds are effective indicators of mercury in terrestrial ecosystems. The objective of this study was to investigate the trophic transfer of mercury through songbird diet analysis using DNA meta-barcoding paired with songbird blood mercury. We collected songbird fecal and blood samples in June and July of 2018 from two sites on Mount Desert Island in Acadia National Park, Maine. At Hunter's Brook (n = 8), Hg concentrations in songbird blood ranged from 149 – 297 ppb wet weight. At Marshall Brook (n = 8), Hg concentrations in songbird blood ranged from 29 – 336 ppb wet weight. Data show a correlation between a diet of insects and songbird blood mercury levels. Next generation sequencing of songbird fecal samples revealed that songbird diets consisted mostly of the order Coleoptera at both Marshall Brook and Hunter's Brook sites.

INTRODUCTION

Mercury is a toxic pollutant that is spread around the earth through both natural and anthropogenic deposition. Natural modes in which mercury is scattered include volcanic eruptions, forest fires, and degassing from water surfaces. Mercury is introduced into the atmosphere by humans through gold mining (Mitchell et al., 2008), as well as the combustion of coal and biomass (Zhang et al. 2018). These particulates of mercury are then transported by wind, resulting in contaminated watersheds through the processes of precipitation and deposition (Morel et al. 1998). Wetlands, superficial sediments, and anoxic waters provide physical and biological conditions in which the methylation of mercury can occur (Windham-Myers et al. 2014). Methylmercury (MeHg) is organic and therefore has a greater bioavailability, trophic biomagnification, and overall effects on wildlife than its elemental form (Eagles-Smith et al. 2016).

Because MeHg has high bioavailability, exposure to wildlife predominantly occurs through trophic transfer (diet). As low trophic organisms are exposed to environments where methylation occurs, the organic mercury can become integrated into their biomass. This mercury is then assimilated into their predator's tissue through digestion. Moving up the food chain, the assimilation of mercury is preserved (Ackerman et al., 2015). This process of bioaccumulation concentrates mercury in organisms of higher trophic levels, as they are inherently exposed to the toxins of both primary and secondary prey items (Cristol et al. 2008). Because MeHg is eliminated slowly, high trophic organisms can have mercury concentrations many orders of magnitude higher than primary consumers (Wolfe et al., 1998).

Adult breeding songbirds (of the order Passeriformes) have been shown to be effective sentinels of mercury in terrestrial ecosystems due to their integration and bioaccumulation of MeHg from their prey items (Tsui et al. 2018). Mercury can have both lethal and sub-lethal effects on songbirds including altered song quality (Hallinger et al., 2010), decreased flight performance (Carlson et al., 2014), decreased reproductive success (Varian-Ramos et al., 2014), nervous system dysfunctions (Wolfe et al., 1998) and immunological disruption (Wada et al., 2009). These effects ultimately lower the fitness of songbirds who have been exposed to mercury.

Diet analysis is paramount to understanding the flow of mercury through an ecosystem. Bird diet has been evaluated using several methods, including manual identification of stomach contents (Williams & Batzli, 1979) or fecal matter (Hagar et al., 2012), visual observations of foraging (Nakano & Murakami, 2001), and stable isotope analysis of blood, breath, or tissue samples (Hatch et al., 2002; Herrera et al., 2003). In this study, DNA meta-barcoding will be used to identify songbird diet items.

DNA sequencing technology has drastically changed in the past 15 years (Valentini et al. 2009). Next generation high-throughput sequencing technology has allowed ecologists to utilize non-invasive molecular techniques to answer questions regarding trophic ecology (Vesterinen et al. 2013). Previous studies have shown that it is possible to obtain high quality DNA from avian fecal samples, amplify barcode genes, and reference those sequences back to representative databases to identify prey items up to the species level (Vo & Jedlicka, 2014). While many barcode genes exist (Jedlicka et al., 2013), the cytochrome c oxidase subunit 1 (CO1) mitochondrial gene is a popular meta-barcoding region in arthropods due to its small size, high conservation amongst taxa, and its interspecific variation (Hebert et al. 2003; Rach et al. 2017). The objective of this study was to investigate the trophic transfer of mercury in two sites in Acadia National Park, Maine through DNA meta-barcoding diet analysis of songbirds and songbird blood mercury.

METHODS

Study Area

Our two sample sites are streams located on Mount Desert Island in Acadia National Park, Maine. Marshall Brook (44.28498, -68.346107) is a stream surrounded by wetlands with dragonfly larvae presenting high Hg levels (462 ppb on average over three years). Hunter's Brook (44.30064, -68.218417) is a fast, clear, cold stream with dragonfly larvae presenting low Hg (283 ppb on average over three years) (Dragonfly Mercury Project, U.S. National Park Service). The source of mercury pollution is unknown for both sites, but is most likely atmospheric deposition from the Midwest (Driscoll et al., 2013). See Figure 1 for an aerial image of the location of both sites. See Figure 2 for the Dragonfly mercury project data for each site.

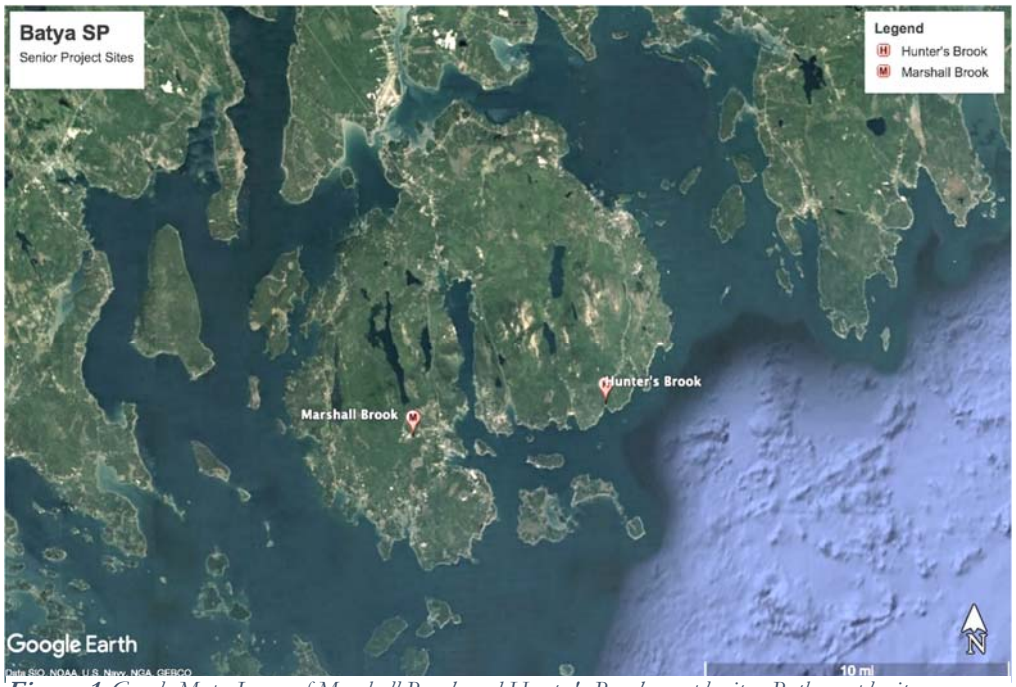

Figure 1*. Google Maps Image of Marshall Brook and Hunter's Brook sample sites. Both sample sites are located on Mount Desert Island in Acadia National Park, Maine.*

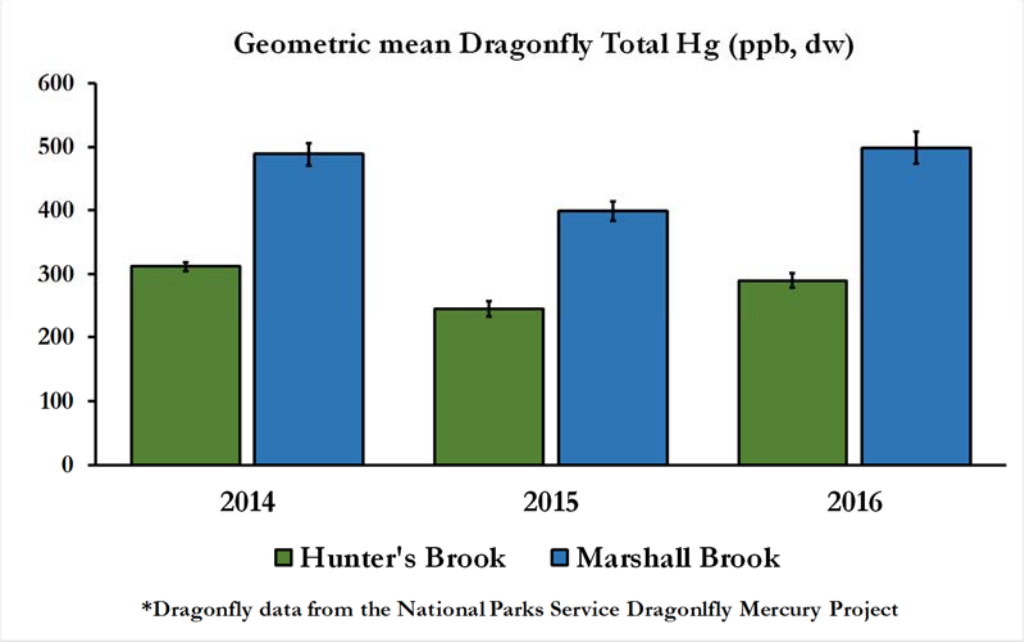

Figure 2. *Mean dragonfly nymph total mercury (ppb dw) comparing Hunter's Brook and Marshall Brook data over three years.*

Sampling Regime and Mercury Analysis

In the months of June and July 2018, songbirds were captured using mesh mist nets at each site and identified by external characteristics. Playback recordings of conspecific songs were used to capture the songbirds in the mist nets. As birds were captured, they were placed in cloth bags for blood and stool collection. The cloth bags were sanitized between collections by soaking for 24 hours in a bleach solution. Fecal samples were stored fully submerged in 95% ethanol and kept cool in the field until frozen at -20˚C for long term storage before analysis. Blood samples of each bird were taken from the brachial ulnar vein, using 27 gauge needles (BD PrecisionGlide, Fisher Scientific) and heparinized microhematocrit capillary tubes (Fisherbrand, Fisher Scientific). Samples were capped with Critocaps™ (Leica Microsystems) and stored in sealed plastic bags on ice in the field until they could be transferred to a freezer at -20˚C (within 6 hours of sampling). No more than 1% of a bird's body weight of blood was collected from each individual, usually between 20 µl and 100 µl. All samples were collected under authority of appropriate scientific collection permits, including: USGS master bander permit to Allyson Jackson #24142, National Park Service Scientific Research and Collecting Permit #ACAD-2018-SCI-0018, State of Maine Department of Inland Fisheries and Wildlife Scientific Collection Permit 2018-536, and Purchase College Institutional Animal Care and Use Permit #2018-AJ01. Songbird blood samples were run on a Nippon MA-3000 Hg Analyzer (Nippon Instruments, College Station, Texas, USA) at Weber State University.

Metagenomics Sequencing

Ethanol was removed from fecal samples with an Eppendorf vacufuge on V-AL mode. The wet weights of each fecal sample were recorded. A combination of QIAamp DNA Stool Kit (Qiagen) and Quick-DNA Fecal/Soil Microbe Microprep Kit (Zymo) were used to extract DNA from the fecal samples. For extraction with the Qiagen kit, DNA isolation followed Crisol-Martínez et al. 2016 with modifications from Zeale et al. 2011 for EtOH sample preservation. These modifications included treatment with proteinase K. Default manufacturer's protocols were followed for isolations with the Zymo Microprep kit. Following isolation, DNA was quantified with a Qubit fluorometer with the accompanying 1X dsDNA HS Assay Kit (Thermo Fisher Scientific).

A number of different PCR master mixes, primers, and amplification conditions were used to amplify various barcode genes from the fecal samples. See appendix for more information regarding amplification experimentation. The extracted DNA samples were pooled by site, prepared into a 5x dilution, and amplified by PCR with AmpliTaq GOLD 360 Master Mix (Thermo Fisher Scientific) and mlCOIintF (Leray et al., 2013) and jgHCO2198 (Geller et al., 2013) primers for a 333 bp section of the CO1 barcode gene. 25 μl reactions were prepared with 12.5 μl of master mix, 1 μl of forward primer, 1 μl of reverse primer, and a total 10.5 μl of DNA and nuclease free distilled H_2O. PCR conditions were as follows: 95°C for 10 minutes, 38 cycles of: 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for one minute, with a final extension at 72°C for 7 minutes.

Amplification efficiency was visualized with gel electrophoresis on 2% agarose gels. Gels were prepared with 1 gram of Molecular Biology Agarose and 50ml of 1x TAE buffer (Bio Rad). The agarose and buffer were microwaved in a 250 ml Erlenmeyer flask for a total of 90 seconds, stirring at 30 second intervals until the solution became clear and no strands of agarose could be seen. The gel solution was left to cool until the flask reached a temperature that could be handled. 5 μl of Midori Green Advance DNA Stain (Nippon Genetics) was added to the gel solution and stirred in before the gel was poured. Each gel was loaded with a ladder in the first well and a combination of 5 μl of PCR product and 1 μl of Nucleic Acid Loading Buffer 5x (Bio Rad) in subsequent wells. Both EZ Load 1kb Ladder (Bio Rad) and 100 bp DNA Ladder (Thermo Fisher Scientific) were used. Each gel was run at 120 volts for approximately 30 minutes or until the

loading dye had reached halfway down the gel. Gels were visualized on an Embitec Sapphire Blue LED Illuminator.

Sequencing was done with an Oxford Nanopore Technologies (ONT) MinION sequencer with the PCR barcoding kit (SQK-PBK004) and a MIN106 flow cell with R9.4 chemistry (Oxford Nanopore Technologies). Each pooled site was given a separate barcode. Sequencing was performed on March 22, 2019 and April 2, 2019. The MinION was run for 24 hours on both sequencing days. Two barcodes were used for the Hunter's Brook site, and three barcodes were used for Marshall Brook.

Bioinformatics

Guppy v2.3.7 basecaller and barcoder were used for basecalling and demultiplexing. Barcodes and adapters were removed with porechop v0.2.3. Reads with quality scores below 8 or lengths below 100 bp were filtered with NanoFilt v2.2.0. The reads for each site were put through a minimap2 v2.16 (Li, 2018) and racon v1.3.2 (Vaser et al., 2017) pipeline, where the reads for each site were mapped to each other and corrected with racon. The corrected reads were then mapped back to the original reads and corrected for a second time. These twice corrected sequences were used in downstream analysis. A database was created from COI nucleotide sequences from NCBI. The consensus sequences were then BLASTed (BLASTn v2.9.0 Altschul et al., 1990) against the COI database. The blast files were imported into MEGAN6 (Huson et al., 2016) and assigned to taxa with the weighted LCA algorithm, covering 80% of reads. Only reads that had expected values below 1E-20 and pairwise percent matches of 97 or above were considered as present. Diet taxa were considered either present or absent by site and relative abundances of prey items were not calculated. Diet was analyzed by diversity of prey items by family, with the exception of arachnids, which were assigned to a single class.

Statistical Analysis

All statistical tests were done with R version 3.5.3 "Great Truth" (R Core Team, 2019). SeqKit v0.10.0 was used for sequence statistics (Shen et al., 2016), and FastQC v0.11.8 was used for sequence visualizations (Andrews, 2014). Percent insectivore data was collected from papers found in the Cornell Lab of Ornithology Birds of North America website (https://birdsna-org.bnaproxy.birds.cornell.edu), where each species was assigned a percentage insectivore diet based on breeding period data.

RESULTS

Sampling and Study Area

A total of 20 individual songbirds belonging to 10 species were used for this study. A total of 16 fecal samples and 16 blood samples were collected. See Table 1 for a complete breakdown of songbird species and mercury levels.

Site	Species Code	Common Name	Name	Family	Blood Hg (ppb ww)
Hunter's Brook, Acadia	AMRE	American Redstart	Setophaga ruticilla	Parulidae	297
Hunter's Brook, Acadia	BTNW	Black Throated Green Warbler	Setophaga virens	Parulidae	247
Hunter's Brook, Acadia	BTNW	Black Throated Green Warbler	Setophaga virens	Parulidae	266
Hunter's Brook, Acadia	BTNW	Black Throated Green Warbler	Setophaga virens	Parulidae	185
Hunter's Brook, Acadia	SOSP	Song Sparrow	Melospiza melodia	Passerellidae	163
Hunter's Brook, Acadia	RBNU	Red-Breasted Nuthatch	Sitta canadensis	Sittidae	182
Hunter's Brook, Acadia	RBNU	Red-Breasted Nuthatch	Sitta canadensis	Sittidae	149
Hunter's Brook, Acadia	SWTH	Swainson's Thrush	Catharus ustulatus	Turdidae	297
Marshall Brook, Acadia	CEDW	Cedar Waxwing	Bombycilla cedrorum	Bombycillidae	29
Marshall Brook, Acadia	CEDW	Cedar Waxwing	Bombycilla cedrorum	Bombycillidae	36
Marshall Brook, Acadia	BCCH	Black-capped Chickadee	Poecile atricapillus	Paridae	107
Marshall Brook, Acadia	COYE	Common Yellowthroat	Geothlypis trichas	Parulidae	104
Marshall Brook, Acadia	COYE	Common Yellowthroat	Geothlypis trichas	Parulidae	336
Marshall Brook, Acadia	YEWA	Yelllow Warbler	Setophaga petechia	Parulidae	83
Marshall Brook, Acadia	SWSP	Swamp Sparrow	Melospiza georgiana	Passerellidae	138
Marshall Brook, Acadia	SOSP	Song Sparrow	Melospiza melodia	Passerellidae	50

Table 1*. List of sampled songbirds with data including: site, species code, common name, scientific name, family, and blood mercury levels (ppb ww).*

Sequencing Results

See Figures 3, 4, 5, 6, and Table 2 for information relating to sequence lengths, quality scores, and quantities before and after quality control and consensus calling.

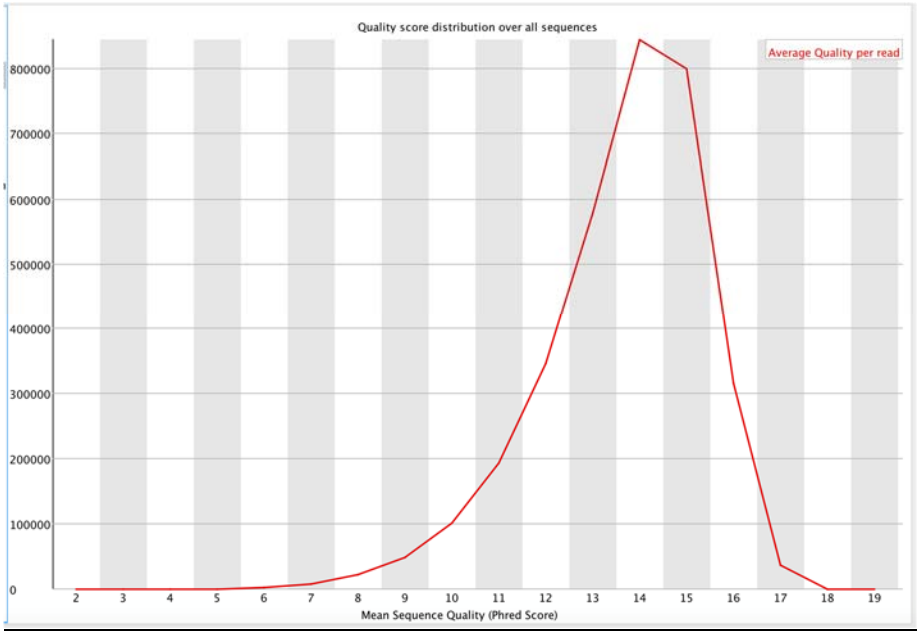

Figure 3. *Quality score distribution over all sequences. Graph made in FastQC.*

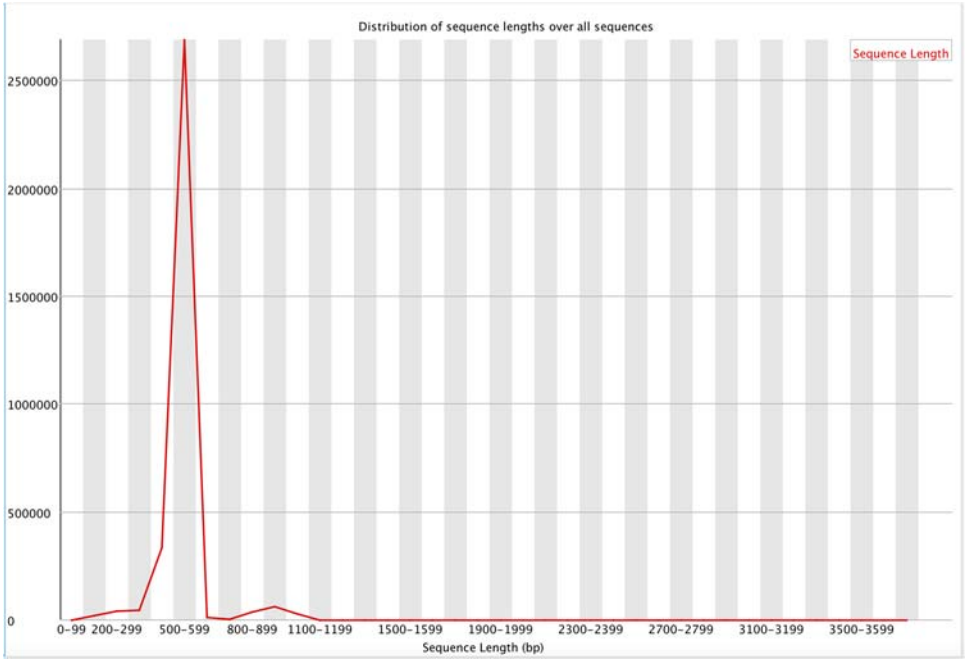

Figure 4*. Distribution of sequence lengths over all sequences. Graph made with FastQC.*

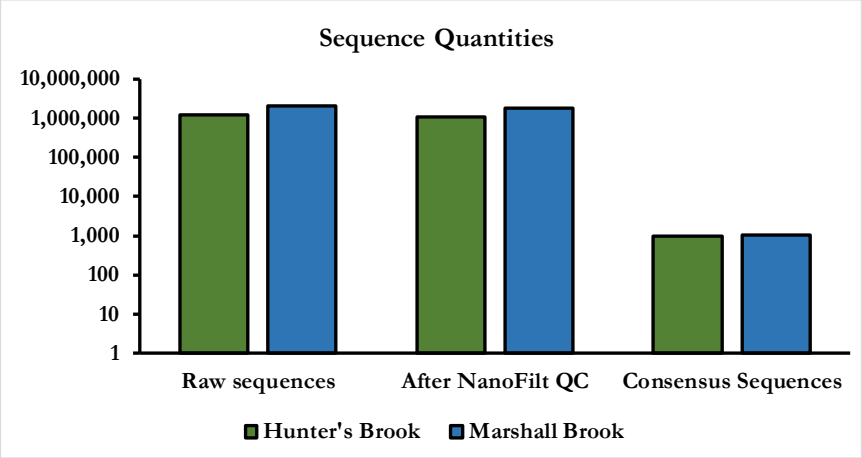

Figure 5. *Sequence Quantities by site before quality control, after NanoFilt, and after Minimap2 and Racon.*

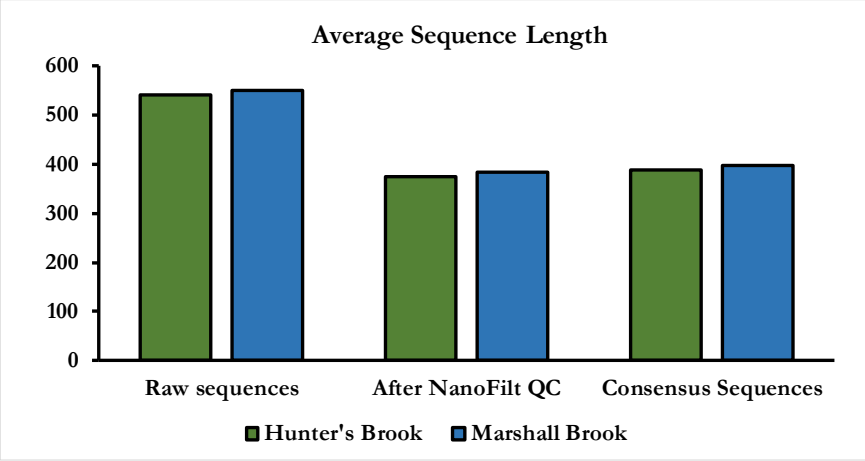

Figure 6. *Sequence Lengths by site before quality control, after NanoFilt, and after Minimap2 and Racon.*

	Hunter's Brook			Marshall Brook		
	Raw sequences	**After NanoFilt QC**	**Consensus Sequences**	**Raw sequences**	**After NanoFilt QC**	**Consensus Sequences**
Quantity	1,230,305	1,081,174	979	2,073,165	1,808,804	1,065
Average Length	541	374	388	550	383	398
Length Range	46-2902	100-2690	143-1138	48-3772	100-2535	137- 1519

Table 2. *Quantities, Average Lengths, and Length Ranges of sequences by site before quality control, after NanoFilt, and after Minimap2 and Racon.*

Diet Taxonomy

Overall, songbird diet items from 13 taxa were identified. See table 3 for a complete list of diet items. The overwhelming majority of songbird diet consisted of beetles, with over 80% of diet species assigned to the order Coleoptera. See Figure 8 for a chart of diet diversity by family. The Shannon-Weaver diversity of diet items was 0.257 and 2.244 for Hunter's Brook and Marshall Brook respectively. One unclassified arachnid was assigned to species level at Hunter's Brook, and two unclassified arachnid species were assigned to Marshall Brook. See Table 4 for the top 10 BLAST results for each site. 18 unique accessions were identified at Hunter's Brook, and 51 unique accessions were identified at Marshall Brook. See Figure 7 for a rarefaction curve of sequences by species.

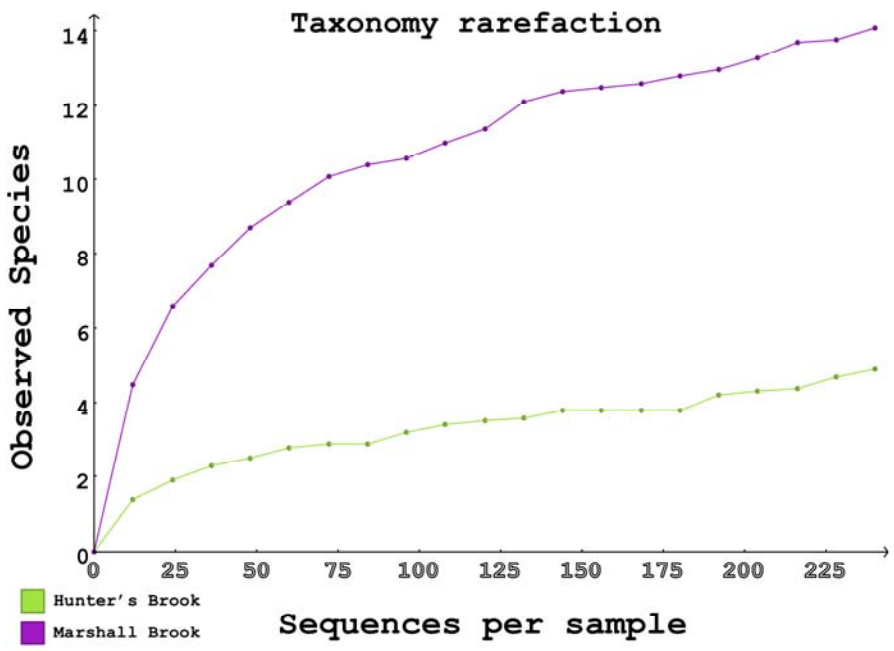

Figure 7*. Taxonomy rarefaction graph by site. Graph made in MEGAN6.*

Songbird Diet Items by Site

Hunter's Brook	Common Name	Family
Isomira quadristriata	Comb-clawed Beetle	Tenebrionidae (Darkling Beetles)
Curculionidae sp. BOLD:ACL9794	True weevil	Curculionidae (Snout and Bark Beetles)
Otiorhynchus singularis	Raspberry Weevil	Curculionidae (Snout and Bark Beetles)
Arachnida sp. BOLD:ACM8542	unclassified Arachnid	unclassified Arachnid
Agriotes fucosus	Click Beetle	Elateridae (Click Beetles)
Sciaphilus asperatus	Broad-nosed Weevil	Curculionidae (Snout and Bark Beetles)
Zonotrichia albicollis*	White-throated Sparrow	Pasillerdae (songbird)

Marshall Brook	Common Name	Family
Isomira quadristriata	Comb-clawed Beetle	Tenebrionidae (Darkling Beetles)
Curculionidae sp. BOLD:ACL9794	True weevil	Curculionidae (Snout and Bark Beetles)
Otiorhynchus singularis	Raspberry Weevil	Curculionidae (Snout and Bark Beetles)
Arachnida sp. BOLD:ACM8542	unclassified Arachnid	unclassified Arachnid
Oedancala dorsalis	seed bug	Pachygronthidae
Arachnida sp. BOLD:ACK4438	unclassified spider	unclassified Arachnid
Anthribus nebulosus	fungus weevil	Anthribidae (Fungus Weevils)
Plateumaris germari	aquatic leaf beetle	Chrysomelidae (Leaf Beetles)
Trachysida mutabilis	Flower longhorn beetle	Cerambycidae (Long-horned Beetles)
Trachysida sp. BOLD:AAB7401	Flower longhorn beetle	Cerambycidae (Long-horned Beetles)
Polydrusus cervinus	Broad-nosed Weevil	Curculionidae (Snout and Bark Beetles)
Sitta canadensis*	Red-breasted Nuthatch	Sittidae (songbird)
Geothlypis trichas*	Common Yellowthroat	Parulidae (songbird)
Melospiza georgiana*	Swamp Sparrow	Passerellidae (songbird)
Poecile atricapilla*	Black-capped Chickadee	Paridae (songbird)
Bombycilla cedrorum*	Cedar Waxwing	Bombycillidae (songbird)
Homo sapiens[α]	Human	Hominidae
Peromyscus leucopus[α]	White-footed mouse	Cricetidae

Table 3. *Songbird Diet Item, common name, and family by site. Items marked with * were determined to be host species. Items marked with a were determined to be contaminants.*

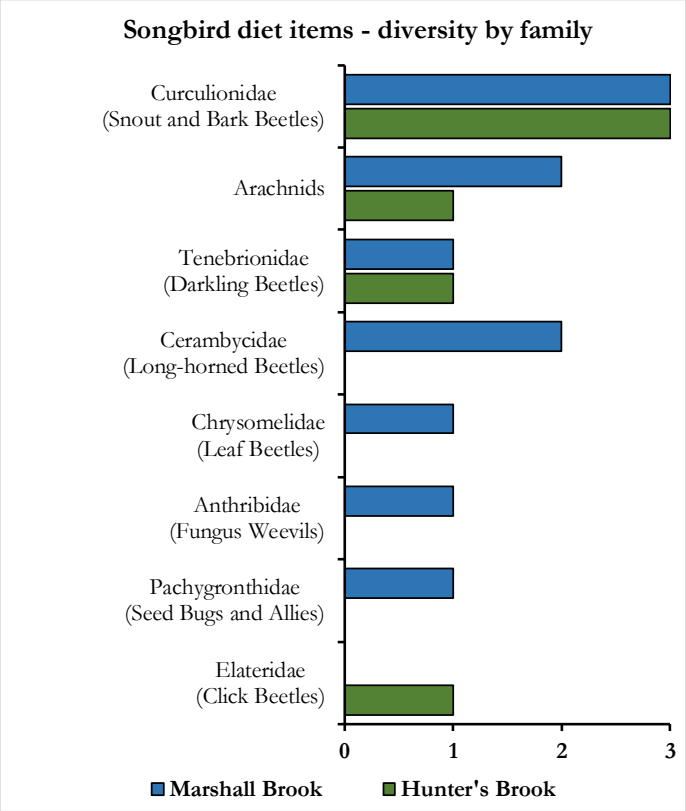

Figure 8*. Songbird diet diversity by family and site.*

Hunter's Brook Top 10 Blast Results							
Accession	**Species**	**Pairwise Identity**	**Length**	**Mismatch**	**E-value**	**Bitscore**	**Gaps**
KJ965711.1	Otiorhynchus singularis	100.0	306	0	1.66E-159	566	0
KU909975.1	Otiorhynchus singularis	99.4	317	0	1.11E-161	573	2
MG058796.1	Agriotes fucosus	98.5	342	3	1.45E-170	603	2
KR485149.1	Isomira quadristriata	98.5	341	3	1.12E-169	601	2
KR102538.1	Arachnida sp. BOLD:ACM8542	97.9	239	3	2.6E-113	412	2
KR121182.1	Curculionidae sp. BOLD:ACL9794	97.8	272	4	5.43E-130	468	2
KJ962310.1	Sciaphilus asperatus	97.6	335	6	1.14E-161	573	2
DQ434238.1	Zonotrichia albicollis	97.4	341	8	2.38E-163	579	1
JN034120.1	Homo sapiens*	96.6	356	10	1.07E-166	590	2
KM845995.1	Ctenicera resplendens*	96.5	315	11	4.06E-146	521	0

Marshall Brook Top 10 Blast Results							
Accession	**Species**	**Pairwise Identity**	**Length**	**Mismatch**	**E-value**	**Bitscore**	**Gaps**
KM441656.1	Polydrusus cervinus	99.7	314	0	1.16E-161	573	1
KJ965711.1	Otiorhynchus singularis	99.7	313	0	4.02E-161	571	1
KR102538.1	Arachnida sp. BOLD:ACM8542	99.1	218	2	3.07E-107	392	0
KM846222.1	Plateumaris germari	99.0	291	1	1.41E-145	520	2
JF456988.1	Peromyscus leucopus	98.8	340	2	3.98E-171	604	2
KU909975.1	Otiorhynchus singularis	98.7	318	0	2.41E-158	562	4
KR121182.1	Curculionidae sp. BOLD:ACL9794	98.5	267	2	1.6E-130	470	2
KR096491.1	Arachnida sp. BOLD:ACK4438	98.4	306	5	4.07E-151	538	0
KR485149.1	Isomira quadristriata	98.2	341	5	2.61E-168	595	1
KR032566.1	Oedancala dorsalis	98.2	341	5	2.37E-168	595	1

Table 4. *Top ten BLAST results by site. Items marked with * did not meet identification criteria.*

Comparative Analyses

Songbird blood mercury concentrations were significantly greater in Hunter's Brook ($t(15)=2.76$, $p=0.01$ Fig. 9). ANOVA tests comparing bird blood mercury concentrations by songbird species and family were not significant (see Figures 11 and 12) ($F=1.9$ $p=0.2$, $F=2.5$ $p=0.09$ respectively). Four diet species were unique to Marshall Brook, while only one unique species was found in Hunter's Brook. A linear regression identified a significant positive correlation between songbird insectivory during breeding season and blood mercury levels ($r=0.4272$, $p=0.001$, Fig. 10).

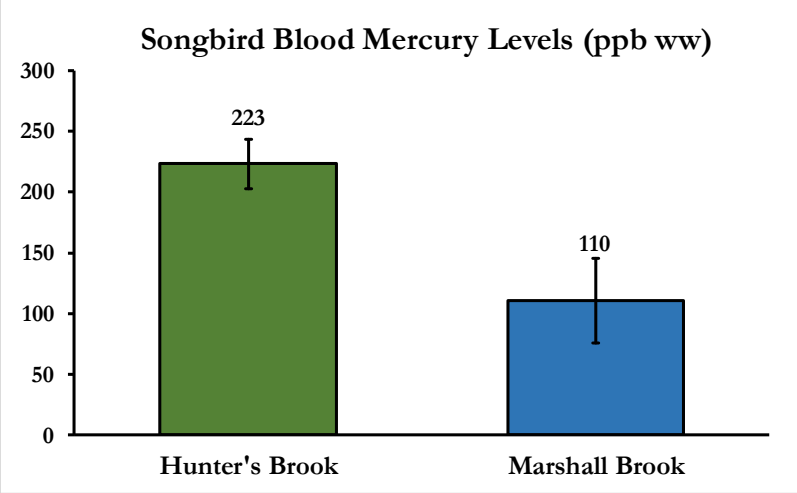

Figure 9. *Songbird blood mercury levels (ppb ww) by site.*

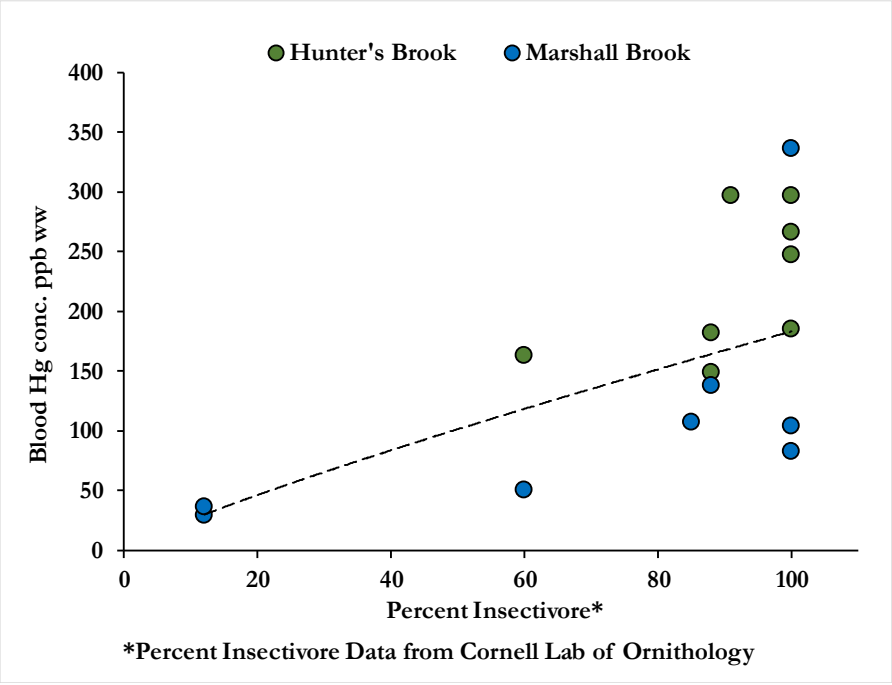

Figure 10*. Percent Insectivore vs. Songbird blood mercury (ppb ww).*

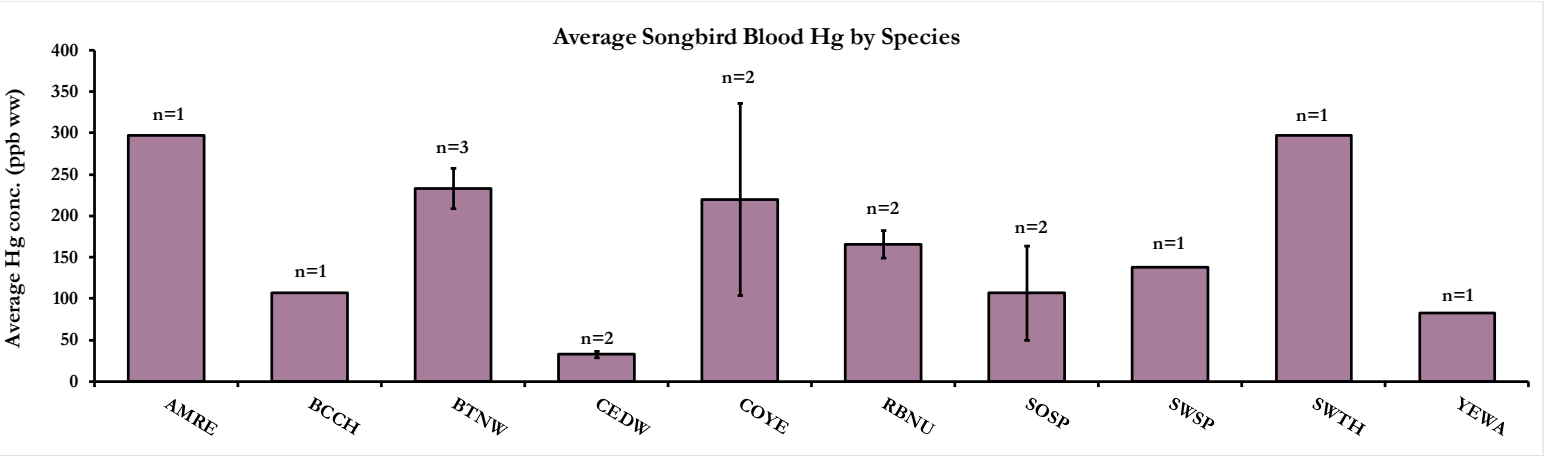

Figure 11. *Average Songbird blood mercury (ppb ww) by songbird species.*

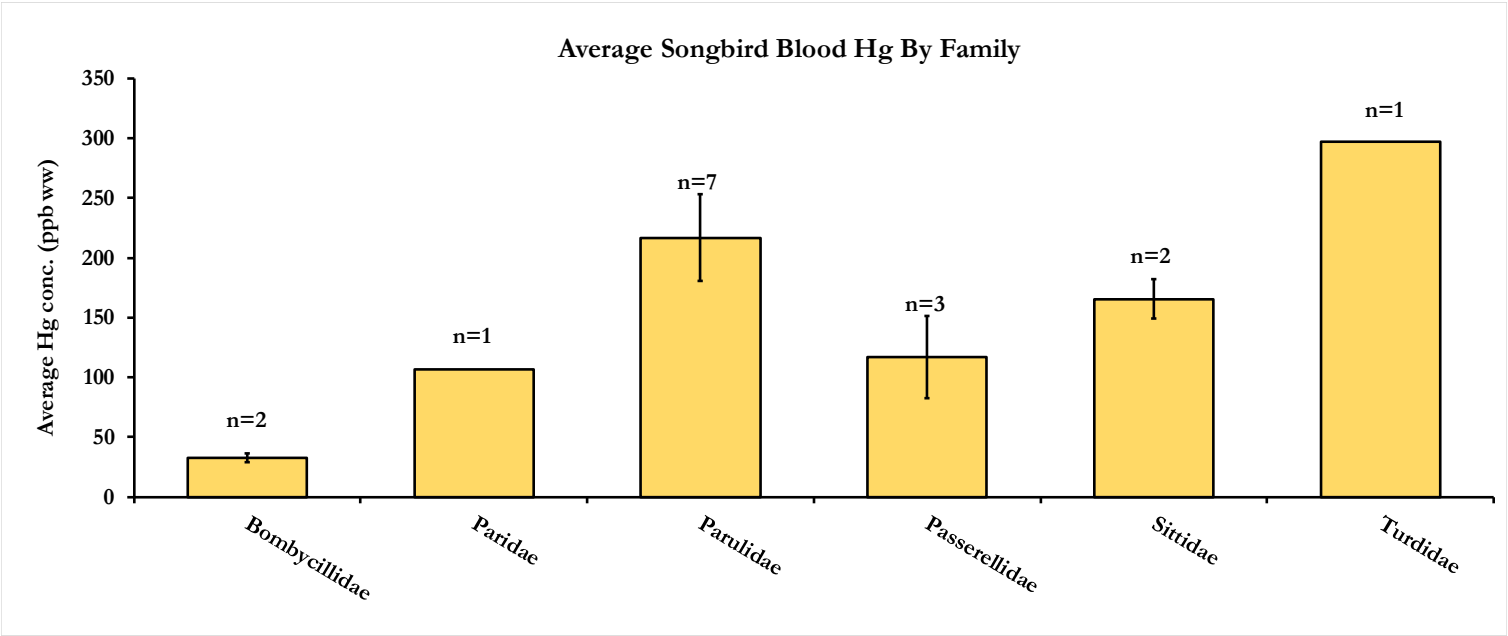

Figure 12. *Average Songbird blood mercury (ppb ww) by songbird family.*

DISSCUSION

Mercury

While ANOVA tests did not show a significant difference in songbird blood mercury levels by bird species or family, this may be due to small sample size. Likewise, fecal DNA extracts were pooled by site and statistical tests showed no significant difference in songbird blood mercury by diet items. We were able to identify two Arachnids in the diet of songbirds at both sites (1 at Hunter's Brook, and 2 and Marshall Brook), however we could not investigate the trophic level of these diet items because both accessions were not identified to species level in BOLD or the NCBI nucleotide database.

The source of discrepancy between the National Park Service Dragonfly Mercury Project data, which show Marshall Brook having consistently higher dragonfly nymph mercury levels than Hunter's Brook, and our songbird blood mercury showing songbird blood mercury concentrations at Hunter's Brook to be significantly higher than those at Marshall Brook is unclear. We were unable to identify any emergent aquatic insects in our songbird diet analysis, which may suggest that the mercury from the water at Marshall Brook was not reaching the songbirds during the months of our sampling (June and July). Studies have shown that birds subsidize terrestrial prey with aquatic prey in the winter months (Nakano & Murakami, 2001), which would call for songbird sampling over seasons to track terrestrial vs. aquatic prey items. Alternatively, Hunter's Brook proximity to the ocean may suggest marine mercury subsidies at that site.

The mean songbird blood mercury levels of species from this study are consistent with data from the Atlantic Highlands, Appalachian Forest and Atlantic mixed woods ecoregions across the northeast (Jackson et al., 2015; Rimmer et al., 2005). Using risk assessment criteria from Ackerman et al., 2016, all songbird blood mercury levels fit in the categories of no to low risk of adverse effects of mercury exposure. While all songbirds sampled can be categorized as no to low risk, over 30% of our samples are over the lowest mercury levels of documented effects (0.2 ppm ww) (Custer et al., 2000). This suggests that the songbirds at Hunter's Brook and Marshall Brook are not experiencing a significant mercury load compared to other songbirds in the northeastern United States, but still may be facing adverse physiological effects due to mercury exposure.

Songbird Diet Analysis and Fecal DNA Meta-barcoding

Depending on methods, PCR inhibitors could be co-extracted during DNA isolation steps (Jedlicka et al., 2013). This may require cleaning steps like DNA precipitations or dilutions for barcode bands to be visualized on gels. Due to the potential taxonomic bias of PCR (Clarke et al., 2014), any read counts reported for meta-barcoding of fecal samples may not be representative of the true ratios of diet taxa. Due to the nature of digestion, soft bodied diet items may be under reported or may be too degraded to be picked up by PCR (Fokidis et al., 2012), which may lead to false negatives. The sequencing taxonomy rarefaction curve (Fig. 7) did not reach an asymptote, indicating that our results are representative of only a portion of songbird diet items. This is most likely due to our bioinformatics pipeline being too conservative, rather than a need for deeper sequencing.

Other studies that used bird fecal DNA meta-barcoding to identify diet items (Wong et al., 2014; Crisol-Martínez et al., 2016; Jedlicka et al., 2013; Trevelline et al., 2016) were able to classify a range of 48-108 diet species, with an average of 1.4 diet species per fecal sample. In this study, we classified an average of 0.8 diet species per fecal sample. Many factors can influence this ratio including species identification criteria. Our conservative Minimap2 and racon bioinformatics pipeline most likely over corrected our reads resulting in artificially reduced prey diversity.

Future Research

Bird fecal samples can show the past 30mins - 4hrs of bird diet (Oehm et al., 2011), however re-sampling after 50 minutes can show dissimilar diets (Jedlicka et al., 2016), which calls for variation in sampling time throughout the day. Other studies show that bird diet can reflect insect phenology (Orłowski et al. 2014), calling for sampling throughout seasons according to research questions. More recent studies suggest the use of stable sulfur isotopes for detection of marine diet items. Further research is needed to determine the presence of sulfur and sulfur reducing microbes in bird habitats. Sulfur reducing microbes may correlate with mercury methylation, or the genes required for mercury methylation in the gut microbiomes of songbirds (Elliott & Elliott, 2016; Parks et al., 2013; Podar et al., 2015). Finally, a meta-barcoding bioinformatics pipeline is needed which addresses the MinION's relatively high error rate (Laver et al., 2015; Rang et al., 2018) while accurately maintaining community assemblages.

REFERENCES

Ackerman, J. T. ., jackerman@usgs. go., Hartman, C. A., Eagles-Smith, C. A. ., Herzog, M. P. ., Davis, J., Ichikawa, G., & Bonnema, A. (2015). Estimating Mercury Exposure of Piscivorous Birds and Sport Fish Using Prey Fish Monitoring. *Environmental Science & Technology*, *49*(22), 13596–13604. doi: 10.1021/acs.est.5b02691

Ackerman, J. T., Eagles-Smith, C. A., Herzog, M. P., Hartman, C. A., Peterson, S. H., Evers, D. C., … Bryan, C. E. (2016). Avian mercury exposure and toxicological risk across western North America: A synthesis. *Science of the Total Environment*, *568*, 749–769. doi: 10.1016/j.scitotenv.2016.03.071

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410. doi: 10.1016/S0022-2836(05)80360-2

Andrews, S. (2014). FastQC A Quality Control tool for High Throughput Sequence Data. (Version 0.11.8). Retrieved from http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Carlson, J., Cristol, D., & Swaddle, J. (2014). Dietary mercury exposure causes decreased escape takeoff flight performance and increased molt rate in European starlings (Sturnus vulgaris). *Ecotoxicology*, *23*(8), 1464–1473. doi: 10.1007/s10646-014-1288-5

Choon Kiang Wong, Ming-Chih Chiu, Yuan-Hsun Sun, Shiao-Yu Hong, & Mei-Hwa Kuo. (2014). Using molecular scatology to identify aquatic and terrestrial prey in the diet of a riparian predator, the Plumbeous Water Redstart Phoenicurus fuliginosa. *AJOB Empirical Bioethics*, *5*(3), 368–376. doi: 10.1080/00063657.2015.1032888

Clarke, L. J., Soubrier, J., Weyrich, L. S., & Cooper, A. (2014). Environmental metabarcodes for insects: in silico PCR reveals potential for taxonomic bias. *Molecular Ecology Resources*, *14*(6), 1160–1170. doi: 10.1111/1755-0998.12265

Crisol-Martínez, E., Moreno-Moyano, L. T., Wormington, K. R., Brown, P. H., & Stanley, D. (2016). Using Next-Generation Sequencing to Contrast the Diet and Explore Pest-Reduction Services of Sympatric Bird Species in Macadamia Orchards in Australia. *PLoS ONE*, *11*(3), 1–19. doi: 10.1371/journal.pone.0150159

Cristol, D. A., Brasso, R. L., Condon, A. M., Fovargue, R. E., Friedman, S. L., Hallinger, K. K., … White, A. E. (2008). The Movement of Aquatic Mercury Through Terrestrial Food Webs. *Science*, *320*(5874), 335–335.

Custer, T. W., Custer, C. M., Hines, R. K., Sparks, D. W., Melancon, M. J., Hoffman, D. J., … Wickliffe, J. K. (2000). Mixed-Function Oxygenases, Oxidative Stress, and Chromosomal Damage Measured in Lesser Scaup Wintering on the Indiana Harbor Canal. *Archives of Environmental Contamination and Toxicology*, *38*(4), 522–529. doi: 10.1007/s002449910068

Dragonfly Mercury Project Data (U.S. National Park Service). (n.d.). Retrieved March 26, 2019, from https://www.nps.gov/articles/dragonflymercury-map.htm

Driscoll, C. T., Mason, R. P., Chan, H. M., Jacob, D. J., & Pirrone, N. (2013). Mercury as a global pollutant: sources, pathways, and effects. *Environmental Science & Technology*, *47*(10), 4967–4983. doi: 10.1021/es305071v

Eagles-Smith, C. A., Wiener, J. G., Eckley, C. S., Willacker, J. J., Evers, D. C., Marvin-DiPasquale, M., … Ackerman, J. T. (2016). Mercury in western North America: A synthesis of environmental contamination, fluxes, bioaccumulation, and risk to fish and wildlife. *Science of the Total Environment*, *568*, 1213–1226. doi: 10.1016/j.scitotenv.2016.05.094

Elliott, K. H., & Elliott, J. E. (2016). Origin of Sulfur in Diet Drives Spatial and Temporal Mercury Trends in Seabird Eggs From Pacific Canada 1968–2015. *Environmental Science & Technology*, *50*(24), 13380–13386. doi: 10.1021/acs.est.6b05458

Fokidis, H. B., des Roziers, M. B., Sparr, R., Rogowski, C., Sweazea, K., & Deviche, P. (2012). Unpredictable food availability induces metabolic and hormonal changes independent of

food intake in a sedentary songbird. *Journal of Experimental Biology*, *215*(16), 2920–2930. doi: 10.1242/jeb.071043

François M. M. Morel, author, Anne M. L. Kraepiel, author, & Marc Amyot, author. (1998). The Chemical Cycle and Bioaccumulation of Mercury. *Annual Review of Ecology and Systematics*, 543.

Geller, J., Meyer, C., Parker, M., & Hawk, H. (2013). Redesign of PCR primers for mitochondrial cytochrome c oxidase subunit I for marine invertebrates and application in all-taxa biotic surveys. *Molecular Ecology Resources*, *13*(5), 851–861. doi: 10.1111/1755-0998.12138

Hagar, J. C., Li, J., Sobota, J., & Jenkins, S. (2012). Arthropod prey for riparian associated birds in headwater forests of the Oregon Coast Range. *Forest Ecology and Management*, *285*, 213–226. doi: 10.1016/j.foreco.2012.08.026

Hatch, K. A., Pinshow, B., & Speakman, J. R. (2002). Carbon isotope ratios in exhaled CO(2) can be used to determine not just present, but also past diets in birds. *Journal Of Comparative Physiology. B, Biochemical, Systemic, And Environmental Physiology*, *172*(3), 263–268. (11919707).

Hebert, P. D. N., Ratnasingham, S., & deWaard, J. R. (2003). Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings. Biological Sciences*, *270 Suppl 1*, S96–S99. (12952648).

Herrera, L. G., Hobson, K. A., Rodríguez, M., & Hernandez, P. (2003). Trophic partitioning in tropical rain forest birds: insights from stable isotope analysis. *Oecologia*, *136*(3), 439–444. (12802673).

Huson, D. H., Beier, S., Flade, I., Górska, A., El-Hadidi, M., Mitra, S., … Tappu, R. (2016). MEGAN Community Edition - Interactive Exploration and Analysis of Large-Scale Microbiome Sequencing Data. *PLoS Computational Biology*, *12*(6), 1–12. doi: 10.1371/journal.pcbi.1004957

Jackson, A. K., Evers, D. C., Adams, E. M., Cristol, D. A., Eagles-Smith, C., Edmonds, S. T., … Tear, T. (2015). Songbirds as sentinels of mercury in terrestrial habitats of eastern North America. *Ecotoxicology*, (2), 453. doi: 10.1007/s10646-014-1394-4

Jedlicka, J. A., Sharma, A. M., & Almeida, R. P. P. (2013). Molecular tools reveal diets of insectivorous birds from predator fecal matter. *Conservation Genetics Resources*, (3), 879. doi: 10.1007/s12686-013-9900-1

Jedlicka, J. A., Vo, A.-T. E., & Almeida, R. P. P. (2016). Molecular scatology and high-throughput sequencing reveal predominately herbivorous insects in the diets of adult and nestling Western Bluebirds (Sialia mexicana) in California vineyards. *La Escatología Molecular y La Secuenciación de Alto Rendimiento Revelan El Predominio de Insectos Herbívoros En Las Dietas de Adultos y Polluelos de Sialia Mexicana En Los Viñedos de California*, (1), 116. doi: 10.1642/AUK-16-103.1

Joseph B. Williams, & George O. Batzli. (1979). Winter Diet of a Bark-Foraging Guild of Birds. *The Wilson Bulletin*, *91*(1), 126.

Kelly K. Hallinger, author, Daniel J. Zabransky, author, Katherine A. Kazmer, author, & Daniel A. Cristol, author. (2010). Birdsong Differs between Mercury-polluted and Reference Sites. *The Auk: A Quarterly Journal of Ornithology*, (1), 156. doi: 10.1525/auk.2009.09058

Laver, T., Harrison, J., O'Neill, P. A., Moore, K., Farbos, A., Paszkiewicz, K., & Studholme, D. J. (2015). Assessing the performance of the Oxford Nanopore Technologies MinION. *Biomolecular Detection and Quantification*, *3*, 1–8. doi: 10.1016/j.bdq.2015.02.001

Leray, M., Yang, J. Y., Meyer, C. P., Mills, S. C., Agudelo, N., Ranwez, V., … Machida, R. J. (2013). A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents. *Frontiers in Zoology*, (1). doi: 10.1186/1742-9994-10-34

Li, H. (2018). Minimap2: pairwise alignment for nucleotide sequences. *Bioinformatics*, *34*(18), 3094–3100. doi: 10.1093/bioinformatics/bty191

Mitchell, C. P. J., Branfireun, B. A., & Kolka, R. K. (2008). Spatial characteristics of net methylmercury production hot spots in peatlands. *Environmental Science & Technology*, *42*(4), 1010–1016. (18351065).

Nakano, S., & Murakami, M. (2001). Reciprocal subsidies: Dynamic interdependence between terrestrial and aquatic food webs. *Proceedings of the National Academy of Sciences*, *98*(1), 166–170. doi: 10.1073/pnas.98.1.166

Oehm, J., Juen, A., Nagiller, K., Neuhauser, S., & Traugott, M. (2011). Molecular scatology: how to improve prey DNA detection success in avian faeces?: IMPROVING PREY DNA DETECTION IN AVIAN FAECES. *Molecular Ecology Resources*, *11*(4), 620–628. doi: 10.1111/j.1755-0998.2011.03001.x

Orłowski, G., Karg, J., & Karg, G. (2014). Functional invertebrate prey groups reflect dietary responses to phenology and farming activity and pest control services in three sympatric species of aerially foraging insectivorous birds. *PloS One*, *9*(12), e114906. doi: 10.1371/journal.pone.0114906

Parks, J. M., Johs, A., Podar, M., Bridou, R., Hurt, R. A., Smith, S. D., … Liang, L. (2013). The genetic basis for bacterial mercury methylation. *Science (New York, N.Y.)*, *339*(6125), 1332–1335. doi: 10.1126/science.1230667

Podar, M., Gilmour, C. C., Brandt, C. C., Soren, A., Brown, S. D., Crable, B. R., … Elias, D. A. (2015). Global prevalence and distribution of genes and microorganisms involved in mercury methylation. *Science Advances*, *1*(9), e1500675. doi: 10.1126/sciadv.1500675

R Core Team. (2019). *R: A Language and Environment for Statistical Computing*. Retrieved from https://www.R-project.org/

Rach, J., Bergmann, T., Paknia, O., DeSalle, R., Schierwater, B., & Hadrys, H. (2017). The marker choice: Unexpected resolving power of an unexplored CO1 region for layered DNA barcoding approaches. *Plos One*, *12*(4), e0174842–e0174842. doi: 10.1371/journal.pone.0174842

Rang, F. J., Kloosterman, W. P., & de Ridder, J. (2018). From squiggle to basepair: computational approaches for improving nanopore sequencing read accuracy. *Genome Biology*, *19*(1). doi: 10.1186/s13059-018-1462-9

Rimmer, C. C., Mcfarland, K. P., Evers, D. C., Miller, E. K., Aubry, Y., Busby, D., & Taylor, R. J. (2005). Mercury concentrations in Bicknell's thrush and other insectivorous passerines in Montane forests of northeastern North America. *Ecotoxicology (London, England)*, *14*(1–2), 223–240. (15931968).

Shen, W., Le, S., Li, Y., & Hu, F. (2016). SeqKit: A Cross-Platform and Ultrafast Toolkit for FASTA/Q File Manipulation. *PLOS ONE*, *11*(10), e0163962. doi: 10.1371/journal.pone.0163962

Trevelline, B. K., Latta, S. C., Marshall, L. C., Nuttle, T., & Porter, B. A. (2016). Molecular analysis of nestling diet in a long-distance Neotropical migrant, the Louisiana Waterthrush (Parkesia motacilla)/Analisis molecular de la dieta de los polluelos de Parkesia motacilla, un ave migrante neotropical de larga distancia. *The Auk*, (3), 415. doi: 10.1642/AUK-15-222.1

Tsui, M. T.-K., Adams, E. M., Jackson, A. K., Evers, D. C., Blum, J. D., & Balogh, S. J. (2018). Understanding sources of methylmercury in songbirds with stable mercury isotopes: Challenges and future directions. *Environmental Toxicology and Chemistry*, (1), 166. doi: 10.1002/etc.3941

Valentini, A., Pompanon, F., & Taberlet, P. (2009). Review: DNA barcoding for ecologists. *Trends in Ecology & Evolution*, *24*, 110–117. doi: 10.1016/j.tree.2008.09.011

Varian-Ramos, C. W., Swaddle, J. P., & Cristol, D. A. (2014). Mercury Reduces Avian Reproductive Success and Imposes Selection: An Experimental Study with Adult- or Lifetime-Exposure in Zebra Finch. *PLoS ONE*, *9*(4), e95674. doi: 10.1371/journal.pone.0095674

Vaser, R., Sović, I., Nagarajan, N., & Šikić, M. (2017). Fast and accurate de novo genome assembly from long uncorrected reads. *Genome Research*, *27*(5), 737–746. doi: 10.1101/gr.214270.116

Vesterinen, E. J., Lilley, T., Laine, V. N., & Wahlberg, N. (2013). Next Generation Sequencing of Fecal DNA Reveals the Dietary Diversity of the Widespread Insectivorous Predator Daubenton's Bat (Myotis daubentonii) in Southwestern Finland. *PLoS ONE*, *8*(11), 1–1. doi: 10.1371/journal.pone.0082168

Vo, A.-T. E., & Jedlicka, J. A. (2014). Protocols for metagenomic DNA extraction and Illumina amplicon library preparation for faecal and swab samples. *Molecular Ecology Resources*, *14*(6), 1183–1197. doi: 10.1111/1755-0998.12269

Wada, H., Cristol, D. A., McNabb, F. M. A., & Hopkins, W. A. (2009). Suppressed adrenocortical responses and thyroid hormone levels in birds near a mercury-contaminated river. *Environmental Science & Technology*, *43*(15), 6031–6038. (19731714).

Windham-Myers, L., Fleck, J. A., Ackerman, J. T., Marvin-DiPasquale, M., Stricker, C. A., Heim, W. A., … Alpers, C. N. (2014). Mercury cycling in agricultural and managed wetlands: A synthesis of methylmercury production, hydrologic export, and bioaccumulation from an integrated field study. *Science of the Total Environment*, *484*, 221–231. doi: 10.1016/j.scitotenv.2014.01.033

Wolfe, M. F., Sulaiman, R. A., & Schwarzbach, S. (1998). Effects of mercury on wildlife: a comprehensive review. *Environmental Toxicology & Chemistry*, *17*(2), 146. doi: 10.1002/etc.5620170203

Zeale, M. R. K., Butlin, R. K., Barker, G. L. A., Lees, D. C., & Jones, G. (2011). Taxon-specific PCR for DNA barcoding arthropod prey in bat faeces. *Molecular Ecology Resources*, (2), 236.

Zhang, N., Zhou, C., Xia, W., & Nguyen, A. V. (2018). Volatilization of mercury in coal during conventional and microwave drying and its potential guidance for environmental protection. *Journal of Cleaner Production*, *176*, 1–6. doi: 10.1016/j.jclepro.2017.12.131

APPENDIX

PCR experimentation

A total of 5 pairs of primers were used, each having variations in order of degeneracy and target taxon. See table 5 for a complete list of primers used. A total of 5 PCR Master Mixes were used including: Phusion® High-Fidelity PCR Master Mix with HF Buffer (NEB), Taq Master Mix For PCR (Bio Rad #1665009EDU), REDTaq® ReadyMix™ PCR Reaction Mix (Sigma-Aldrich), REPLI-g UltraFast Mini Kit (Qiagen), and AmpliTaq Gold™ 360 Master Mix (Thermo Fisher Scientific). 5 different master mixes were used in experimentation including Phusion® High-Fidelity PCR Master Mix with HF Buffer (NEB), Taq Master Mix For PCR (Bio Rad), REDTaq ReadyMix™ (Sigma-Aldrich), REPLI-g UltraFast Mini Kit (Qiagen), and AmpliTaq Gold™ 360 Master Mix (Thermo Fisher). Denaturation, annealing, and extension temperatures were adjusted for each polymerase and primer set. A total of 81 combinations of template DNA concentrations, master mixes, primers, and thermocycler settings were tested.

Primer Name	Sequence (5' -3')	Target Taxon	DNA region	Target Length (bp)	Reference
LCO 1490(F)	TTTCTGTTGGTGCTGATATTGCGGTCAACAAATCATAAAGATATTGG	Invertabrates	COI	658	Folmer et al., 1994
HCO 2198 (R)	ACTTGCCTGTCGCTCTATCTTCTAAACTTCAGGGTGACCAAAAAAT	Invertabrates	COI	658	Folmer et al., 1994
dgLCO_ONT_F	TTTCTGTTGGTGCTGATATTGCGGTCAACAAATCATAAAGAYATYGG	Invertabrates	COI	658	Meyer et al., 2003
dgHCO_ONT_R	ACTTGCCTGTCGCTCTATCTTCTAAACTTCAGGGTGCCAAARAAYCA	Invertabrates	COI	658	Meyer et al., 2003
A49425_ trnL5_F	TTTCTGTTGGTGCTGATATTGCCGAAATCGGTAGACGCTACG	Plants	trnL	450	Taberlet et al., 2007
B49863_ trnL3_R	ACTTGCCTGTCGCTCTATCTTCGGGGATAGAGGGACTTGAAC	Plants	trnL	450	Taberlet et al., 2007
ITS-U1 F *	TTTCTGTTGGTGCTGATATTGCGGAAGKARAAGTCGTAACAAGG	Fungi	ITS	800	Cheng et a., 2016
ITS-U4 R *	ACTTGCCTGTCGCTCTATCTTCRGTTTCTTTTCCTCCGCTTA	Fungi	ITS	800	Cheng et a., 2016
MetaF_mlCOIintF	AAG ACG AGG WAC WGG WTG AAC WGT WTA YCC YCC	Invertabrates	COI	333	Leray et al., 2013
MetaR_jgHCO2198	AAG ACG ATA NAC YTC NGG RTG NCC RAA RAA YCA	Invertabrates	COI	333	Geller et al., 2013

Table 5. *List of primers, their sequences, target taxa, region, target length, and references. ONT barcode tags are in red.*

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Ethics Statement

The permits acquired for this work were as follows:
USGS master bander permit to Allyson Jackson #24142, National Park Service Scientific Research and Collecting Permit #ACAD-2018-SCI-0018, State of Maine Department of Inland Fisheries and Wildlife, Wildlife Scientific Collection Permit 2018-536, and Purchase College Institutional Animal Care and Use Permit #2018-AJ01

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