Exposure Science to Investigate Factors Influencing Mercury Concentrations in Non-piscivorous Birds

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Abstract

Mercury (Hg) is a ubiquitous environmental contaminant that poses a significant threat to the health of humans, wildlife, and ecosystems. Efforts to reduce anthropogenic Hg emissions, such as the United Nations Environmental Programme Minamata Convention on Mercury, are vital to the protection of human and ecosystem health. Mercury exposure assessments of bioindicator species, such as birds, are critical to monitoring Hg in the environment and understanding how reductions in Hg emissions relate to its bioavailability within ecosystems and food webs, and ultimately its risk. The overall objective of this work was to increase understanding of factors that influence blood and feather Hg concentrations in non-piscivorous bird species to improve Hg exposure assessments. The specific objectives included: (1) identifying factors that influence Hg concentrations of blood and feathers of Arctic-breeding shorebirds, (2) developing and validating a novel method to assess Hg exposure in birds using dried blood spots (DBS), and (3) investigating key biochemical processes, Hg speciation and subcellular distribution of Hg, to better understand Hg distribution across tissues of embryos and hatchling birds. To achieve these objectives, I conducted field studies, including a large-scale, multi-species Hg exposure assessment at sites across the North American Arctic, and laboratory studies, including methylmercury (MeHg) dosing of White Leghorn Chicken (*Gallus gallus domesticus*) and Zebra Finch (*Taeniopygia guttatato*). Field studies indicated that overall, Arctic-breeding shorebirds were at low risk of adverse effects of Hg exposure, but some individuals may exceed exposures that could result in adverse health effects. Blood Hg concentrations for Arctic-breeding shorebirds were influenced by species, breeding site, and the interaction between site and predominant foraging habitat moisture classification, but not by year. Feather Hg concentrations

were influenced by species, sex, and year, but not breeding site. Laboratory studies indicated that DBS, analyzed using a direct Hg analyzer, are a good method to evaluate Hg exposure in birds. Mercury concentrations for entire chicken DBS were stable when subjected to time, temperature, and humidity storage treatments, with mean DBS Hg concentrations within ± 8 % of the whole blood for all treatments (n = 10). Further laboratory studies found the distributions of total Hg among liver subcellular fractions were similar among early-developmental time points for chicken dosed with MeHg via egg injection. Distributions were also similar between embryonic Ring-billed Gulls (*Larus delawarensis*), exposed to maternally-deposited MeHg, and chicken. This research aims to improve Hg exposure assessments with the development of a novel sampling method for using DBS to determine avian blood Hg concentrations, that can aid in reducing logistic burdens and costs of collecting and analyzing field collected samples. Additionally, this work advances the understanding of the extrinsic and intrinsic factors that influence Hg toxicokinetics in wild birds and a model avian species exposed to MeHg via egg injection.

Résumé

Le mercure (Hg) est un contaminant global qui menace de la santé des humains et des écosystèmes. Les efforts pour réduire les émissions anthropiques du Hg, tel que la Convention de Minamata sur le Mercure, sont essentiels à la conservation des écosystèmes et à la protection des humains. L'évaluation de l'exposition au Hg chez les espèces bio-indicatrices, comme les oiseaux, est un outil nécessaire à l'étude du Hg dans l'environnement et de la relation entre la réduction des émissions de Hg et sa biodisponibilité dans les écosystèmes et les chaines alimentaires. Ultimement, l'évaluation de l'exposition au Hg permettrait de mieux comprendre les risques. Les objectifs de cette recherche étaient de mieux comprendre les facteurs influençant les concentrations de Hg dans le sang et les plumes des oiseaux non-piscivores afin améliorer les évaluations d'exposition au Hg. Plus précisément, les objectifs étaient de: 1) identifier les facteurs qui influencent les concentrations de Hg dans le sang et les plumes des oiseaux de rivages d'Arctique, 2) développer et valider une méthode d'évaluation de l'exposition au Hg chez les oiseaux utilisant des tâches de sang séché (TSS) et 3) étudier les principaux processus biochimiques, la spéciation du Hg et sa répartition subcellulaire, afin de mieux comprendre la distribution du Hg dans les tissus d'embryons d'oiseaux et de nouveau-nés. Pour ce faire, j'ai mené des études de terrain, incluant l'évaluation de l'exposition du Hg à grande échelles chez plusieurs espèces à travers l'Arctique Nord-Américain, ainsi que des études de laboratoires, incluant le dosage au méthylmercyre (MeHg) de modèles d'espèce, soit la poule Livourne (*Gallus gallus domesticus*) et le diamant mandarin (*Taeniopygia guttatato*). Les études de terrains ont indiqué, en général, que les oiseaux de rivage d'Arctique présentaient un faible risque d'effets indésirables de l'exposition au Hg. Toutefois, certains individus peuvent faire face

à une exposition dépassant les seuils de toxicité générant des effets néfastes pour leur santé. Les concentrations de Hg dans le sang des oiseaux de rivages d'Arctiques variaient dépendamment de l'espèce, du site de reproduction, ainsi que de l'interaction entre le site de reproduction et l'humidité de l'habitat d'alimentation, mais ne variaient pas avec l'année. Les concentrations de Hg dans le sang ont également été influencées dans une moindre mesure par certains facteurs physiologiques comme le sexe, la masse corporelle et le métabolisme du Hg, alors que la mue des plumes n'avait aucun effet. Les études de laboratoires ont démontré que les TSS, analysées avec un analyseur direct de Hg, représentaient une méthode efficace pour évaluer l'exposition au Hg chez les oiseaux. Les concentrations de Hg chez les poules Livourne obtenues avec les TSS étaient constantes pour divers traitements de l'entreposage. Les concentrations de Hg obtenues avec cette méthode se situaient à ± 8 % des concentrations du sang total pour tous les traitements (n=10). De plus, en ce qui a trait aux poules injectées au MeHg à travers leur coquille d'œuf, la répartition du Hg total dans les fractions subcellulaires était similaire entre les stades du développement. La répartition subcellulaire du Hg total était aussi semblable entre les goélands à bec cerclés (*Larus delawarensis*) exposés au Hg par transmission maternelle et les poules Livourne. Cette recherche permet d'améliorer l'évaluation de l'exposition au Hg grâce au développement d'une méthode innovatrice employant les TSS pour déterminer les concentrations de Hg sanguin chez les oiseaux. Cette méthode permet de réduire la charge logistique et les coûts associés à la collecte et l'analyse d'échantillons de sang récoltés sur le terrain. De plus, cette étude représente une avancée importante pour mieux comprendre des facteurs influençant la toxicocinétique du Hg chez les oiseaux sauvages ainsi que chez des modèles d'oiseaux exposés au MeHg par l'injection des œufs.

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This thesis is the result of considerable collaboration, advice, and guidance from researchers and colleagues, without which this work would not have been possible. First and foremost, I would like to express my immense gratitude to my supervisor, Dr. Niladri Basu, for his endless patience, support, and insight throughout my time working with him. Additionally, I am grateful to him for encouraging such a highly collaborative and supportive atmosphere within his lab. I would like to thank all the current and past members of the Basu lab for their generous support, advice, and encouragement throughout this process. Specifically, I thank Jenny Eng and Gordana Martincevic for their technical assistance; Dr. Matthew Alloy, Krittika Mittal, Dr. Theresa Johnston, and Dr. Kimmo Mäenpää for help with egg injection studies; and Dr. Benjamin Barst, Krittika Mittal, and Jessica Pawly for editorial support. Additional thanks to Justine Hadrava for providing the French translation of the abstract.

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Preface and Contribution of the Authors

This thesis fills important knowledge gaps and contributes to the advancement of knowledge as follows:

Chapter 2 is a large-scale, multi-species Hg exposure assessment conducted at study sites across the North American Arctic. This chapter investigated factors influencing mercury (Hg) concentrations of blood and feathers of Arctic-breeding shorebirds and identified regions and species at the greatest risk of Hg exposure. This chapter fills an important knowledge gap in Arctic Hg exposure research by conducting a Hg exposure assessment on a group of terrestrial avian invertivores for which information on Hg exposure has been lacking. Additionally, since the shorebird species sampled in this study forage in Arctic tundra wetlands and occupy the same foraging guild we were able to investigate factors influencing Hg concentrations in non-piscivorous birds without having to account for ecoregion and foraging guild differences.

Chapter 3 developed and validated a novel method to assess Hg exposure in birds using dried blood spots (DBS). This is the first study to our knowledge to use direct Hg analysis to determine Hg concentrations in DBS. This study greatly expands upon previous studies on the use of DBS in birds by investigating non-homogenous spread of Hg across DBS and the stability of Hg in DBS subjected to short-term, field simulated collection and storage conditions and multiple long-term storage conditions. This is also the first study to our knowledge that has collected whole blood and created DBS using a capillary tube in the field to determine Hg exposure in birds.

Chapter 4 investigated Hg speciation and subcellular distribution of Hg in tissues of embryonic and hatchling White Leghorn Chicken (*Gallus gallus domesticus*) dosed with

methylmercury (MeHg) via egg injection and embryonic Ring-billed Gulls (*Larus delawarensis*) exposed to maternally-deposited MeHg. We also adapted and verified a partitioning procedure for separating avian liver tissue into operationally defined subcellular fractions with high effectiveness. To our knowledge, this is the first study that has used MeHg egg injection to investigate subcellular distribution of Hg in bird liver and compared the results with those from a wild avian species exposed to maternally-deposited MeHg.

This thesis consists of 3 chapters intended for publication. Chapter 2 was designed and developed by the degree candidate in conjunction with Drs. David Evers, Lisa Ferguson (currently at the Wetlands Institute), and Iain Stenhouse from the Biodiversity Research Institute. Samples were collected by various collaborators from the Arctic Shorebird Demographics Network (ASDN) and analyzed by the Biodiversity Research Institute. The candidate was responsible for analysis and interpretation of the data, discussion of the results, and preparation of the manuscript and was provided advice and guidance by the candidate's supervisor Dr. Niladri Basu. This chapter is co-authored by the candidate's supervisor Dr. Niladri Basu and project collaborators Drs. Iain Stenhouse and Richard Lanctot (ASDN), with planned submission to *Environmental Science & Technology*. Additional ASDN collaborators will also be included as co-authors, however, the determination of co-authors is still on-going. For chapter 3, the candidate was responsible for the study design and development, sample collection and analysis, analysis and interpretation of the data, discussion of the results, and preparation of the manuscript. A portion of the samples were collected by Spencer Morran from Simon Fraser University and Dr. JF Lamarre from Université du Québec à Rimouski. This chapter is co-authored by the candidate's supervisor Dr. Niladri Basu, who provided guidance and advice on all aspects of the study, with planned submission to *Environmental Pollution*. For chapter 4, the

candidate was responsible for the study design and development, sample collection and analysis, analysis and interpretation of the data, discussion of the results, and preparation of the manuscript. This chapter is co-authored by the candidate's supervisor Dr. Niladri Basu, Dr. Benjamin Barst, and Justine Hadrava. Drs. Niladri Basu and Benjamin Barst provided guidance and advice on all aspects of the study. Justine Hadrava assisted in method development, sample analysis, and manuscript preparation. This chapter was provisionally accepted for publication in *Environmental Toxicology and Chemistry* on 1 May 2017 and is formatted in the style of this journal.

List of Abbreviations

AIC	Akaike's Information Criteria
ASDN	Arctic Shorebird Demographics Network
BRI	Biodiversity Research Institute
CCO	Cytochrome C Oxidase
DBS	Dried Blood Spot
dw	Dry Weight
fw	Fresh Weight
GLM	Generalized Linear Model
GLMM	Generalized Linear Mixed-effects Model
Hg	Mercury
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IoHg	Inorganic Mercury
IPR	Initial Precision and Recovery
LDH	Lactate Dehydrogenase
LOD	Limit of Detection
LOQ	Limit of Quantification
MeHg	Methylmercury
MeHgCl	Methylmercury Chloride
OHg	Organic Mercury
OPR	Ongoing Precision and Recovery
PMDL	Practical Method Detection Limit

RPD	Relative Percent Difference
RSD	Relative Standard Deviation
SRM	Standard Reference Material
THg	Total Mercury
TMDL	Theoretical Method Detection Limit
UNEP	United Nations Environmental Programme
ww	Wet Weight

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Chapter 1

1 Introduction and Literature Review

1.1 General Introduction

Mercury (Hg) is a ubiquitous environmental contaminant that poses a significant threat to the health of humans, wildlife, and ecosystems (UNEP 2013). Efforts to reduce anthropogenic Hg emissions are essential to the protection of human and ecosystem health, with the United Nations Environmental Programme (UNEP) Minamata Convention on Mercury being the largest effort to date to reduce new Hg emissions (UNEP 2015). Assessing and monitoring Hg exposure in humans, wildlife, and the environment is vital to gauge the effectiveness of the Minamata Convention. Therefore, Hg exposure assessments of bioindicator species, such as birds, are critical to understanding how reductions in Hg emissions relate to the bioavailability of Hg within ecosystems and food webs. Non-destructive sampling of bird blood and feathers is increasingly preferred for avian Hg exposure assessments. Insights into the extrinsic and intrinsic factors influencing bird blood and feather Hg concentrations are needed to help clarify among and within species variability observed in these concentrations, and thus help stakeholders (e.g., regulators, risk assessors, scientists) better use and interpret Hg exposure data. Blood collection for Hg analysis may be prohibitive or logistically unfeasible, particularly in remote or resource-limited regions. The development of a novel blood collection method that can reduce logistic burdens and costs is needed to further encourage large-scale Hg exposure assessments. The overall objective of this work is to improve Hg exposure assessments by increasing the understanding of extrinsic and intrinsic factors that influence blood and feather Hg

concentrations in non-piscivorous bird species and by developing a novel blood collection method.

1.1.1 Specific Aims

Aim1: Identify factors that influence Hg concentrations of bird blood and feathers using a large-scale, multi-species Hg exposure assessment of Arctic-breeding shorebirds.

- Establish current Hg concentrations in shorebird blood and feathers that can be used to evaluate the efficacy of future regulations to reduce global Hg emissions.
- Identify Arctic shorebird breeding regions and species at the greatest risk for high Hg.
- Evaluate the influence of species, preferred foraging habitat moisture classification, study site, molt pattern, sex, body condition, and Hg turnover in blood (as reflected by capture day) on bird blood and feather Hg concentrations.

Aim 2: Develop and validate a method to assess total Hg (THg) exposure in birds using dried blood spots (DBS).

- Develop an accurate and precise method to determine blood Hg concentrations using entire DBS and sub-sampled DBS from a model avian species, White Leghorn Chicken (*Gallus gallus domesticus*).
    - Estimate blood volume of DBS sub-samples and DBS with unknown volumes by determining the relationship between DBS area and volume
    - Investigate the accuracy and precision of entire DBS and sub-sampled DBS
    - Examine intra-DBS variation
- Investigate Hg stability of DBS prepared with blood from chicken dosed with methylmercury (MeHg) via egg injection exposed to time, temperature, and humidity treatments.

    - Examine three pathogen decontamination heat treatments
    - Examine short-term (≤ 3 months) field collection and storage conditions, including high heat and humidity, freeze-thaw cycle, and UV light exposure
    - Examine long-term (1 year) laboratory storage conditions including ambient temperature, low humidity, refrigerator, freezer, and ultracold freezer
- Apply the developed method to DBS created with blood collected using standard field methods from Zebra Finch (*Taeniopygia guttatato*) dosed with MeHg in the laboratory and American Golden-Plover (*Pluvalis dominica*) sampled at an Arctic field site.

Aim 3: Investigate key biochemical processes, Hg speciation and subcellular distribution of Hg, in tissues of embryo and hatchling birds.

- Evaluate THg and MeHg concentrations of brain and liver tissues of embryonic and blood, brain, and liver tissues of hatchling chicken dosed with MeHg via egg injection, as well as liver tissues of embryonic Ring-billed Gulls exposed to maternally deposited Hg.
    - Compare Hg speciation in blood, brain, and liver tissues across embryonic and hatchling developmental time points
    - Compare Hg speciation in liver tissues between embryonic chicken and Ring-billed Gulls
- Evaluate subcellular distribution of Hg in liver tissues of embryonic and hatchling chicken dosed with MeHg via egg injection and embryonic Ring-billed Gulls exposed to maternally deposited Hg.
    - Compare Hg subcellular distribution in liver tissue of chicken across embryonic and hatchling developmental time points

- Compare Hg subcellular distribution in liver tissues between embryonic chicken and Ring-billed Gulls

1.2 Literature Review

1.2.1 Mercury in the Environment

1.2.1.1 Sources, Cycling, and Fate

Anthropogenic Hg emissions, such as those from mining and the release of Hg as a by-product of burning coal and other fossil-fuels, are a major source of Hg mobilization throughout the global environment (UNEP 2013). Recent assessments estimate that 1875 metric tons per year of Hg are emitted globally from anthropogenic sources (AMAP/UNEP 2015). Mercury is a highly mobile pollutant, transported readily around the globe by air, ocean, and river currents. Atmospheric Hg emissions can travel great distances from where they originated, resulting in relatively large Hg depositions in remote ocean and polar regions (AMAP 2011, AMAP/UNEP 2015). Once deposited, a portion can be sequestered in sediments or mineral compounds (UNEP 2013). However, most deposited Hg continues to cycle through the environment, with 60 percent of annual Hg emissions coming from re-mobilized, previously deposited anthropogenic Hg.

Mercury deposited across the landscape can also be incorporated into biotic communities. Specifically, Hg can be converted into its more toxic and biologically available form, methylmercury (MeHg), by bacterial processes (Wiener et al. 2003). Methylmercury can readily cross cell membranes and accumulate and magnify through food webs, as seen in increasing concentrations in predatory species (Wiener et al. 2003, Clarkson and Magos 2006). Since Hg methylation occurs readily under anoxic conditions, Hg is often more biologically available in aquatic and wetland habitats where anaerobic conditions are common (Wiener et al. 2003). Due

to increased production of MeHg in aquatic environments, Hg exposure assessments of wildlife have traditionally focused on aquatic and piscivorous species (Evers et al. 2008, Seewagen 2010). This has been particularly warranted since fish consumption is one of the main sources of Hg exposure for humans. While research has shown that Hg exposure and biomagnification also occur in terrestrial and invertebrate-based food webs (Rimmer et al. 2005, Cristol et al. 2008), the extent of Hg contamination and bioavailability beyond aquatic and wetland habitats warrants further study.

1.2.1.2 Mercury in the Arctic

Globally, atmospheric Hg releases are on the rise and emissions from East Asia, the principal source of atmospheric Hg deposited across the Arctic, are predicted to increase (Durnford et al. 2010, AMAP 2011, Dastoor et al. 2015). Predictions based on current atmospheric Hg deposition rates indicate Hg concentrations in the eastern North Pacific Ocean will double by 2050 (Sunderland et al. 2009). Further, Hg sequestered in permafrost, representing deposition from past centuries, has the potential to be rapidly released into the environment as climate change increases temperatures (Schuster et al. 2011, Stern et al. 2011). Increasing Arctic temperatures may also accelerate Hg methylation rates (Stern et al. 2011), making Hg more bioavailable to Arctic wildlife. Arctic wildlife are at risk of Hg contamination, with many species exposed to Hg at concentrations associated with adverse health effects (Scheuhammer et al. 2015), though our understanding of risk to Arctic species is relatively unknown compared to other geographic regions due to the many challenges associated with northern-based research.

1.2.1.3 Mercury Effects in Birds

In birds and other wildlife, chronic exposure to MeHg can cause sub-lethal effects, including impaired physiology, behavior, and reproductive success, ultimately having population level

effects (Evers et al. 2008, Seewagen 2010, Scheuhammer et al. 2011, Ackerman et al. 2016b). These negative effects have been well documented in piscivorous birds, such as the Common Loon (*Gavia immer*; Burgess and Meyer 2008; Evers et al. 2008, 2011), however, less is known about the effects of Hg on avian invertivores. Laboratory studies have shown that Hg sensitivity can vary among bird species. For example, Heinz et al. (2009) used MeHg egg injections to rank 23 bird species into categories of low, medium, and high sensitivity based on dose–response curves and median lethal doses (LC50), with a 4-fold difference in dose between the low and high sensitivity species. This dosing study indicated that avian invertivores are at greater risk for negative health effects of Hg exposure than avian piscivores. Field-based studies on invertivore songbirds have associated adverse health effects with Hg exposure, for example, studies show reduced reproductive success and suppressed immune function is related to Hg exposure in Tree Swallows (*Tachycineta bicolor*, Brasso and Cristol 2008, Hawley et al. 2009, Hallinger and Cristol 2011). Additionally, research on the Carolina Wren (*Thryothorus ludovicianus*) at two contaminated rivers in Virginia, indicated reproductive impairment was related to known Hg adult body burdens (Jackson et al. 2011). However, since field and laboratory studies to determine adverse effect levels are difficult and costly to conduct, the Hg sensitivity for most wild bird species is unknown. Overall, the substantial body of literature indicates that birds are sensitive to MeHg risk but that this risk varies greatly among species. Therefore, uniform guidelines are not appropriate and there is considerable need for increased understanding of inter-species differences in Hg exposure and ultimately risk.

1.2.1.4 Minamata Convention on Mercury

Efforts to reduce anthropogenic Hg emissions are vital to the protection of human and ecosystem health. These efforts are increasing in many regions (USEPA 2012, CCME 2014), with the

UNEP Minamata Convention on Mercury being the largest effort to date to reduce new Hg emissions (UNEP 2015). As of 4 March 2017, the Minamata Convention has been signed by 128 countries and ratified by 38, though ratification is still ongoing (UNEP 2015). The Minamata Convention (i.e., Articles 19 and 22) requires monitoring Hg concentrations in wildlife and the environment (Evers et al. 2016, Gustin et al. 2016) to assess whether reductions in Hg emissions result in decreased exposures. The use of exposure science to assess and monitor Hg pollution across the landscape will increase the understanding of the global Hg cycle and help regulators make informed decisions regarding environmental Hg reductions.

1.2.1.5 Mercury Exposure Assessments

Exposure science has been used successfully to identify populations and systems at risk from contaminants and to better inform policy and regulations (Graham 2010). It has played a prominent role in understanding the effectiveness of regulations and policies aimed at reducing contaminants in the environment (NRC 2012). For instance, lead exposure assessments helped identify sources of lead exposure and were vital to verify the efficacy of the Lead Contamination Control Act of 1988 (Grant 2010). Similarly, Hg exposure assessments may aid in identifying Hg contaminated systems, gauging current contamination levels, and identifying future reductions (Schmeltz et al. 2011). Improvements and expansions to Hg exposure assessments are needed to aid policy makers and researchers in determining the efficacy of new regulations enacted under the Minamata Convention.

Due to the complex behavior of Hg within the natural system, including bioaccumulation and biomagnification within food webs, abiotic Hg concentrations are often not associated with those in biota (Evers et al. 2016, Gustin et al. 2016). Therefore, Hg exposure assessments of bioindicator species are critical to understanding how reductions in Hg emissions relate to the

bioavailability of Hg within ecosystems and food webs (Evers et al. 2016, Gustin et al. 2016). Birds are considered to be effective bioindicator species and have been used to provide reference levels for Hg risk in a variety of systems, including marine habitats, coastal, estuarine, and freshwater wetlands, and upland forests (Goodale et al. 2008, Warner et al. 2010, Jackson et al. 2015). Exposure assessments are a vital and regularly used tool to determine population risks from MeHg. Additionally, birds have been used to determine biological Hg hotspots, as well as species and populations at greatest Hg exposure risk (Evers et al. 2007, Ackerman et al. 2016b, Perkins et al. 2016). However, the relationship between biomarker Hg concentrations and adverse effects from Hg exposure can vary among species (Evers et al. 2008, Heinz et al. 2009, Jackson et al. 2011), which makes it challenging to determine risk based solely on tissue concentrations.

1.2.2 Mercury in Wild Birds

1.2.2.1 Birds as Bioindicators

Bioindicator species are defined as organisms that can accumulate a contaminant and provide a reference level for contaminant risk in a system (Beeby 2001). A good bioindicator species should be abundant in the system, easily identified, sampled and aged, have a well-defined range, assimilate and accumulate the contaminant rapidly from known sources, and the factors that influence contaminant assimilation should be well known (Beeby 2001). Since birds are widespread and tend to have well-defined home ranges during the breeding season, they have the potential to provide vital information about Hg contamination in remote or under represented regions. Birds assimilate and accumulate Hg rapidly in multiple tissues (Bearhop et al. 2000), including blood and feathers, both of which can be sampled with minimum risk to populations.

1.2.2.1.1 Biomarker Tissues

Bird liver and other internal tissues such as kidney, brain, and muscle (by destructive sampling) have commonly been used as Hg biomarkers (Burger et al. 2013, 2014, Hall et al. 2014), with previous field studies indicating strong correlations in THg concentrations among internal tissues for multiple species of juvenile and adult birds (Scheuhammer et al. 2008, Eagles-Smith et al. 2008, Rutkiewicz et al. 2011). Internal tissues often represent long-term Hg body burdens, for example, laboratory and field studies indicate that the liver is important for Hg sequestration in embryonic, juvenile, and adult birds (Henny et al. 2002, Kenow et al. 2007, Rutkiewicz and Basu 2013). Additionally, determining Hg concentrations of internal tissues can supply further information about Hg risk, since some tissues are integral to MeHg detoxification and mobility (ie. blood and liver), while others are more sensitive to the adverse effects of MeHg (ie. liver and brain). Despite these benefits, the toxicokinetics of MeHg within avian tissues is poorly understood, which hinders proper risk assessments within the field. Further, sampling internal tissues require sacrificing animals which pose many logistical and ethical concerns, particularly for birds of conservation concern.

Bird blood and feathers are the most frequently used Hg biomarkers, as these tissues are easily sampled and analyzed for Hg concentrations with minimum risk to populations (i.e. live, non-destructive sampling). Previous studies have found hatchling birds have greater THg concentrations in growing feathers than in internal tissues (Kenow et al. 2007, Eagles-Smith et al. 2008, Rutkiewicz and Basu 2013), indicating that Hg sequestration in growing feathers is an important detoxifying method for hatchling birds. Therefore, feather Hg concentrations are often suggested to reflect exposure from the local environment during feather growth as well as overall body burdens (Wolfe et al. 1998; Evers et al. 2005), making comparisons to local capture

environments difficult. Additionally, considerable variability in Hg concentrations has been observed among and within species for both blood and feathers (Bond and Diamond 2008, Eagles-Smith et al. 2008, Hargreaves et al. 2011). Differences among bird species and individuals in MeHg toxicokinetics, the assimilation and behavior of MeHg within the body, may play a role in the variability observed in biomarker tissue concentrations. Research indicates that both extrinsic and intrinsic factors can influence these concentrations (Evers et al. 2005). A better understanding of how avian MeHg toxicokinetics effects biomarker tissue concentrations is needed for birds to be useful bioindicator species.

1.2.2.1.2 Blood Sampling

Blood is considered to provide a good biomarker of recent dietary exposure to Hg, as studies on birds fed a MeHg dosed diet show that blood Hg concentrations markedly increase within the first weeks of dosing (Bearhop et al. 2000, Fournier et al. 2002, Bennett et al. 2009). Researchers have recently started using large-scale datasets of blood or blood-equivalent THg concentrations of birds to determine Hg contamination and risk throughout North America (Evers et al. 2011, Jackson et al. 2015, Ackerman et al. 2016b). Therefore, blood may be an ideal biomarker tissue to sample when using birds as bioindicators for Hg contamination. Blood sampling for Hg analysis generally does not result in any lasting effects on the sampled birds when conducted using established protocols (Sheldon et al. 2008). However, researchers should be properly trained and care should be taken to reduce handling time and the quantity of blood sampled (Fair et al. 2010). Sample sizes should be limited to no more than 1 % of the birds' body weight, though this volume should be reduced when possible, especially during energetically demanding or stressful time periods (Fair et al. 2010). After collection, Hg concentrations in blood are stable for several years when stored frozen soon after collection (Liang et al. 2000, Varian-Ramos et al.

2011). However, Hg loss has been seen for human blood samples subjected to repeated freeze-thaw cycles (Horvat and Byrne 1992). Therefore, established blood collection and storage methods for Hg analysis require samples to be kept cool during collection and shipping and be stored frozen until analysis (Evers 2008). Due to this, blood collection for Hg analysis can require heavy or bulky supplies such as blood storage tubes, coolers, and ice packs, which may be burdensome to transport to remote sampling locations. The availability of a freezer or cyroshipper during sampling may be required and samples need to be transported or shipped under cool conditions. For samples collected at remote locations, equipment and shipping costs may be prohibitive or logistically unfeasible. Additionally, in order to reduce the spread of avian disease to birds used in domestic agriculture, bird blood collected from countries with the presence of H5N1 subtype of Highly Pathogenic Avian Influenza or Exotic Newcastle Disease need to be treated prior to importation into the United States (Paul 2005). Generally, treatment of samples is not possible using current blood collection and storage methods.

Developing a simple, accurate, and cost-effective technique for collecting bird blood in the field will allow for Hg exposure assessments at remote, resource-limited locations where data is currently lacking. Dried blood spots (DBS) are a method for collecting blood by applying it to specialized filter paper and allowing it to air dry. Dried blood spots were developed in the 1960s as a method to detect metabolic diseases in newborns (Guthrie and Susi 1963). They are still used today as part of the Newborn Screening Quality Assurance Program, with standardized methods and materials for collecting and analyzing blood samples (Mei et al. 2001). In addition, more recent studies have used DBS to determine blood concentrations of heavy metals and other elements in both humans (Chaudhuri et al. 2008, Lehner et al. 2013) and birds (Shlosberg et al. 2011, 2012). Standardized filter paper used for DBS is small and light weight, and during

collection, DBS samples do not need to be kept cold or stored frozen. This can reduce cost, weight, and the number of blood sampling supplies needed during field collection, as well as reduce shipping costs. Dried blood spot use has been standardized for a variety of human health related analyses, however collection, storage, and analysis methods have not been well established for Hg assessment of bird blood.

1.2.2.2 Mercury Toxicokinetics in Birds

Sampling avian biomarker tissues for Hg analysis can be useful for providing a reference level for Hg risk in a system. However, to best utilize avian biomarker tissues, it is critical to understand how MeHg behaves within the body. In addition, it is vital to understand how extrinsic and intrinsic factors may influence avian MeHg toxicokinetics. Given the considerable variability found in biomarker tissue Hg concentrations among and within bird species, such information is needed to better understand how biomarker tissue Hg concentrations relate to the bioavailability of Hg within the environment.

Birds are exposed to Hg through their local food sources (Scheuhammer et al. 2007, Cristol et al. 2008, Rimmer et al. 2010). Factors such as foraging habitat and foraging guild have been shown to be predictors of both blood and feather Hg concentrations (Evers et al. 2005, Keller et al. 2014, Jackson et al. 2015). Mercury methylation occurs readily under anoxic conditions, therefore, Hg is often more biologically available in aquatic environments where anaerobic conditions are common (Wiener et al. 2003). Due to this, Hg exposure assessments of birds have traditionally focused on aquatic species (Evers et al. 2008, Seewagen 2010). Less is known about Hg exposure in terrestrial bird species. Recent studies focused on Hg exposure in invertebrate-eating (invertivore) songbirds suggest that within the terrestrial environment, wetter foraging habitats are related to greater blood and feather Hg concentrations (Keller et al. 2014,

Jackson et al. 2015). MeHg also accumulates and magnifies through the food web, where increasing concentrations occur in predatory species (Rimmer et al. 2010, Lavoie et al. 2013). Due to this, blood and feather Hg concentrations also differ among foraging guilds, generally based on trophic position (Evers et al. 2005, Keller et al. 2014, Jackson et al. 2015). Overall, piscivorous and carnivorous bird species have higher Hg concentrations compared to omnivores and herbivores (Ackerman et al. 2016b). However, avian invertivores can also have high Hg concentrations.

Mercury exposure is generally in the form of MeHg, since this form easily moves across membranes and throughout organisms, which is not the case for inorganic Hg (IoHg, Wiener et al. 2003, Clarkson and Magos 2006). Once MeHg is ingested, it enters blood through diffusion across the gastrointestinal epithelium and is then circulated through the body (Nichols et al. 2010). Blood Hg concentrations increase rapidly over time for birds fed a diet dosed with MeHg (Bearhop et al. 2000, Fournier et al. 2002, Bennett et al. 2009), with the majority of THg within bird blood in the form of MeHg (Rimmer et al. 2005). However, researchers have found the half-life of MeHg in blood to vary by species, age, sex, and molt pattern (Bearhop et al. 2000, Monteiro and Furness 2001, Fournier et al. 2002). For example, Lavoie et al. (2014) found that blood Hg concentrations showed carry over from the winter season to the breeding season for Double-crested Cormorants (*Phalacrocorax auritus*) and Caspian Terns (*Hydroprogne caspia*), though the amount of carry over differed between the two species.

Birds are able to eliminate ingested MeHg through excretion in droppings, feather molt, and egg laying (Lewis and Furness 1993, Bearhop et al. 2000, Nichols et al. 2010). Blood MeHg concentrations are quickly influenced by both the onset of egg laying and feather molt (Bearhop et al. 2000, Bennett et al. 2009), with the majority of THg within eggs and feathers in the form of

MeHg (Rimmer et al. 2005, Ackerman et al. 2013). Due to MeHg deposition in eggs, females can have lower blood Hg concentrations than males during the breeding season (Robinson et al. 2012), however, the proportion of MeHg that females deposit into eggs can differ by species and with increasing Hg exposure (Ackerman et al. 2016a). During molt, MeHg can be transferred from the blood to growing feathers quickly and feather concentrations can represent blood MeHg concentrations at the time of growth (Bearhop et al. 2000). In addition, MeHg can be remobilized from other body tissues and deposited in feathers (Honda et al. 1986, Braune 1987, Agusa et al. 2005). However, MeHg is deposited unevenly among both body and flight feathers, for instance in birds that molt their flight feathers sequentially, greater Hg is deposited in the first flight feathers molted (Furness et al. 1986, Braune 1987, Bond and Diamond 2008). This variability limits the accuracy of feather Hg concentrations to represent Hg exposure in birds.

Mercury speciation studies on avian liver tissue have shown the ratio of MeHg to THg is consistently less than 100 % (Kim et al. 1996, Henny et al. 2002, Eagles-Smith et al. 2009, Robinson et al. 2011), indicating hepatic MeHg demethylation (the extent to which MeHg is converted to IoHg in vivo) occurs. These studies indicate that MeHg demethylation ability differs among species, by sex and age, and with Hg exposure. Methylmercury demethylation in the liver may influence both biomarker tissue concentrations and Hg risk. For instance, research indicates that both blood and feather Hg concentrations may be influenced by the ability to demethylate MeHg in the liver. Henny et al. (2002) found a strong correlation between liver MeHg and blood concentrations for both adult and fledgling birds from multiple species, while liver IoHg was not correlated with blood. Additionally, a reduction in the proportion of Hg excreted into feathers has been found for species that have a greater hepatic MeHg demethylation ability (Wolfe et al. 1998). Methylmercury demethylation in the liver is also considered to be a

detoxification scheme. Research suggests that IoHg in the liver (and its likely sequestration with selenium) is no longer able to negatively affect the nervous, cardiovascular, or other systems (Henny et al. 2002, Scheuhammer et al. 2011). Therefore, species or individuals with higher ability to demethylate MeHg may be at lower risk of negative health effects from Hg exposure.

Recent studies have used a differential centrifugation procedure of tissues to investigate how Hg is distributed among subcellular fractions in fish liver (Bebianno et al. 2007, Araújo et al. 2015, Barst et al. 2016, Peng et al. 2016) and other tissues (Bebianno et al. 2007, Onsanit and Wang 2011, Peng et al. 2016), invertebrates (Dang and Wang 2010), and marine mammal liver (Ikemoto et al. 2004b). These procedures allow for the distinction between metal binding to potentially sensitive target molecules (enzymes and organelles) and metal accumulation in detoxified fractions such as heat-stable proteins and metal-rich granules (Campbell and Hare 2009). A greater proportion of Hg bound to metal-sensitive fractions may indicate an increased health risk. Species differences in the distribution of Hg among subcellular fractions have been noted for invertebrates (Dang and Wang 2010) and marine mammals (Ikemoto et al. 2004a). However, only one species of bird, a marine foraging, piscivorous species (Black-footed Albatross, *Diomedea nigripes*, n = 5), has been investigated for subcellular distribution of Hg (Ikemoto et al. 2004a, 2004b).

1.2.3 Focal Species

1.2.3.1 Arctic-breeding Shorebirds

Shorebirds may represent an ideal group for Arctic Hg exposure research, as shorebirds are widespread across the Arctic during the breeding season. Most shorebirds inhabit similar nesting and foraging habitats in Arctic tundra wetlands and occupy the same foraging guild (invertivores,

Table 2.1, Colwell 2010, Rodewald 2015). However, species can differ in their migratory routes and wintering grounds, mating systems, and molt patterns. Recent studies have documented distinct differences in blood Hg concentrations among shorebird species sampled at the same Arctic breeding site (Hargreaves et al. 2010, 2011, Perkins et al. 2016). A comprehensive Hg exposure assessment of Arctic-breeding shorebirds can help to identify factors influencing Hg concentrations in shorebirds and identify populations at the greatest risk for Hg exposure (Rimmer et al. 2005, Jackson et al. 2015).

Shorebirds forage primarily on invertebrates in wet habitats, where Hg is likely to be more bioavailable (Driscoll et al. 2007, Colwell 2010). Additionally, many species are long-distance migrants that stop-over and winter in regions of Central and South America that are at particular risk of increasing Hg contamination due to artisanal small-scale gold mining (UNEP 2013). Arctic-breeding shorebirds may face further risk due to the increasing deposition and methylation of Hg in the Arctic (Durnford et al. 2010, AMAP 2011, Stern et al. 2011, UNEP 2013). Hargreaves et al. (2010, 2011) found high blood Hg concentrations in some shorebird species breeding in the eastern Canadian Arctic and indicated that this exposure may be related to reduced reproductive success. Perkins et al. (2016) also found high blood Hg concentrations in breeding shorebirds sampled in northern Alaska. These studies indicate that high Hg exposure in breeding shorebirds may be widespread across the North American Arctic.

1.2.3.2 Model Avian Species

1.2.3.2.1 White Leghorn Chicken

White Leghorn Chicken (*Gallus gallus domesticus*) have been used previously to investigate avian MeHg toxicokinetics (Rutkiewicz and Basu 2013), as well as adverse health effects and sensitivity to Hg exposure (Heinz et al. 2009, Rutkiewicz et al. 2013). These studies indicate that

MeHg egg injection is a useful method for studying toxicity in Hg exposed birds. Chickens are large-bodied, granivorous birds and chicken physiology has been well documented. Based on MeHg egg injection studies, chickens are considered to be moderately sensitive to Hg exposure (Heinz et al. 2009). Additionally, fertilized chicken eggs are easily obtained commercially and can be successfully incubated and hatched under artificial conditions. Therefore, MeHg egg injection of chicken embryos can provide a useful model for understanding avian MeHg toxicokinetics and risk within a laboratory setting.

1.2.3.2.2 Zebra Finch

Zebra Finch (*Taeniopygia guttatato*) have been used as a model songbird species to investigate the effects of Hg exposure on reproduction, behavior, and physiology (Lewis et al. 2013, Moore et al. 2014, Varian-Ramos et al. 2014, Kobiela et al. 2015). They are small, granivorous birds that do well in captivity and are able to breed continuously with adequate resources. Simon Fraser University, Department of Biological Sciences currently has a captive breeding population which is used to further investigate negative health effects of Hg exposure on songbirds using both egg injection and chick dosing methods (Morran 2016, Yu et al. 2016).

1.2.3.3 Ring-billed Gull

Ring-billed Gulls (*Larus delawarensis*) are medium sized birds that are wide-spread in North America. They breed in colonies, laying their eggs in sparse ground nests. Ring-billed Gulls generally lay 3 eggs per clutch and do not fully incubate eggs until after the clutch is complete, with an incubation period ranging from 20 – 31 days (Pollet et al. 2012) In the wild, embryos are exposed to maternally-deposited Hg in the form of MeHg (Ackerman et al. 2013). Ring-billed Gulls are considered to be carnivorous year-round and insectivorous during the breeding season (De Graaf et al. 1985), however, they eat a broad diet that includes fish, earthworms and other

invertebrates, and grains (Pollet et al. 2012). There is an easily accessible nesting colony located near Beauharnois, QC, Canada (45.316052, -73.906218 degrees decimal).

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Preface to Chapter 2

Chapter 2 describes a large-scale, multi-species field-based study which examines mercury (Hg) exposure in breeding shorebird species sampled across the North American Arctic. Moving beyond simply reporting Hg concentrations in blood and feathers, an emphasis was placed on identifying factors that influence the concentrations of these biomarker tissues in non-piscivorous bird species to improve Hg exposure assessments. This was accomplished by comparing generalized linear mixed-effects models and generalized linear models of variables such as species, foraging habitat moisture classification, breeding site, sex, flight feather molt, body condition, and Hg turnover in blood (as reflected by capture day). This study also established current Hg concentrations in shorebird blood and feathers that can be used to evaluate the efficacy of future regulations to reduce global Hg emissions and identified regions and species at the greatest risk for Hg exposure.

This chapter is co-authored by the candidate's supervisor Dr. Niladri Basu, Dr. Iain Stenhouse, and Dr. Richard Lanctot, with the likely inclusion of additional project collaborators. It is planned for submission to *Environmental Science & Technology*.

Chapter 2

2 Factors Influencing Mercury Exposure in Arctic-breeding Shorebirds

2.1 Abstract

Mercury (Hg) is a pervasive environmental contaminant that presents a significant health hazard to exposed birds. Globally, atmospheric Hg releases are on the rise and Hg deposition in the Arctic is expected to increase if emissions are not reduced. Arctic-breeding shorebirds may represent an ideal group to use as bioindicators of Hg within the terrestrial Arctic. This research intends to identify regions within the terrestrial Arctic and shorebird species at the greatest risk for Hg exposure and to elucidate factors influencing Hg concentrations in shorebirds. We analyzed 2,478 blood and feather samples collected from 12 breeding shorebird species during 2012 and 2013. Sampling locations included five sites in Alaska located near Nome, Cape Krusenstern, Barrow, and the Ikpikpuk and Colville rivers; and four sites in Canada located at the Mackenzie River Delta, Bylot Island, Igloolik, and East Bay. Blood Hg concentrations in individual shorebirds ranged from 0.01 – 3.52 μg/g, with an overall mean of 0.30 ± 0.27 μg/g. They were influenced by species and study site, but not year, with birds sampled at Barrow having the greatest concentrations. Blood Hg concentrations were also influenced by foraging habitat moisture classification at the Barrow and East Bay study sites but there was no clear trend for the influence of foraging habitat between sites. We saw a weak decreasing relationship for blood Hg concentrations and Julian day and body mass. For a subset of species, blood Hg

concentrations for males were approximately 11 % greater than for females. We did not see a relationship between blood Hg concentrations and flight feather molt in Dunlin (*Calidris alpina*). Feather Hg concentrations were generally higher than blood Hg concentrations, ranging from 0.07 – 12.14 μg/g in individuals, with a mean of 1.14 ± 1.18 μg/g. Feather Hg concentrations were influenced by species and year. Mean feather Hg concentrations for males range from 4 % to 35 % greater than females based on species. For Long-billed Dowitchers (*Limnodromus scolopaceus*), mean feather Hg was 31 % greater in females than males. Most Arctic-breeding shorebirds had blood and feather Hg concentrations related to either background or low risk categories for adverse effects of Hg exposure. The greatest proportion of individuals sampled from higher risk categories were seen at the Barrow study site and among Long-billed Dowitchers and Pectoral Sandpipers (*Calidris melanotos*). Overall, this study indicated that some Arctic-breeding shorebirds may exceed concentrations that could result in impaired health.

2.2 Introduction

Mercury (Hg) is a pervasive environmental contaminant that presents a significant health hazard to exposed wildlife (UNEP 2013). Anthropogenic Hg emissions continue to rise globally and are highly mobile in the atmosphere, traveling great distances to be deposited in remote regions such as the Arctic (AMAP 2011, UNEP 2013, AMAP/UNEP 2015). Current projections predict a 5 % increase in atmospheric Hg deposition in the Canadian Arctic by 2020 if emissions are not reduced (Dastoor et al. 2015). Mercury deposited across the landscape can be converted into methylmercury (MeHg) by bacterial processes under anoxic conditions (Wiener et al. 2003). Methylmercury is a more toxic and biologically available form of Hg that magnifies through the food web (Rimmer et al. 2010, Lavoie et al. 2013). In birds and other wildlife, chronic Hg

exposure is a significant health risk, resulting in impaired physiology, behavior, and reproductive success (Evers et al. 2008, Seewagen 2010, Scheuhammer et al. 2011, Ackerman et al. 2016b).

The Minamata Convention on Mercury aims to reduce new Hg emissions (UNEP 2015) and evaluating its effectiveness (i.e., Articles 19 and 22) requires Parties to commit towards collaborative efforts to monitor Hg concentrations in biota (Evers et al. 2016, Gustin et al. 2016). Mercury exposure assessments in birds may aid in identifying regions with Hg contamination, gauging current contamination levels, and identifying future reductions (Schmeltz et al. 2011). Birds are considered to be effective bioindicator species and have been used to provide reference levels for Hg risk in a variety of systems, including marine habitats, coastal, estuarine, and freshwater wetlands, and upland forests (Goodale et al. 2008, Warner et al. 2010, Jackson et al. 2015). Bird blood and feathers are easily sampled with minimum risk to populations and Hg assimilates and accumulates rapidly in these tissues (Bearhop et al. 2000). The majority of total Hg (THg) within bird blood and feathers is in the form of MeHg (Rimmer et al. 2005, Edmonds et al. 2010).

Avian blood Hg concentrations are often suggested to reflect recent exposure as studies show that concentrations increase rapidly over time for birds fed a diet dosed with MeHg (Bearhop et al. 2000, Fournier et al. 2002, Bennett et al. 2009). During molt, MeHg can be transferred from blood to growing feathers quickly and feather concentrations can represent blood MeHg concentrations at the time of growth (Bearhop et al. 2000). In addition, MeHg can be remobilized from other body tissues and deposited in feathers (Honda et al. 1986, Braune 1987, Agusa et al. 2005). Due to this, feather Hg concentrations are often suggested to reflect exposure from the local environment during feather growth as well as overall body burdens (Wolfe et al. 1998; Evers et al. 2005). These differences in Hg assimilation may offer

information on Hg exposure at different time points and locations if both blood and feathers are sampled for migratory birds.

A large-scale Hg exposure assessment of Arctic-breeding shorebirds offers a unique opportunity to investigate Hg contamination and risk across an extensive geographic range. Shorebirds may represent an ideal group for Arctic Hg exposure research, as shorebirds are widespread across the Arctic during the breeding season. Most shorebirds inhabit similar nesting and foraging habitats in Arctic tundra wetlands and occupy the same foraging guild (invertivores, Table 2.1, Colwell 2010, Rodewald 2015). Mercury research on terrestrial avian invertivores is lacking in the Arctic (Scheuhammer et al. 2015), therefore an investigation of shorebird Hg concentrations fills an important knowledge gap in Arctic Hg exposure research. In addition, many species of North American shorebirds are declining, making them a group of notable conservation concern that may be at risk from Hg exposure (Brown et al. 2001, Morrison et al. 2006, Bart et al. 2007). Hargreaves et al. (2010, 2011) found high blood Hg concentrations in some shorebird species breeding in the eastern Canadian Arctic and indicated that this exposure may be related to reduced reproductive success. Perkins et al. (2016) also found high blood Hg concentrations in breeding shorebirds sampled in northern Alaska. These studies indicate that high Hg exposure in breeding shorebirds may be widespread across the North American Arctic.

This study aimed to establish current Hg concentrations in shorebird blood and feathers that can be used to evaluate the efficacy of future regulations to reduce global Hg emissions. Additionally, we aimed to identify species and regions within the terrestrial Arctic that are at the greatest risk for negative health effects of Hg. Finally, considerable variability in bird blood and feather Hg concentrations has been observed among and within species (Bond and Diamond 2008, Hargreaves et al. 2011). Therefore, this research aimed to identify factors that influence

Hg concentrations of bird blood and feathers. We predicted that factors such as moisture content of preferred foraging habitat within the Arctic tundra would influence shorebird blood Hg concentrations. Perkins et al. (2016) found higher blood Hg concentrations in Alaskan shorebirds that forage predominantly in wet habitats compared to those foraging predominantly in upland areas. Additionally, we predicted that sex, flight feather molt, body condition, and Hg turnover in blood (as reflected by capture day) would be associated with blood Hg concentrations. A better understanding of the factors that influence Hg concentrations in terrestrial invertivores will aid in the interpretation of how these concentrations relate to environmental Hg contamination.

2.3 Methods

2.3.1 Study Species and Sites

This study was in collaboration with the Arctic Shorebird Demographics Network (ASDN), a large-scale network currently researching shorebirds across the North American Arctic, and the Biodiversity Research Institute (BRI, Portland, Maine, USA), a non-profit organization specializing in Hg exposure in wildlife. Collaborating study sites in 2012 included five sites in Alaska located near Nome, Cape Krusenstern, Barrow, the Ikpikpuk River, and the Colville River, and three sites in Canada near the Mackenzie River Delta, Bylot Island, and East Bay (Figure 2.1). An additional Canadian study site, Igloolik, was included with the previously sampled sites in 2013. The ASDN biologists collected blood and feather samples for Hg analysis from adult shorebirds (hatched the prior summer or earlier) captured while conducting routine fieldwork during the breeding season. Shorebird nests were found using systematic area searches and behavioral cues of adults, as well as rope-dragging in suitable tundra areas (Naves et al. 2008). Most shorebirds were captured using bownets while birds were sitting on their nests,

incubating eggs (Bub 1995). A small number were sampled during pre-laying and while brooding. Additional capture methods were also used, including walk-in traps and mist nets (Bub 1995). Both individuals of mated pairs were sampled when possible. Additional data were collected by ASDN biologists for each shorebird sampled, where applicable, including date of capture, sex, flight feather molt pattern at sampling, and morphometric measurements. The latitude and longitude of each capture location were also recorded. A total of 12 species were sampled; the species and number of individuals sampled at each site were determined by species availability and nesting abundance (Table 2.2 and 2.3).

2.3.2 Sample Collection

Blood and feather samples were collected using standard protocols (Evers 2008). Approximately 50 to 100 µl of whole blood was collected in heparinized capillary tubes via puncture of the brachial vein using a small gauge needle. No more than 1 % of the birds' body weight in blood was collected during sampling (Fair et al. 2010). During field collection, whole blood samples were stored in coolers with ice packs. For sites with electric freezers (Cape Krusenstern, Barrow, Colville River, Mackenzie Delta, and Bylot Island), each capillary tube was sealed firmly on both ends with Critocaps (Leica Microsystems, Inc., Concord, Ontario, Canada), placed in a labeled 6 ml plastic vacutainer (BD, Franklin Lakes, New Jersey, USA) to prevent breakage, and stored in the freezer. At field sites with no freezer access (Nome, Ikpikpuk River, East Bay, and Igloolik), blood from each capillary tube was transferred to a labeled cryovial tube and stored in a dry-nitrogen filled cryoshipper that kept samples at -40 °C. One 10^{th} secondary feather was collected for all species except Dunlin (*Calidris alpina*), for Dunlin 5 black breast feathers were collected. The majority of feather samples were collected by removing the entire feather, however, a small number of feathers were cut at the superior umbilicus. All feather samples were

placed in a clean paper envelope and stored at ambient temperature until analysis. All samples collected in 2012 and 2013 were shipped to BRI's Mercury Laboratory at the end of each field season. Blood samples were shipped on ice in coolers and stored frozen (-20 °C) prior to analysis.

For all samples used in this research, capture, handling, and sampling of animals took place under applicable state and federal permits for wildlife research under BRI, the U.S. Geological Survey, and the Canadian Wildlife Service. All sampling followed protocols approved by the Institutional Animal Care and Use Committees or Animal Use Protocol Approval at the U.S. Fish and Wildlife Service and all associated universities. Samples from Canadian study sites were shipped to BRI under a U.S. Fish and Wildlife Service import permit (MB083478).

2.3.3 Sample Analysis

Whole blood and feather samples collected in 2012 and 2013 were analyzed for THg concentrations at BRI's Mercury Laboratory. Blood samples were transferred to a sample boat and weighed prior to analysis. Feather samples were wiped clean of visible debris and the entire feather sample was weighed and placed in a sample boat for analysis. Since the feathers were not washed prior to analysis there is a possibility of external surface Hg contamination, however, external contamination should be minimal compared to internal Hg sources (Goede and DeBruin 1986). All samples were analyzed with a direct Hg analyzer (DMA-80, Milestone Inc., Shelton, Connecticut, USA) using the U. S. Environmental Protection Agency Method 7473 (U.S. EPA 2007). Quality control samples were analyzed before and after every set of 30 samples and included one sample each of two Standard Reference Materials (SRM, DORM-3 fish protein and DOLT-4 fish liver; National Research Council of Canada), two method blanks, and one sample blank. A duplicate shorebird blood sample was analyzed after every set of 20 samples (n = 37).

A single secondary feather was collected from each individual shorebird and the entire feather was analyzed for THg, therefore no feather tissue remained for duplicate feather sample analysis for the majority of shorebirds sampled. Since multiple Dunlin breast feathers were collected, duplicate feather samples were analyzed for a small number of individual Dunlin (n = 8). Duplicate samples were averaged for all statistical analyses. All quality control analyses were considered acceptable if within ± 10 % of the expected Hg concentration. Total Hg concentrations for all sampled shorebirds are reported in micrograms per gram (μg/g) wet weight (ww) for blood and μg/g fresh weight (fw) for feathers.

2.3.4 Assigning Foraging Habitat, Sex, and Molt Score

All species sampled in this study can be assigned to the same foraging guild (i.e., invertivores). Additionally, these species tend to forage on similar major prey items across the Arctic tundra during the breeding season (Table 2.1). However, the preferred foraging habitat of these species can differ by moisture content. We grouped the shorebird species sampled in this study by the moisture content of their predominant foraging habitat during the breeding season, as indicated by the Birds of North America species accounts and field observations (Rodewald 2015, R.B. Lanctot personal observation). Foraging habitat moisture classification groups consisted of: 1) upland, which included Black-bellied Plover (*Pluvialis squatarola*) and American Golden-Plover (*Pluvalis dominica*), 2) intermediate, which included Ruddy Turnstone (*Arenaria interpres*), Black Turnstone (*Arenaria melanocephala*), Dunlin, Pectoral Sandpiper (*Calidris melanotos*), Semipalmated Sandpiper (*Calidris pusilla*), and Western Sandpiper (*Calidris mauri*), and 3) wet to aquatic, which included Long-billed Dowitcher (*Limnodromus scolopaceus*), Red-necked Phalarope (*Phalaropus lobatus*), and Red Phalarope (*Phalaropus fulicarius*, Table 2.1).

The sex of each shorebird was determined using either 1) breeding characteristics such as egg laying, brood patch, cloacal protuberance, and known sex of mate, 2) plumage and morphometric measurements, as outlined in Birds of North America species accounts and other literature (Sandercock 1998, Rodewald 2015), or 3) discriminant function equations or molecular analysis (Gates et al. 2013). Due to nest attendance behavior, samples were only obtained from male Red-necked Phalaropes and Red Phalaropes and female Pectoral Sandpipers; for all other species both sexes were sampled. Due to plumage similarities and overlap in morphometric measurements between sexes, we were unable to identify the sex for all individuals.

Dunlin were the only species sampled that molt their flight feathers during the breeding the season (Holmes 1966). Molt scores for each flight feather were determined based on the growth stage of the feather. Scores for individual feathers range from 0 to 5, with a score of 0 indicating the presence of an old feather (i.e. no molt occurring) and a score of 5 indicating a completely grown, new feather (Ginn and Melville 1983) The molt score of each primary, secondary, and tertiary feather was determined for Dunlin sampled at the Barrow and Colville River study sites. For each Dunlin, we summed the molt scores for all flight feathers to determine an overall individual flight feather molt score, where a higher score indicated a greater amount of flight feather molt had occurred at the time of sampling.

2.3.5 Statistical Analyses

2.3.5.1 General Overview

We report the mean, standard deviation, and range of Hg concentrations for blood and feathers from shorebird species sampled during each year at each location. We determined that the lognormal distribution best fit the blood and feather Hg data. Therefore, we fit generalized linear

mixed-effects models (GLMM) and generalized linear models (GLM) with lognormal distribution on non-transformed Hg concentrations. For all models, we started with the full model and models were compared and selected using Akaike's information criteria (AIC). To estimate the strength of the relationship for each model, we used explained deviance, a log-likelihood proxy for explained variance, calculated as: ((Null deviance - Residual deviance) / Null Deviance)*100 (Zuur et al. 2009)). Tukey's HSD post hoc tests are used as appropriate. Separate models were fit for blood and feather Hg concentrations and each model is described in further detail below. For model results, we present Hg concentrations as least squares means with standard errors or 95 % confidence intervals. Additionally, since only two Baird's Sandpipers (*Calidris bairdii*) were sampled, resulting in 1 blood and 1 feather sample, this species was excluded from all statistical analyses. Analyses were performed using R (R Core Team 2017) and significance was determined if $P < 0.05$.

2.3.5.2 Blood Hg Concentrations

2.3.5.2.1 Variability among species, study sites, and year

We investigated the influence of the three-way interaction term for species, study site, and year on blood Hg concentrations using GLM with lognormal distribution. We did not consider the effect of sex in this analysis since the sex sampled varied with species or was unknown for some individuals.

2.3.5.2.2 Influence of foraging habitat and study site

We used GLMM to assess the influence of foraging habitat moisture classification and study site on blood Hg concentrations of Arctic-breeding shorebirds. Using the Laplace Approximation, we fit models with lognormal distribution, since this is a preferred method for modeling mixed

effects in non-normal data with small means (means < 5, Bolker et al. 2009). We used the glmer function in package lme4 (Bates et al. 2016) in R (version 3.3.3, R Core Team 2017) to find the best-fitting model. We fit the full model using blood Hg concentration as the dependent variable, with foraging habitat, study site, and the interaction between foraging habitat and study site as fixed factors, and species and year as random factors.

2.3.5.2.3 Influence of capture day and body mass

We also used GLMM to assess the influence of capture day and body mass on blood Hg concentrations using the Laplace Approximation, with lognormal distribution. We fit the full model using blood Hg concentration as the dependent variable, with Julian capture day and body mass as fixed factors, and species and study site as random factors. Since Arctic-breeding shorebirds tend to have low lipid stores during the sampling period, we use body mass as an indicator of body condition (Jacobs et al. 2012). Julian capture day and body mass were tested for collinearity and were not correlated (correlation coefficient < 0.20).

2.3.5.2.4 Influence of sex

We investigated the influence of sex on blood Hg concentrations using only species for which both sexes were sampled and for which we could identify ≥ 10 males and females each. Therefore, we included American Golden-Plover, Dunlin, Semipalmated Sandpiper, Western Sandpiper, and Long-billed Dowitcher for this analysis. We used GLM with blood Hg concentration as the dependent variable and included sex, species, the interaction between sex and species in the full model.

2.3.5.2.5 Influence of flight feather molt in Dunlin

Additionally, we investigated the influence of flight feather molt on blood Hg concentrations for Dunlin sampled at the Barrow and Colville River study sites. We used GLM with blood Hg

concentration as the dependent factor, with flight feather molt score, study site, sex, and year included as variables in the full model.

2.3.5.3 Feather Hg Concentrations

2.3.5.3.1 Variability among species, study sites, and year

We investigated the influence of the three-way interaction term for species, study site, and year on feather Hg concentrations using GLM with lognormal distribution. We did not consider the effect of sex in this analysis since the sex sampled varied with species or was unknown for some individuals.

2.3.5.3.2 Variability between Dunlin subspecies

We sampled body feathers of two subspecies of Dunlin in this study: *C. a. arcticola* sampled at the Barrow, Ikpikpuk River, and Colville River study sites and *C. a. pacifica* sampled at Cape Krusenstern and Nome. These subspecies winter in different regions of the world, with *C. a. arcticola* wintering in East Asia and *C. a. pacifica* along the Pacific Coast of North America. We compared feather Hg concentrations between these two subspecies of Dunlin to investigate the influence of wintering region on feather Hg concentrations in Dunlin. We used feather Hg concentrations in a GLM that included subspecies, year, sex and the interaction between sex and subspecies.

2.3.5.3.3 Influence of sex

We investigated the influence of sex on feather Hg concentrations using GLM with feather Hg concentration as the dependent variable and sex, species, the interaction between sex and species in the full model. We included American Golden-Plover, Dunlin, Semipalmated Sandpiper,

Western Sandpiper, and Long-billed Dowitcher in this analysis since we were able to identify ≥ 10 males and females each for these species.

2.3.6 Mercury Risk Categories

Adverse effects of Hg exposure on birds have been well documented (Evers et al. 2008, Seewagen 2010, Scheuhammer et al. 2011, Ackerman et al. 2016b). However, laboratory studies have shown that Hg sensitivity can vary among bird species (Heinz et al. 2009), and risk benchmarks for most species, including shorebirds, are unknown. A comprehensive summary of Hg exposure benchmarks (translated to blood Hg-equivalent units) and risk categories, using wild bird and laboratory studies, was compiled by Ackerman et al. (2016b). We applied these risk categories to blood Hg concentrations found for Arctic-breeding shorebirds. We added an additional risk category based on findings from Jackson et al. (2011), which determined blood Hg concentrations of 0.7 μg/g were associated with a 10 % reduction in nest success for the Carolina Wren (*Thryothorus ludovicianus*). Therefore, we grouped shorebirds into 4 blood Hg risk categories: 1) <0.2 μg/g, background exposure levels; 2) 0.2 to <0.7 μg/g, low risk; 3) 0.7 to <1.0 μg/g, low to moderate risk; and 4) >1.0 μg/g, moderate risk. Only one individual, a Ruddy Turnstone sampled at Colville River, had a blood Hg concentration (3.52 μg/g) which would fall in the high risk category (>3.0 μg/g) identified by Ackerman et al. (2016b). This individual was included in the moderate risk category (>1.0 μg/g). We determined the percentage of individual shorebirds with blood Hg concentrations that correspond to each risk category for shorebirds sampled at each site.

Risk benchmarks based on feather Hg concentrations are even more limited. Jackson et al. (2011) also modeled the association of nest success and feather Hg concentrations for Carolina Wren, using concentrations for both body and tail feathers. They determined that Carolina Wren

body and tail feather Hg concentrations of 2.4 and 3.0 μg/g were associated with a 10% reduction in nest success, respectively. Additionally, body and tail feather Hg concentrations of 6.2 and 9.1 μg/g were associated with a 50% reduction in nest success. We sampled secondary flight feathers for most species, making comparisons with tail feather concentrations tenuous, however, we use these concentrations as a best estimate for flight feather Hg risk benchmarks. Therefore, we grouped shorebirds into 3 flight feather Hg risk categories: 1) <3.0 μg/g, low risk; 2) 3.0 to <9.1 μg/g, moderate risk; 3) >9.1 μg/g, high risk. We sampled body feathers for Dunlin only and grouped this species into body feather Hg risk categories: 1) <2.4 μg/g, low risk; 2) 2.4 to <6.2 μg/g, moderate risk; 3) >6.2 μg/g, high risk. We determined the percentage of individual shorebirds with feather Hg concentrations that correspond to each risk category for shorebirds sampled at each site.

2.4 Results

For this study, a total of 1472 Arctic-breeding shorebirds were sampled across the North American Arctic from 20 May to 22 July 2012, and again from 28 May to 23 July 2013 (Table 2.2 and 2.3). This resulted in Hg analysis of 1094 blood samples (578 in 2012 and 516 in 2013) and 1384 feather samples (673 in 2012 and 711 in 2013) from 12 shorebird species sampled at 9 study sites (Figure 2.1). We sampled the greatest number of individuals (n = 563) and the greatest number of species (n = 8) at the Barrow study site in northern Alaska, USA. Relatively lower sample sizes and species numbers were obtained for the study sites in eastern Canada, Bylot Island (n = 53, 2 species), Igloolik (n = 28, 3 species), and East Bay (n = 47, 3 species).

2.4.1 Blood Hg Concentrations

For blood Hg analyses, SRM sample concentrations were within ± 10 % of the expected Hg concentrations. Analytical precision for shorebirds blood samples, calculated as mean RPD, was 1.9 ± 1.6 % (n = 37). All quality control analyses were of acceptable quality according to U.S. Environmental Protection Agency guidelines (U.S. EPA. 2007). Blood Hg concentrations in individual Arctic-breeding shorebirds ranged between 0.01 μg/g (Dunlin sampled at Ikpikpuk River) and 3.52 μg/g (Ruddy Turnstone sampled at Colville River, Table 2.2). The mean blood Hg concentration for all shorebirds sampled in this study was 0.30 ± 0.27 μg/g. The mean blood Hg concentration in shorebirds from the Barrow study site (0.48 ± 0.29 μg/g) was 4.8 times greater than for the Ikpikpuk River site (0.10 ± 0.06 μg/g). Among species, the mean blood Hg concentration for Long-billed Dowitchers (0.74 ± 0.25 μg/g) was over 4.9 times greater than for American Golden-Plovers (0.15 ± 0.07 μg/g).

2.4.1.1 Variability Among Species, Study Sites, and Year

We examined the influence of the three-way interaction for species, study site, and year on blood Hg concentrations. We found that year was not a significant explanatory variable (P = 0.53) and the inclusion of this term did not improve the model. The model including the two-way interaction between species and study site had an explained deviance of 49 % and indicated differences among species and study sites (Figure 2.2). At the Barrow study site, we saw large differences among the species sampled, with Long-billed Dowitchers having an approximately 4.0 times greater least squares mean blood Hg concentration than that for American Golden-Plover and 2.3 times greater concentration than for Dunlin. Indeed, we saw that at each site where they were sampled, both American Golden-Plover and Dunlin had the lowest least square mean blood Hg concentration. At the Igloolik study site, the least squares mean blood Hg

concentration was approximately 2.2 times greater for Semipalmated Sandpipers than for American Golden-Plovers.

We also found that shorebird species sampled at the Barrow study had greater Hg exposure than those sampled at other study sites. Red-necked Phalaropes, sampled across 6 study sites, had a least squares mean blood Hg concentration at Barrow approximately 4.2 times greater than at Ikpikpuk River and 2.7 times greater than at Nome. We saw even greater differences in least squares mean blood Hg concentrations for Semipalmated Sandpipers, with those sampled at Barrow having 5.7 times and 3.9 times greater concentrations than those sampled at Ikpikpuk River and Nome, respectively. Additional comparisons for Semipalmated Sandpipers indicated that the least squares mean blood Hg concentration at Barrow was approximately 3.6 and 3.4 times greater than that at Cape Krusenstern and Mackenzie River Delta, respectively.

2.4.1.2 Influence of foraging habitat and study sites

We found that the full model including foraging habitat moisture classification, study site, and the interaction between foraging habitat and study site as fixed factors, and species and year as random factors best explained blood Hg concentrations in Arctic-breeding shorebirds. A model with the interaction term removed, indicated that foraging habitat was not a significant factor influencing blood Hg concentrations across all study sites (post-hoc comparisons: upland/intermediate, $P = 0.34$; wet/intermediate, $P = 0.98$; wet/upland, $P = 0.37$). However, when the interaction term was included, we observed that blood Hg concentrations were influenced by foraging habitat at the Barrow and East Bay study sites only (Figure 2.3). This interaction indicated there is not a clear trend for the influence of foraging habitat on blood Hg concentrations for the Arctic-breeding shorebirds. The model results show the least squares mean

blood Hg concentrations are greatest for the wet habitat classification at East Bay (0.56 µg/g, 95 % CI: 0.28 - 1.12), but greatest for the intermediate habitat classification at Barrow (0.61 µg/g, 95 % CI: 0.40 - 0.91). Additionally, the inclusion of the interaction term also indicated that blood Hg concentrations differed among study sites in relation to foraging habitat. We found that the Barrow site had the greatest least squares mean blood Hg concentration for species foraging in intermediate habitat, while East Bay had the greatest concentrations for both upland (0.34 µg/g, 95 % CI: 0.17 - 0.69) and wet foraging species. Bylot had the lowest least squares mean blood Hg concentration for the upland habitat (0.11 µg/g, 95 % CI: 0.05 - 0.26) while Ikpikpuk River, followed by Nome, had the lowest blood Hg concentrations for the intermediate (0.11 µg/g, 95 % CI: 0.07 - 0.20 and 0.19 µg/g, 95 % CI: 0.12 - 0.32, respectively) and wet habitats (0.11 µg/g, 95 % CI: 0.05 - 0.26 and 0.19 µg/g, 95 % CI: 0.09 - 0.40, respectively).

2.4.1.3 Influence of capture day and body mass

We investigated the influence of Julian capture day and body mass on blood Hg concentrations, with a full model that included Julian capture day and body mass as fixed factors, and species and study site as random factors. This model indicated a significant, decreasing relationship for blood Hg concentrations and both Julian day ($P = 0.003$) and body mass ($P = 0.03$) for shorebirds sampled in this study. We found that for both Julian day and body mass, the decrease in blood Hg concentrations differed by species and by study site. However, the relationship for this model was relatively weak, with an explained deviance of approximately 2 %.

2.4.1.4 Influence of sex

We examined the influence of sex on blood Hg concentrations for American Golden-Plover, Dunlin, Semipalmated Sandpiper, Western Sandpiper, and Long-billed Dowitcher. We found the interaction between sex and species was not significant in the full model. The best model

included sex and species as the explanatory variables, with an explained deviance of 28 %. Overall, blood Hg concentrations for males were approximately 11 % greater than for females, though within each species, sexes did not differ significantly (Figure 2.4).

2.4.1.5 Influence of flight feather molt in Dunlin

The overall model investigating the influence of flight feather molt on blood Hg concentrations for Dunlin was significant, however, neither flight feather molt score ($P = 0.61$) nor year ($P = 0.21$) were explanatory variables within the model. The best model explaining blood Hg concentrations for Dunlin sampled at Barrow and Colville River included only study site and sex, with an explained deviance of 23 %. From this model, we determined that the least squares mean blood Hg concentration for Dunlin was approximately 47 % greater at Barrow (0.32 ± 0.01 μg/g) than at Colville River (0.17 ± 0.02 μg/g, $P < 0.0001$). Additionally, least squares mean blood Hg concentration was approximately 15 % greater in male Dunlin (0.25 ± 0.02 μg/g) than in females (0.21 ± 0.02 μg/g, $P = 0.03$).

2.4.2 Feather Hg Concentrations

For feather Hg analyses, SRM sample concentrations were within ± 10 % of the expected Hg concentrations. Analytical precision for Dunlin feather samples, calculated as mean RPD, was 15.1 ± 11.7 % ($n = 8$). All quality control analyses were of acceptable quality according to U.S. Environmental Protection Agency guidelines. Feather Hg concentrations for Arctic-breeding shorebirds were generally greater than those seen for blood, with a mean feather Hg concentration of 1.14 ± 1.18 μg/g for all samples analyzed. We found feather Hg concentrations to be wide-ranging across individual shorebirds, with the lowest concentration of 0.07 μg/g seen for a Semipalmated Sandpiper and the highest concentration of 12.14 μg/g for a Long-billed

Dowitcher, both sampled at the Barrow study site (Table 2.3). As with blood Hg concentrations, mean feather Hg concentrations were greatest at the Barrow study site (1.35 ± 1.48 μg/g) and lowest at the Ikpikpuk River site (0.75 ± 0.61 μg/g), with concentrations 1.8 times greater at the Barrow site. Feather Hg concentrations also differed by species, with the mean feather Hg concentration for Pectoral Sandpipers (2.58 ± 1.76 μg/g) over 4.3 times greater than for Red Phalaropes (0.60 ± 0.44 μg/g).

2.4.2.1 Variability Among Species, Study Sites, and Year

We examined the influence of the three-way interaction for species, study site, and year on feather Hg concentrations. We found that the model was not improved by the inclusion of the study site, and this variable was removed from the model. The model including the two-way interaction between species and year had an explained deviance of 21 %. This model indicated that variability in feather Hg concentrations between 2012 and 2013 differed by species sampled (Figure 2.5). In both 2012 and 2013, Pectoral Sandpipers had the greatest least squares mean feather Hg concentration, while Red Phalaropes had the lowest, though the extent of the difference varied by year. In 2012, Pectoral Sandpipers had approximately 3.6 times greater least squares mean feather Hg concentration than Red Phalaropes, while in 2013 the concentration was 5.3 times greater for Pectoral Sandpipers.

2.4.2.2 Variability between Dunlin subspecies

We examined the influence of wintering region on body feather Hg concentrations for two subspecies of Dunlin, *C. a. arcticola* that winter in East Asia and *C. a. pacifica* that winter along the Pacific Coast of North America. We found the least squares mean feather Hg concentration was greater for *C. a. arcticola* (male: 1.57 ± 0.10 μg/g, female: 1.45 ± 0.11 μg/g) than for *C. a. pacifica* (male: 1.18 ± 0.20 μg/g, female: 1.36 ± 0.22 μg/g). However, neither subspecies (P =

0.69), sex (P = 0.39), nor the interaction between subspecies and sex (P = 0.38) were significant explanatory variables within the model. The best model explaining feather Hg concentrations for Dunlin included year only, though the relationship was weak, with an explained deviance of 4 %. The least squares mean feather Hg concentration was approximately 21 % greater in 2013 than in 2012 for Dunlin sampled in this study.

2.4.2.3 Influence of sex

We examined the influence of sex on feather Hg concentrations for American Golden-Plover, Dunlin, Semipalmated Sandpiper, Western Sandpiper, and Long-billed Dowitcher. We found the best model included sex, species, and the interaction between sex and species as the explanatory variables, with an explained deviance of 9 %. Overall, least squares mean feather Hg concentrations for males range from 4 % greater than for females in Dunlin to 35 % greater than for females for Western Sandpiper (Figure 2.6). Long-billed Dowitcher was the only species for which the females least squares mean feather Hg concentration was greater than males, with a concentration approximately 31 % greater for females.

2.4.3 Mercury Risk Categories

Overall, most Arctic-breeding shorebirds had blood Hg concentrations related to the background and low risk categories for adverse effects of Hg exposure, with approximately 45 % of individuals falling into the background category and 47 % falling into the low risk category (Table 2.2). We found a low proportion of individuals with blood Hg concentrations within the low to moderate and the moderate risk categories, approximately 5 % and 3 % respectively, with the greatest proportion sampled at the Barrow study site (Figure 2.7). The Cape Kruesenstern, Ikpikpuk River, Mackenzie River Delta, and Bylot Island sites did not have any individual

shorebirds with blood Hg concentrations falling within these risk categories. Long-billed Dowitcher had the greatest proportion of individuals that fell in the low to moderate risk and moderate risk categories, with approximately 22 % and 17 %, respectively (Table 2.2). Pectoral Sandpiper also had approximately 15 % and 8 % of individuals within these respective categories. While individual American Golden-Plover, Baird's Sandpiper, Black-bellied Plover, and Black Turnstone were found to have blood Hg concentrations only within the background and low risk categories.

Feather Hg concentrations also indicated that the majority of Arctic-breeding shorebirds sampled in this study were at low risk for adverse effects from Hg exposure. Approximately 6 % of individuals had feather Hg concentrations that fell within the moderate and high risk categories, with 94 % of individuals falling into the low risk category (Table 2.3). Only the Cape Krusenstern and Barrow study sites had individual shorebirds with feather Hg concentrations in the high risk category (Figure 2.8). Four species had individuals with feather Hg concentrations in the high risk category: Dunlin (<1 %), Long-billed Dowitcher (~3 %), Semipalmated Sandpiper (<1 %), and Western Sandpiper (~1 %, Table 2.3). Pectoral did not have any individuals within the high risk category but had the greatest proportion of individuals within the moderate risk category (27 %). While individual Baird's Sandpiper, Black-bellied Plover, and Red-necked Phalarope had feather Hg concentrations in the low risk category only.

2.5 Discussion

We utilized a large-scale collaborative network, to collect and analyze nearly 2500 blood and feather samples over two breeding seasons. This dataset provides Hg concentrations for shorebird blood and feathers that can serve as baseline measurements to evaluate future

reductions in global Hg emissions. Previous large-scale Hg exposure assessments of birds have converted Hg concentrations from various tissues to blood-equivalent units for comparisons, or have used differing sample collection, preparation, and analysis methods (Jackson et al. 2015, 2016, Ackerman et al. 2016b). The current study used standardized sample collection methods, analysis methods, and quality control practices for all blood and feather samples, offering an advantage for comparisons among samples. In addition, previous studies have found that foraging guild and ecoregion (ie. freshwater wetlands, upland forests, salt marsh, etc.) are important factors influencing blood and feather Hg concentrations (Keller et al. 2014, Jackson et al. 2015, Ackerman et al. 2016b). Since the shorebird species sampled in this study forage in Arctic tundra wetlands and occupy the same foraging guild (invertivores, Table 2.1, Colwell 2010, Rodewald 2015), we were able to investigate additional factors influencing Hg concentrations without having to account for ecoregion and foraging guild differences.

2.5.1 Blood Hg Concentrations

Overall, shorebirds sampled in this study had low blood Hg concentrations, though we found elevated exposure at some study sites and for certain species. A previous study investigated Hg exposure in breeding shorebirds at the Barrow study site during 2008 and 2009 (Perkins et al. 2016). In comparison to the current study, mean blood Hg concentrations were similar for American Golden-Plovers (2009: 0.18 ± 0.20 µg/g) and Red Phalaropes, though concentrations were variable among years for this species (2008: 0.66 ± 0.36 µg/g, 2009: 0.43 ± 0.34 µg/g). Pectoral Sandpipers and Semipalmated Sandpipers sampled in the current study had approximately 18 % (2009: 0.68 ± 0.66 µg/g) and 31 % (2009: 0.95 ± 0.62 µg/g,) lower mean blood Hg concentrations, respectively, than those sampled in the previous study. In contrast, Long-billed Dowitchers and Dunlin had approximately 46 % (2009: 0.53 ± 0.20 µg/g) and 56 %

(2009: 0.21 ± 0.09 μg/g) greater mean Hg concentrations, respectively, in the current study. Overall, both studies found elevated blood Hg concentrations for shorebirds sampled at this site.

Blood Hg concentrations were also determined for Arctic-breeding shorebirds at the East Bay study site in 2008 and 2009 (Hargreaves et al. 2010, 2011). In comparison to the current study, these past studies found similar mean blood Hg concentrations for both Black-bellied Plovers (means ranged from 0.34 - 0.43 μg/g) and Red Phalaropes (0.51 ± 0.07 μg/g). However, the previous studies found higher mean blood Hg concentrations for Ruddy Turnstones (means ranged from 0.52 - 0.68 μg/g), with an approximately 36 % greater concentration seen in 2009 compared to 2012. Comparisons with these previous studies indicate similar Hg exposure for shorebirds sampled at these two study sites, however, concentrations for some species varied among years.

2.5.1.1 Variability Among Species, Study Sites, and Year

We saw differences in blood Hg concentrations among species sampled within the same study site. The previous studies investigating blood Hg concentrations at Barrow and East Bay found similar differences among species of Arctic-breeding shorebirds (Hargreaves et al. 2010, 2011, Perkins et al. 2016). In the current study, we saw common trends in blood Hg concentrations among species, regardless of sampling location. For example, at each study site where American Golden-Plover and Dunlin were sampled, they had low mean blood Hg concentrations compared to other species. Similarly, species comparisons indicated Pectoral and Semipalmated sandpipers had greater mean blood Hg concentrations. This study further emphasizes the importance of species, even within the same foraging guild and ecoregion, for using birds as bioindicators of Hg risk in a system. These results suggest that sampling a single species such as American Golden-Plover may not identify potential hotspots for Hg exposure, such as Barrow, since this

species had low concentrations across sites. In contrast, the inclusion of Semipalmated Sandpipers in this study highlighted the potential for high Hg exposure at Barrow.

We found a relationship between blood Hg concentrations and breeding season sampling sites, indicating that breeding site exposure is an important factor influencing blood Hg concentrations. However, differences in blood Hg concentrations among species combined with differences in species sampled at each site make determining Hg exposure risk across study sites difficult. For instance, this study sampled only American Golden-Plover at the Bylot study site, since this species had low Hg concentrations across study sites, we are uncertain of the Hg exposure risk at Bylot. Future sampling of additional species at this site might provide a better understanding of the Hg exposure risk at this site. Additionally, low sample sizes and number of species sampled at both the Igoolik and East Bay site also hinder understanding of Hg exposure risk at these sites. Overall, we found blood Hg concentrations were the greatest for species sampled at the Barrow study site, while the Ikpikpuk site, located near Barrow, had the lowest blood Hg concentrations. Further research is needed to determine why species breeding at the Barrow study site had higher Hg exposure, particularly when compared to the nearby Ikpikpuk site.

2.5.1.2 Influence of foraging habitat and study sites

Mercury methylation occurs readily under anoxic conditions, often making Hg more biologically available in aquatic environments where anaerobic conditions are common (Wiener et al. 2003). Recent studies focused on Hg exposure in invertebrate-eating songbirds suggest that within the terrestrial environment, wetter foraging habitats are related to greater blood and feather Hg concentrations (Keller et al. 2014, Jackson et al. 2015). Therefore, we expected shorebird species foraging more frequently in wetter habitats to have greater blood Hg concentrations. Indeed, the

previous study at Barrow found greater blood Hg concentrations for species foraging predominately in wetter habitats (Perkins et al. 2016). However, in the current study, we found moisture content of preferred foraging habitat influenced blood Hg concentrations at the Barrow and East Bay study sites only and there was no consistent pattern between sites. While upland foraging species at Barrow had the lowest blood Hg concentrations, intermediate foraging species had the lowest blood Hg concentrations at East Bay. Differences in the species sampled between the Barrow (n = 8 species) and Easy Bay (n = 3 species) study sites may have played a role in the conflicting pattern seen between the sites. These results indicate that other foraging differences among shorebird species may have a greater influence on blood Hg concentrations than foraging habitat moisture content.

While shorebirds sampled in this study generally forage on similar major prey items (Table 2.1), differences in foraging strategy among species may influence blood Hg concentrations. Little is known about the distribution of MeHg concentrations among invertebrates at the Arctic study sites, however, MeHg concentrations in invertebrates have been shown to differ by developmental stage, foraging behavior, trophic level, and feeding depth (Chetelat et al. 2008, Sizmur et al. 2013, Clayden et al. 2014). Therefore, differences among shorebird species in foraging and specific foods taken may play a role in blood Hg concentrations. For instance, Long-billed Dowitchers, which had the greatest blood Hg concentrations, forage by probing with their long bills (Takekawa and Warnock 2000) and may forage on invertebrates located deeper in sediments. Sizmur et al. (2013) found polychaete worms in the Bay of Fundy located deeper in sediments had greater MeHg concentrations than those closer to the sediment surface. In contrast, American Golden-Plover forage by sight using the "run-stop-peck" strategy to take prey near the substrate surface (Johnson and Connors 2010).

It is currently unknown how these foraging differences may influence blood Hg concentrations of Arctic-breeding shorebirds. Further research is needed to better understand MeHg distribution within Arctic invertebrate food webs and how shorebird species differ in foraging on these invertebrates during the breeding season.

2.5.1.3 Influence of capture day and body mass

We found a significant, but weak relationship that indicated blood Hg concentrations for Arctic-breeding shorebirds decreased over the sampling period. This result may suggest shorebirds showed carry over of Hg exposure from wintering grounds or stop-over sites to the breeding season and have lower Hg exposure on the Arctic breeding grounds. Previous research found that blood Hg concentrations showed carry over from the winter season to the breeding season for two migratory species, Double-crested Cormorant (*Phalacrocorax auritus*) and Caspian Tern (*Hydroprogne caspia*), though the amount of carry over differed between species (Lavoie et al. 2014). While this may also occur for Arctic-breeding shorebirds, this explanation is contradicted by the much stronger relation found between blood Hg concentrations and breeding site. While migratory connectivity is not well known for most Arctic-breeding shorebirds, a recent study using geolocators on Semipalmated Sandpipers compared migratory pathways and wintering locations for individuals from 6 breeding sites used in the current study (Brown et al. 2017). While sample sizes were low (n = 3) for the Ikpikpuk River site, results indicated that Semipalmated Sandpipers breeding at Barrow and Ikpikpuk River follow similar migration routes and use the same stop-over and wintering sites. Due to this, the large differences in blood Hg concentrations found between Semipalmated Sandpipers sampled at the Barrow and Ikpikpuk River study sites indicate that breeding location is a main factor influencing these concentrations. Additionally, the previous study at the East Bay study site found a significant increasing

relationship between blood Hg concentrations and sampling date (Hargreaves et al. 2011), indicating further study may be needed.

Many aspects of Hg toxicokinetics in wild birds, particularly long-distance migrants, are poorly understood. A recent laboratory study on Zebra Finch (*Taeniopygia guttata*) indicated that tissue catabolism during flight may result in increased blood Hg concentrations (Seewagen et al. 2016). Findings determined an average decrease in lean mass of 12 % resulted in an increase in blood Hg concentrations of over 10 %. Long-distance migrants are likely to have much greater lean mass catabolism during migration. Therefore, the negative relationship between blood Hg concentrations and sampling date observed in the current study could be the result of Hg mobilization due to tissue catabolism during migration. This could result in elevated blood Hg concentrations upon arrival at the breeding grounds. Further research on the effects of long-distance migration on Hg toxicokinetics and blood Hg concentrations in birds may be beneficial for interpreting blood Hg concentrations in migrating birds.

We found a significant, but weak trend indicating that blood Hg concentrations decreased with increasing body mass for shorebirds sampled in this study. Previous studies have also found a negative relationship between body condition measurements and Hg exposure for diving ducks and rails sampled in coastal California (Takekawa et al. 2002, Ackerman et al. 2012). However, the previous study on Arctic-breeding shorebirds at East Bay found no relationship between blood Hg concentrations and body condition (body mass/culmen length, Hargreaves et al. 2010). In the current study, we were not able to ascertain the underlying cause for the observed relationship. There may be a causal effect of Hg exposure on body mass, however, previous research found reduced blood Hg concentrations as body mass increased in growing chicks (Ackerman et al. 2011), though this dilution effect has not been documented in adult birds.

Additionally, sex could be a factor, female shorebirds are generally larger than males and had lower blood Hg concentrations. While we were unable to account for sex in our analysis, future studies should further investigate the influence of sex on this relationship. Overall, we suggest that the relationship between blood Hg concentrations and body mass may be complicated by multiple factors, and should be further investigated.

2.5.1.4 Influence of sex

We investigated the influence of sex on blood Hg concentrations for a subset of species sampled in this study (American Golden-Plover, Dunlin, Semipalmated Sandpiper, Western Sandpiper, and Long-billed Dowitcher). We found males had greater blood Hg concentrations than females. Most shorebirds in this study were sampled during the incubation period and females had recently completed egg laying. Therefore, the lower blood Hg concentrations found in females were likely due to MeHg deposition in eggs (Robinson et al. 2012). A previous study found male Dunlin breeding in Alaska had greater blood Hg concentrations compared to females, but no difference in concentrations was found between sexes for Semipalmated Sandpipers (Perkins et al. 2016). Additionally, blood Hg concentrations for breeding shorebirds sampled at East Bay did not differ significantly by sex, but mean concentrations were greater for males than females for Black-bellied Plovers and Ruddy Turnstone, though not for Semipalmated Plovers (*Charadrius semipalmatus*, Hargreaves et al. 2010). Previous studies have found the proportion of MeHg that females deposit into eggs can differ by species and with increasing Hg exposure (Robinson et al. 2012, Ackerman et al. 2016a). However, we found a similar difference in blood Hg concentrations between males and females for all species included in this analysis. This research further indicates that sex is an important factor influencing blood Hg concentrations and should be considered when using birds as bioindicators of Hg risk in a system.

2.5.1.5 Influence of flight feather molt in Dunlin

Blood Hg concentrations have been shown to quickly decrease during feather molt (Bearhop et al. 2000). Dunlin were the only species undergoing flight feather molt during this study, and the extent of flight feather molt was recorded for Dunlin sampled at the Barrow and Colville River study sites. We examined the influence of flight feather molt on blood Hg concentrations but did not find a significant relationship. In general, Dunlin had low blood Hg concentrations at both study sites, which may affect the influence of molt on blood Hg concentrations. Examining the relationship between flight feather molt and blood Hg concentrations in birds with a broader range of Hg exposure may help to better understand how molt affects Hg concentrations in wild birds. Additionally, the method for determining flight feather molt score is standardized, however, there may have been variability in how molt was scored among researchers that we were unable to account for in this study. Lastly, it is possible that we did not find a relationship because Dunlin acquired Hg from their diet in an amount that offset any decrease in blood Hg concentrations due to feather molt.

2.5.2 Feather Hg Concentrations

This study aimed to sample shorebird feathers that were likely grown on the wintering grounds. Since the shorebirds sampled in this study molt their flight feathers sequentially on the wintering grounds, the 10th secondary was sampled for all species except Dunlin (Rodewald 2015). Dunlin was the only species sampled that molt flight feathers during the breeding the season (Holmes 1966), therefore, we sampled black breast feathers that were likely to have been grown on the wintering grounds. Overall, shorebirds sampled in this study had greater feather Hg concentrations than blood Hg concentrations, with elevated feather concentrations seen for some individuals. The previous study conducted at the Barrow study site determined feather Hg

concentrations for breeding Semipalmated Sandpipers and Red Phalaropes in 2008 (Perkins et al. 2016), and found average feather Hg concentrations approximately 20 % greater for both species than in the current study (Semipalmated Sandpiper: 1.12 ± 0.70 μg/g, Red Phalarope: 0.76 ± 0.50 μg/g). We can also compare mean feather Hg concentrations in Arctic-breeding shorebirds sampled at the East Bay study site in 2008 and 2009 (Hargreaves et al. 2010, 2011). In comparison to the current study, these previous studies also found greater mean feather Hg concentrations for both Ruddy Turnstones (between 41 and 63 % greater) and Red Phalaropes (23 % greater). However, mean feather Hg concentrations for Black-bellied Plover varied widely among years with no trend between the studies. Overall, comparisons with previous studies indicated variable feather Hg concentrations for Arctic-breeding shorebirds sampled at these two study sites.

2.5.2.1 Variability Among Species, Study Sites, and Year

We saw differences in feather Hg concentrations among species and between years for Arctic-breeding shorebirds sampled in this study. Arctic-breeding shorebirds sampled during migratory stop-over in Delaware Bay also indicated differences in feather Hg concentrations among species and sampling years (Burger et al. 2015). Similarly, variability in feather Hg concentrations (breast feathers) among years was observed for Semipalmated Sandpipers migrating through Delaware Bay from 2010 to 2012 (Burger et al. 2014). In the current study, we found different trends among species for blood and feather Hg concentrations. Comparisons among species indicated Pectoral Sandpipers and Long-billed Dowitchers had higher mean Hg concentrations for both blood and feathers. In contrast, Semipalmated Sandpipers had higher blood Hg concentrations, but lower feather Hg concentrations, while we found the opposite trend for Dunlin. Since feather Hg concentrations are often thought to reflect exposure from the local

environment during feather growth (Wolfe et al. 1998; Evers et al. 2005), these results may indicate that some Arctic-breeding shorebird species have different levels of Hg exposure on the wintering grounds compared to the breeding grounds.

We did not see a relationship between feather Hg concentrations and breeding season sampling sites, further indicating that wintering site exposure is an important factor influencing feather Hg concentrations. An example of this may be seen for the phalarope species, both of which had among the lowest feather Hg concentrations seen in this study. These species are pelagic during the non-breeding season and foraging within a low trophic level, planktivorous marine food web (Rubega et al. 2000, Tracy et al. 2002). Therefore, they likely have low Hg exposure during the wintering season, which was reflected in their feather Hg concentrations. Further research on migratory connectivity for species and individuals may provide a better understanding of year-round Hg exposure for Arctic-breeding shorebirds.

2.5.2.2 Variability between Dunlin subspecies

We examined the influence of wintering region on body feather Hg concentrations for two subspecies of Dunlin, *C. a. arcticola* that winter in East Asia and *C. a. pacifica* that winter along the Pacific Coast of North America. While we found mean feather Hg concentrations to be slightly greater for *C. a. arcticola*, there was not a significant relationship between subspecies and feather Hg concentrations. These results indicate that wintering region is not a factor influencing feather Hg concentrations in the Dunlin sampled in this study.

2.5.2.3 Influence of sex

We investigated the influence of sex on feather Hg concentrations for a subset of species sampled in this study (American Golden-Plover, Dunlin, Semipalmated Sandpiper, and Western Sandpiper), and found males had greater feather Hg concentrations than females for most

species. The difference between sexes varied among species and we found Long-billed Dowitcher females had greater feather Hg concentrations than males. The previous study at the East Bay site did not find that feather Hg concentrations differed by sex for Black-bellied Plovers, Ruddy Turnstones, and Semipalmated Plovers. Feather Hg concentrations for Dunlin (*C. a. pacifica*) sampled in British Columbia, Canada, prior to breeding also did not differ by sex (St. Clair et al. 2015). While we assume that the influence of sex on feather Hg concentrations is due to intrinsic differences, extrinsic differences may also play a role, since little is known about how males and females partition resources on the wintering grounds.

2.5.3 Mercury Risk Categories

Mercury exposure in birds has been associated with a variety of adverse health effects, however, the toxicity benchmark at which effects occur can vary among species (Evers et al. 2008, Seewagen 2010, Scheuhammer et al. 2011, Ackerman et al. 2016b). A recent review integrated the available literature on thresholds for adverse effects determined in multiple bird species and created toxicity benchmarks for Hg exposure based on blood-equivalent units (Ackerman et al. 2016b). While the risk categories determined by this review are not specifically developed for shorebirds, they are currently the best available benchmarks for adverse health effects of Hg exposure for the birds sampled in this study. Additionally, we aimed to improve these risk categories by including an additional category specifically based on adverse effects (reduced reproduction) seen for an avian invertivore (Jackson et al. 2011). Overall, Arctic-breeding shorebirds sampled in this study had blood Hg concentrations related to the background and low risk categories for adverse effects of Hg exposure. However, the greatest proportion of individuals at greater risk for Hg exposure were sampled at the Barrow study site, indicating that this may be an area of concern for Arctic-breeding shorebirds. Additionally, certain species such

as Long-billed Dowitchers and Pectoral Sandpipers, may be at greater risk of adverse effects of Hg exposure.

Limited data are available for toxicity benchmarks for Hg exposure based on feather Hg concentrations. Here, we developed risk categories based on adverse effect thresholds determined for a single species of avian intertivore (Jackson et al. 2011), therefore, these risk categories are not well developed for shorebirds. Additionally, we sampled different feather types, making comparisons problematic. However, similar to results found for blood Hg concentrations, we found most shorebirds sampled in this study had feather Hg concentrations related to a low risk of adverse effects of Hg exposure.

2.5.4 Conclusion

This study investigated factors influencing Hg exposure in Arctic-breeding shorebirds by utilizing a large-scale collaborative network to collect and analyze nearly 2500 blood and feather samples. Additionally, this broad data set provides baseline measurements that can aid in evaluating changes in Hg emissions over time. We determined that overall, Arctic-breeding shorebirds were at low risk of adverse effects of Hg exposure. However, blood Hg concentrations were influenced by species and study site and birds sampled at the Barrow study were at the greatest risk for adverse effects of Hg exposure. Differences in concentrations among species indicated that some species were at greater risk of adverse effects of Hg exposure than others. While blood Hg concentrations were influenced by foraging habitat moisture classification for some study sites, no clear trend was determined. We found blood Hg concentrations were also influenced by sex, body mass, and Hg turnover (as reflected by capture day). Feather Hg concentrations were also influenced by species and sex. This research indicates that many factors influence blood and feather Hg concentrations, therefore, care should be taken

when using birds as bioindicators of Hg risk within a system. This study suggests that including multiple species from the same foraging guild and ecoregion in Hg exposure assessments may better elucidate regions of high Hg exposure. The influence of multiple factors on Hg concentrations for a group terrestrial invertivores determined in this study can aid in the interpretation of how these concentrations relate to environmental Hg contamination for future studies.

2.6 Acknowledgments

The research was a large collaborative effort with the Biodiversity Research Institute (BRI) and the Arctic Shorebird Demographics Network (ASDN). We thank BRI collaborators Drs. David Evers, Iain Stenhouse, and Lisa Ferguson, and Jennifer Goyette and Kevin Regan, and the ASDN steering committee, Drs. Stephen Brown, Richard Lanctot, and Brett Sandercock. We thank the many ASDN principle investigators, field coordinators, and technicians that collected samples in remote regions of North American Arctic. River Gates provided help with sample and shipping logistics in 2012. Additionally, Michelle St. Peters, Scot Williamson, and Meghan Gilbart provided help with project logistics. Dr. Jessica O'Connell generously provided guidance on statistical analyses. This project was funded by the U.S. Fish and Wildlife Service (USFWS), via a cooperative agreement (Award # F12AP00704) under the Migratory Bird Project Support: Monitoring, Assessment, Conservation program (15.655) in 2012, and a grant award (FWS 2013-01) through the Avian Health and Disease program (F12PX03583) in 2013. Additional funding was provided by Fulbright Canada (Perkins), as well as the Canada Research Chair program and NSERC Discovery Grant (Basu).

2.7 Tables and Figures

Table 2.1 Overview of breeding season foraging habitat, guild, and main food taken for Arctic shorebird species. Information adapted from The Birds of North America species account for each species (Rodewald 2015), with input on moisture classification of predominate foraging habitat (foraging habitat moisture classification) from R.B. Lanctot (personal observation).

Species	Foraging Habitat (Moisture Classification)	Foraging Habitat Description	Guild	Main Foods Taken	Citation
Black-bellied Plover (*Pluvialis squatarola*)	Upland	Open tundra	Invertivore	Insects: Diptera; Coleoptera; Lepidoptera, Ephemeroptera; Trichoptera Some plant matter	Poole et al. 2016
American Golden-Plover (*Pluvialis dominica*)	Upland	Various types of tundra	Invertivore; primarily terrestrial	Insects: Various adult and larval insects Berries	Johnson and Connors 2010
Ruddy Turnstone (*Arenaria*	Intermediate	Dry to mesic tundra; near to	Invertivore	Insects: Diptera, primarily Chironomidae	Nettleship 2000

interpres)		ponds and streams; wet habitats after hatch		Some plant matter	
Black Turnstone (*Arenaria melanocephala*)	Intermediate	Wet sedge meadows; shrub mat and alpine tundra	Invertivore; aquatic and terrestrial	Insects: Diptera; Coleoptera Some seeds	Handel and Gill 2001
Dunlin (*Calidris alpina*)	Intermediate	Rims and troughs of polygons; hummocks; wet marsh; edges of lakes and ponds	Invertivore; freshwater, marine, and terrestrial	Insects: Diptera, primarily Chironomidae and Tipulidae larvae	Warnock and Gill 1996
Pectoral Sandpiper (*Calidris melanotos*)	Intermediate	Flat, grass-sedge meadows; edges of tundra polygons (wetter than	Invertivore; freshwater, marine, and terrestrial	Insects: Diptera, primarily Chironomidae and Tipulidae	Farmer et al. 2013

		Dunlin)			
Semipalmated Sandpiper (*Calidris pusilla*)	Intermediate	Edges of lakes and ponds	Invertivore; benthic and terrestrial	Insects: Diptera, primarily Chironomidae and Tipulidae Arachnids Some seeds	Hicklin and Gratto-Trevor 2010
Western Sandpiper (*Calidris mauri*)	Intermediate	Wet mud and shallow water Tundra; littoral zone of ponds	Invertivore; benthic and terrestrial	Insects: Diptera, primarily larval and pupal Muscidae, Chironomidae, and Tipulidae; Coleoptera Arachnids; Grazer on biofilms (diatoms)	Franks et al. 2014
Long-billed Dowitcher (*Limnodromus scolopaceus*)	Wet to aquatic	Shallow water of interior wetlands and wet meadows	Invertivore; benthic, aquatic, and terrestrial	Insects: Diptera, primarily Chironomidae larvae; Some plant matter	Takekawa and Warnock 2000

Red-necked Phalarope (*Phalaropus lobatus*)	Wet to aquatic	Aquatic, small pools and ponds	Invertivore; aquatic	Insects: Diptera, primarily adult and larval Chironomidae, and Tipulidae Small invertebrates; Copepods	Rubega et al. 2000
Red Phalarope (*Phalaropus fulicarius*)	Wet to aquatic	Shallow water at pond edges	Invertivore; aquatic and terrestrial	Insects: Diptera, primarily adult and larval Chironomidae, and Tipulidae	Tracy et al. 2002

Table 2.2. Comparisons of blood total Hg concentrations (arithmetic mean, standard deviation (SD), range, and sample size, n) for all Arctic shorebird species, sampled at each study site during 2012 and 2013, with the percent of samples in each Hg risk category (years combined where applicable) adapted from toxicity benchmarks proposed by Ackerman et al. (2016b) and Jackson et al. (2011).

						Hg Exposure Risk Categories			
						Background	Low	Low to Moderate	Moderate
Study Site	Species	Year	n	Blood Hg (SD)	Blood Hg Range	<0.20	0.20 - 0.70	0.70 - 1.0	1.0
Nome	Dunlin	2012	1	0.33	·	0 %	100 %	0 %	0 %
	Pectoral Sandpiper	2012	5	0.23 (0.10)	0.16 - 0.41	40 %	60 %	0 %	0 %
	Semipalmated Sandpiper	2012	21	0.17 (0.10)	0.05 - 0.44	73 %	27 %	0 %	0 %
		2013	16	0.16 (0.09)	0.05 - 0.33				
	Western Sandpiper	2012	15	0.26 (0.09)	0.16 - 0.48	57 %	43 %	0 %	0 %
		2013	13	0.11 (0.07)	0.03 - 0.30				
	Red-necked Phalarope	2012	16	0.11 (0.03)	0.07 - 0.18	91 %	7 %	0 %	2 %
		2013	28	0.19 (0.29)	0.04 - 1.55				
Cape Krusenstern	Black Turnstone	2012	15	0.20 (0.13)	0.11 - 0.63	63 %	38 %	0 %	0 %
		2013	1	0.20	·				
	Dunlin	2012	13	0.15 (0.03)	0.09 - 0.21	94 %	6 %	0 %	0 %
		2013	19	0.13 (0.05)	0.03 - 0.27				
	Semipalmated Sandpiper	2012	34	0.18 (0.04)	0.11 - 0.27	76 %	24 %	0 %	0 %
		2013	8	0.17 (0.04)	0.13 - 0.24				
	Western Sandpiper	2012	28	0.21 (0.05)	0.11 - 0.35	57 %	43 %	0 %	0 %
		2013	21	0.17 (0.04)	0.09 - 0.23				
	Red-necked Phalarope	2012	10	0.17 (0.08)	0.07 - 0.30	70 %	30 %	0 %	0 %
Barrow	American Golden-Plover	2012	20	0.20 (0.07)	0.10 - 0.40	62 %	38 %	0 %	0 %
		2013	14	0.16 (0.07)	0.09 - 0.35				

	Dunlin	2012	32	0.31 (0.14)	0.15 - 0.76	13 %	83 %	3 %	0 %
		2013	57	0.33 (0.14)	0.09 - 0.70				
	Pectoral Sandpiper	2012	34	0.58 (0.36)	0.20 - 2.04	0 %	72 %	18 %	10 %
		2013	26	0.54 (0.29)	0.23 - 1.09				
	Semipalmated Sandpiper	2012	34	0.70 (0.33)	0.18 - 1.53	1 %	59 %	24 %	15 %
		2013	40	0.61 (0.29)	0.25 - 1.56				
	Western Sandpiper	2012	17	0.43 (0.15)	0.27 - 0.73	0 %	94 %	0 %	0 %
		2013	16	0.40 (0.09)	0.24 - 0.58				
	Long-billed Dowitcher	2012	23	0.68 (0.17)	0.39 - 1.01	0 %	61 %	22 %	17 %
		2013	13	0.87 (0.33)	0.49 - 1.49				
	Red-necked Phalarope	2012	10	0.48 (0.35)	0.26 - 1.43	0 %	93 %	0 %	7 %
		2013	4	0.34 (0.08)	0.29 - 0.46				
	Red Phalarope	2012	36	0.42 (0.23)	0.12 - 1.12	10 %	74 %	12 %	4 %
		2013	32	0.54 (0.31)	0.20 - 1.60				
Ikpikpuk River	Dunlin	2012	21	0.06 (0.03)	0.01 - 0.12	100 %	0 %	0 %	0 %
		2013	11	0.11 (0.03)	0.07 - 0.19				
	Semipalmated Sandpiper	2012	46	0.08 (0.04)	0.03 - 0.20	90 %	10 %	0 %	0 %
		2013	31	0.16 (0.06)	0.10 - 0.32				
	Red-necked Phalarope	2012	15	0.07 (0.03)	0.03 - 0.17	96 %	4 %	0 %	0 %
		2013	12	0.15 (0.11)	0.08 - 0.49				
	Red Phalarope	2012	19	0.07 (0.03)	0.04 - 0.16	100 %	0 %	0 %	0 %
		2013	13	0.14 (0.03)	0.09 - 0.19				
Colville River	Ruddy Turnstone	2013	12	0.61 (0.95)	0.13 - 3.52	33 %	50 %	0 %	17 %
	Dunlin	2012	12	0.17 (0.04)	0.13 - 0.24	80 %	20 %	0 %	0 %
		2013	13	0.18 (0.05)	0.13 - 0.31				
	Semipalmated Sandpiper	2012	14	0.25 (0.08)	0.17 - 0.41	38 %	63 %	0 %	0 %
		2013	2	0.18 (0.01)	0.17 - 0.19				
	Red-necked Phalarope	2013	8	0.26 (0.09)	0.13 - 0.39	38 %	63 %	0 %	0 %
	Red Phalarope	2012	13	0.26 (0.09)	0.11 - 0.38	23 %	77 %	0 %	0 %
Mackenzie	Pectoral Sandpiper	2013	7	0.36 (0.21)	0.15 - 0.66	29 %	71 %	0 %	0 %

River Delta	Semipalmated Sandpiper	2012	16	0.19 (0.04)	0.12 - 0.27	64 %	36%	0 %	0 %
		2013	20	0.19 (0.05)	0.11 - 0.28				
	Red-necked Phalarope	2012	15	0.24 (0.08)	0.13 - 0.45	13 %	87 %	0 %	0 %
		2013	23	0.37 (0.10)	0.21 - 0.64				
Bylot Island	American Golden-Plover	2012	23	0.13 (0.05)	0.06 - 0.30	96 %	4 %	0 %	0 %
		2013	24	0.11 (0.03)	0.08 - 0.22				
	Pectoral Sandpiper	2013	1	0.32	·	0 %	100 %	0 %	0 %
Igloolik	American Golden-Plover	2013	9	0.20 (0.06)	0.10 - 0.32	56 %	44 %	0 %	0 %
	Baird's Sandpiper	2013	1	0.11	·	100 %	0 %	0 %	0 %
	Semipalmated Sandpiper	2013	7	0.43 (0.18)	0.26 - 0.79	0 %	86 %	14 %	0 %
East Bay	Black-bellied Plover	2012	6	0.33 (0.06)	0.25 - 0.41	0 %	100 %	0 %	0 %
		2013	2	0.40 (0.06)	0.36 - 0.44				
	Ruddy Turnstone	2012	14	0.38 (0.22)	0.16 - 1.00	13 %	81 %	0 %	6 %
		2013	2	0.50 (0.06)	0.46 - 0.55				
	Red Phalarope	2013	10	0.52 (0.13)	0.28 - 0.66	0 %	100 %	0 %	0 %

Table 2.3. Comparisons of feather total Hg concentrations (arithmetic mean, standard deviation (SD), range, and sample size, n) for all Arctic shorebird species, sampled at each study site during 2012 and 2013, with the percent of samples in each Hg risk category (years combined where applicable) adapted from toxicity benchmarks determined for the Carolina Wren (Jackson et al. 2011).

						Hg Exposure Risk Categories		
						Low	Moderate	High
Study Site	Species	Year	n	Feather Hg (SD)	Feather Hg Range	<3.0	3.0 - 9.1	>9.1
Nome	Dunlin	2012	2	0.88 (0.41)	0.59 - 1.17	100 %	0 %	0 %
	Pectoral Sandpiper	2012	7	1.66 (0.89)	0.66 - 3.40	86 %	14 %	0 %
	Semipalmated Sandpiper	2012	38	0.93 (0.66)	0.28 - 3.66	99 %	1 %	0 %
		2013	35	0.97 (0.66)	0.18 - 2.54			
	Western Sandpiper	2012	38	0.97 (0.58)	0.17 - 2.44	99 %	1 %	0 %
		2013	30	1.03 (0.92)	0.15 - 4.68			
	Red-necked Phalarope	2012	44	1.03 (0.64)	0.30 - 2.42	100 %	0 %	0 %
		2013	33	0.60 (0.43)	0.24 - 2.17			
Cape Krusenstern	Black Turnstone	2012	14	1.69 (1.39)	0.51 - 4.69	84 %	16 %	0 %
		2013	5	1.32 (1.66)	0.39 - 4.27			
	Dunlin	2012	14	1.17 (0.71)	0.47 - 2.54	94 %	3 %	3 %
		2013	19	1.41 (1.32)	0.49 - 6.38			
	Semipalmated Sandpiper	2012	34	0.79 (0.60)	0.17 - 2.70	100 %	0 %	0 %
		2013	27	0.72 (0.50)	0.18 - 2.10			
	Western Sandpiper	2012	27	1.74 (2.83)	0.13 - 10.24	86 %	12 %	2 %
		2013	30	1.46 (1.13)	0.12 - 4.42			
	Red-necked Phalarope	2012	9	0.70 (0.33)	0.34 - 1.33	100 %	0 %	0 %
Barrow	American Golden-Plover	2012	20	0.60 (0.25)	0.37 - 1.41	100 %	0 %	0 %
		2013	16	0.75 (0.38)	0.23 - 1.71			
	Dunlin	2012	24	1.66 (1.20)	0.10 - 5.34	82 %	18 %	0 %

	Pectoral Sandpiper	2013	60	1.72 (0.79)	0.57 - 4.87	70 %	30 %	0 %
		2012	40	2.31 (1.76)	0.56 - 6.67			
		2013	33	3.21 (1.92)	0.94 - 7.37			
	Semipalmated Sandpiper	2012	55	0.96 (1.30)	0.24 - 9.25	99 %	1 %	1 %
		2013	81	0.86 (0.63)	0.07 - 2.90			
	Western Sandpiper	2012	19	1.64 (1.71)	0.19 - 6.21	81 %	17 %	2 %
		2013	33	1.93 (2.40)	0.17 - 10.13			
	Long-billed Dowitcher	2012	22	2.19 (2.59)	0.31 - 12.14	92 %	6 %	3 %
		2013	14	1.07 (0.77)	0.31 - 2.42			
	Red-necked Phalarope	2012	12	0.46 (0.11)	0.24 - 0.71	100 %	0 %	0 %
		2013	5	0.46 (0.21)	0.26 - 0.80			
	Red Phalarope	2012	51	0.68 (0.67)	0.14 - 4.00	99 %	1 %	0 %
		2013	40	0.50 (0.15)	0.21 - 0.84			
Colville River	Ruddy Turnstone	2013	14	2.25 (2.04)	0.84 - 7.29	71 %	29 %	0 %
	Dunlin	2012	12	1.36 (0.47)	0.80 - 2.72	83 %	17 %	0 %
		2013	17	1.92 (1.01)	0.86 - 4.96			
	Semipalmated Sandpiper	2012	14	0.80 (0.49)	0.34 - 1.83	100 %	0 %	0 %
		2013	2	0.83 (0.16)	0.72 - 0.94			
	Red-necked Phalarope	2013	8	0.43 (0.25)	0.27 - 1.02	100 %	0 %	0 %
	Red Phalarope	2012	13	0.51 (0.35)	0.21 - 1.54	100 %	0 %	0 %
Ikpikpuk River	Dunlin	2012	21	1.02 (0.46)	0.49 - 1.87	100 %	0 %	0 %
		2013	11	1.32 (0.29)	0.92 - 1.81			
	Semipalmated Sandpiper	2012	46	0.71 (0.75)	0.18 - 5.18	98 %	2 %	0 %
		2013	36	0.85 (0.77)	0.27 - 4.79			
	Red-necked Phalarope	2012	15	0.50 (0.24)	0.18 - 1.03	100 %	0 %	0 %
		2013	16	0.61 (0.57)	0.24 - 2.30			
	Red Phalarope	2012	19	0.53 (0.27)	0.24 - 1.19	100 %	0 %	0 %
		2013	16	0.54 (0.27)	0.22 - 1.20			
Mackenzie River Delta	Pectoral Sandpiper	2013	8	2.23 (0.90)	0.88 - 3.94	88 %	13 %	0 %
	Semipalmated	2012	17	1.04 (0.61)	0.14 - 2.01	100 %	0 %	0 %

	Sandpiper	2013	20	0.61 (0.38)	0.13 - 1.73			
	Red-necked Phalarope	2013	23	0.68 (0.48)	0.21 - 1.78	100 %	0 %	0 %
Bylot Island	American Golden-Plover	2012	26	0.92 (0.54)	0.31 - 2.27	94 %	6 %	0 %
		2013	25	1.27 (1.13)	0.34 - 4.34			
	Pectoral Sandpiper	2013	1	1.91	·	100 %	0 %	0 %
Igloolik	American Golden-Plover	2013	15	1.41 (1.41)	0.35 - 5.83	87 %	13 %	0 %
	Baird's Sandpiper	2013	1	2.38	·	100 %	0 %	0 %
	Semipalmated Sandpiper	2013	11	0.59 (0.52)	0.30 - 2.10	100 %	0 %	0 %
East Bay	Black-bellied Plover	2012	6	1.70 (0.84)	0.29 - 2.61	100 %	0 %	0 %
		2013	6	0.86 (0.43)	0.31 - 1.55			
	Ruddy Turnstone	2012	14	1.15 (0.87)	0.27 - 3.29	95 %	5 %	0 %
		2013	5	0.73 (0.22)	0.49 - 0.93			
	Red Phalarope	2013	15	0.77 (0.34)	0.37 - 1.33	100 %	0 %	0 %

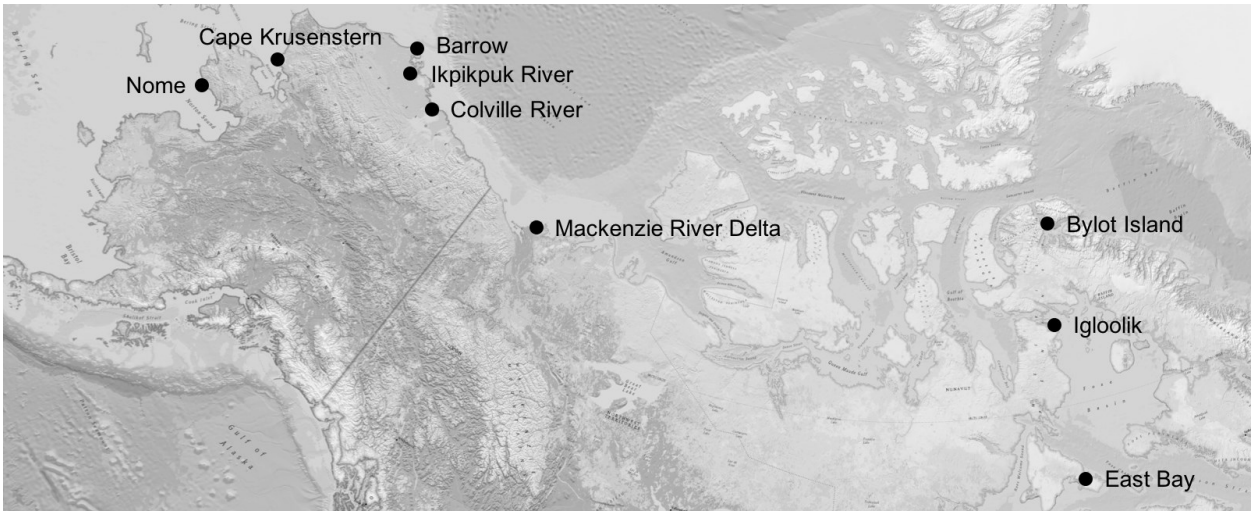
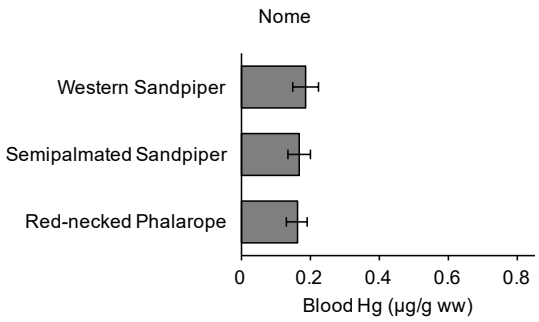
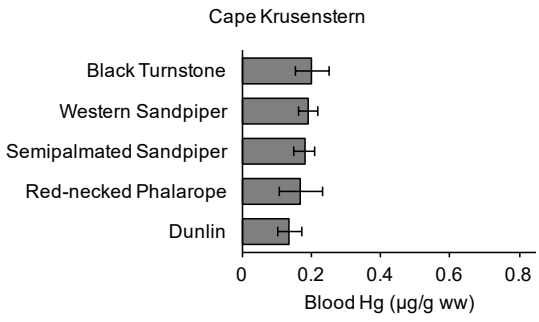
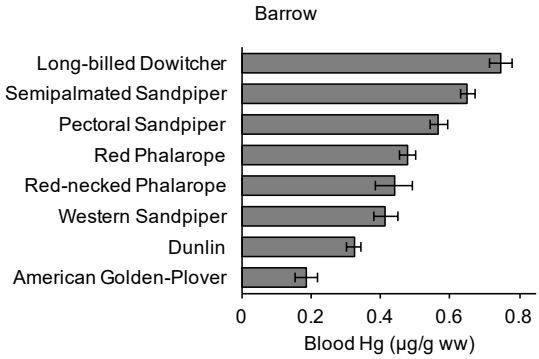
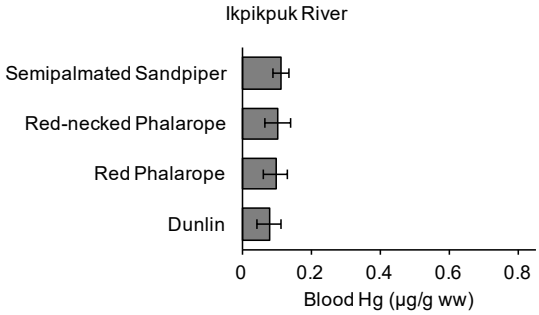


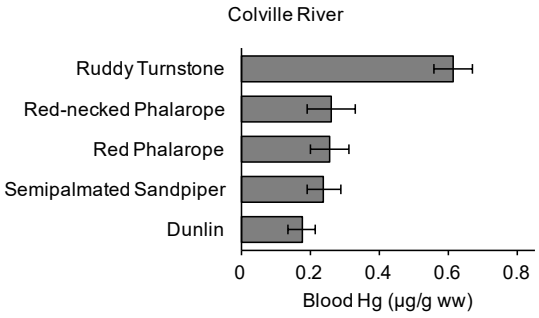
Figure 2.1. Arctic Shorebird Demographics Network shorebird breeding sites where blood and feather samples were collected from adult shorebirds in 2012 and 2013 for Hg analysis.

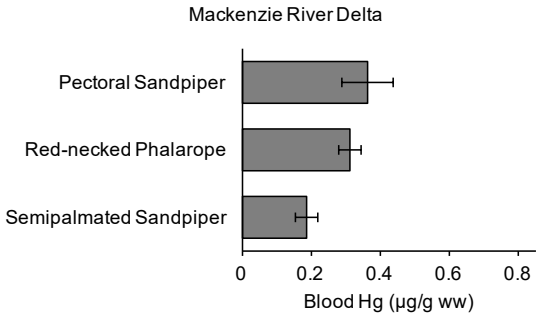

Nome
Western Sandpiper
Semipalmated Sandpiper
Red-necked Phalarope
0
0.2
0.4
0.6
0.8
Blood Hg (µg/g ww)

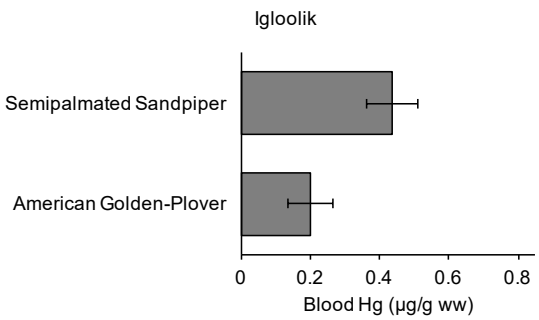

Cape Krusenstern
Black Turnstone
Western Sandpiper
Semipalmated Sandpiper
Red-necked Phalarope
Dunlin
0
0.2
0.4
0.6
0.8
Blood Hg (µg/g ww)


Barrow
Long-billed Dowitcher
Semipalmated Sandpiper
Pectoral Sandpiper
Red Phalarope
Red-necked Phalarope
Western Sandpiper
Dunlin
American Golden-Plover
0
0.2
0.4
0.6
0.8
Blood Hg (µg/g ww)


Ikpikpuk River
Semipalmated Sandpiper
Red-necked Phalarope
Red Phalarope
Dunlin
0
0.2
0.4
0.6
0.8
Blood Hg (µg/g ww)


Colville River
Ruddy Turnstone
Red-necked Phalarope
Red Phalarope
Semipalmated Sandpiper
Dunlin
0
0.2
0.4
0.6
0.8
Blood Hg (µg/g ww)


Mackenzie River Delta
Pectoral Sandpiper
Red-necked Phalarope
Semipalmated Sandpiper
0
0.2
0.4
0.6
0.8
Blood Hg (µg/g ww)


Igloolik
Semipalmated Sandpiper
American Golden-Plover
0
0.2
0.4
0.6
0.8
Blood Hg (µg/g ww)

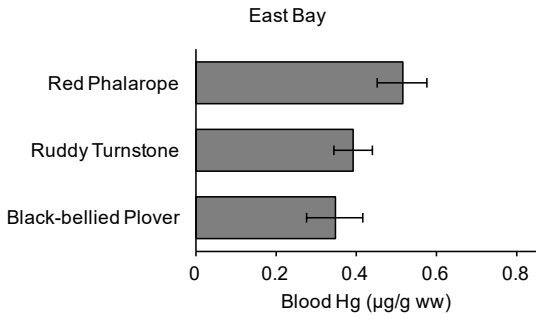

East Bay
Red Phalarope
Ruddy Turnstone
Black-bellied Plover
0
0.2
0.4
0.6
0.8
Blood Hg (µg/g ww)

Figure 2.2. Comparisons of blood total Hg concentrations (least squares mean and standard error) among shorebird species sampled at Arctic-breeding study sites during 2012 and 2013. Sexes and years combined where applicable.

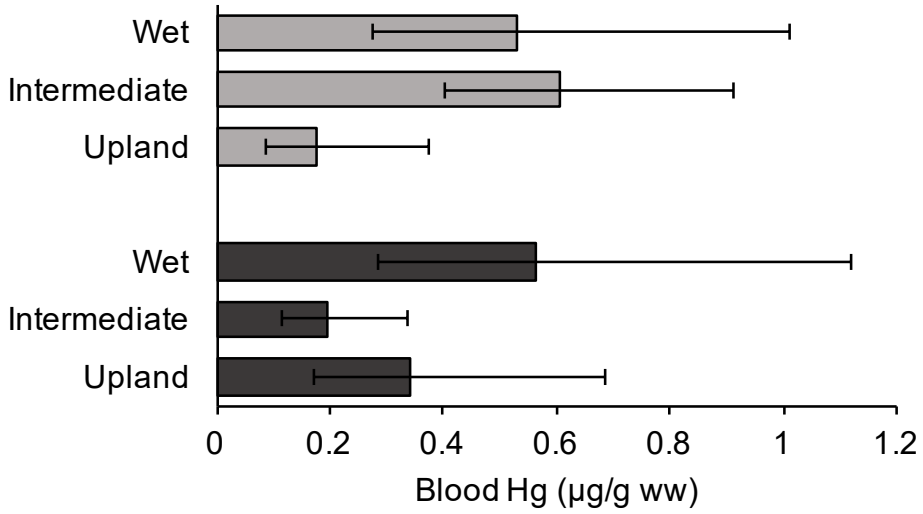


Figure 2.3. Comparisons of blood total Hg concentrations (least squares mean and 95% confidence intervals) by preferred foraging habitat moisture classification for Arctic-breeding shorebirds sampled at the Barrow (light grey) and East Bay (dark grey) study sites.

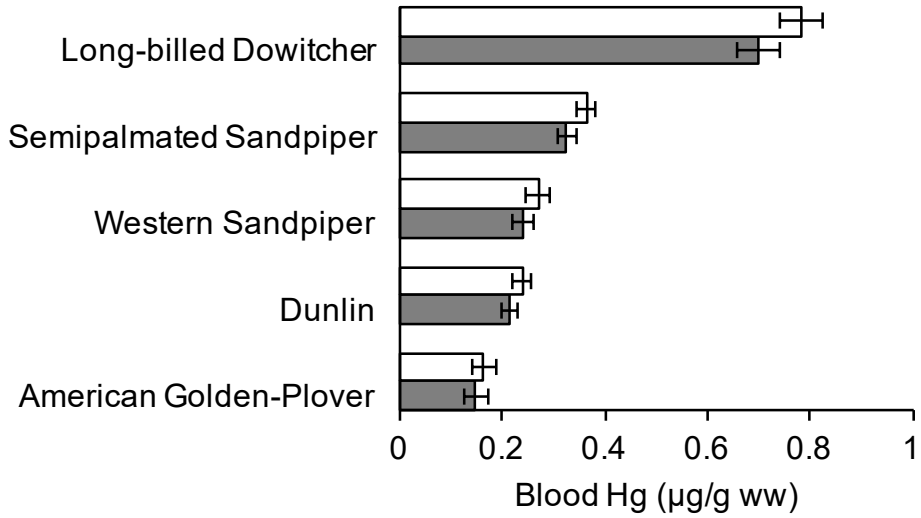


Figure 2.4. Comparisons of blood total Hg concentrations (least squares mean and standard error) between male (white bars) and female (grey bars) shorebirds sampled at Arctic-breeding study sites during 2012 and 2013. Years and study sites combined for all species.

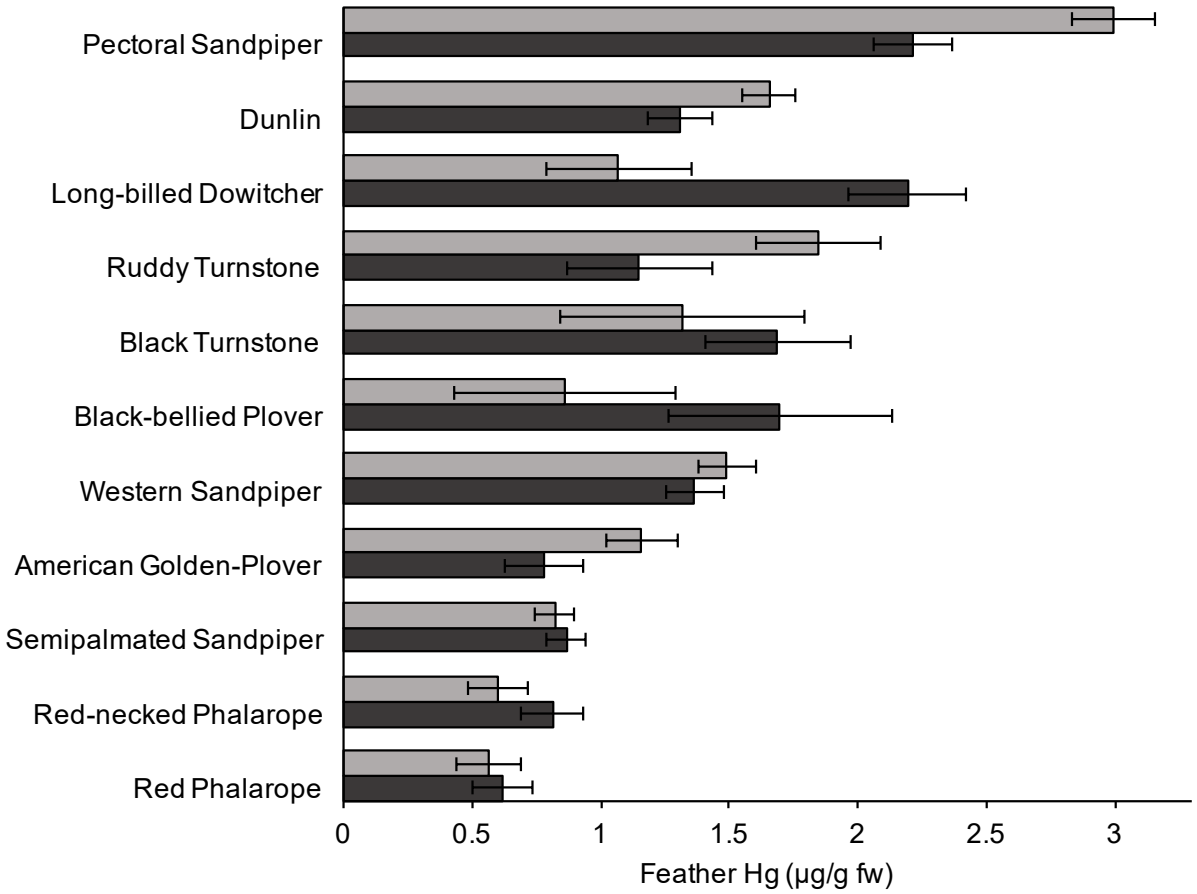


Figure 2.5. Comparisons of feather total Hg concentrations (least squares mean and standard error) among shorebird species sampled at Arctic-breeding study sites during 2012 (dark grey bars) and 2013 (light grey bars). Concentrations differed by species and by year ($P < 0.05$). Study sites and sexes combined where applicable.

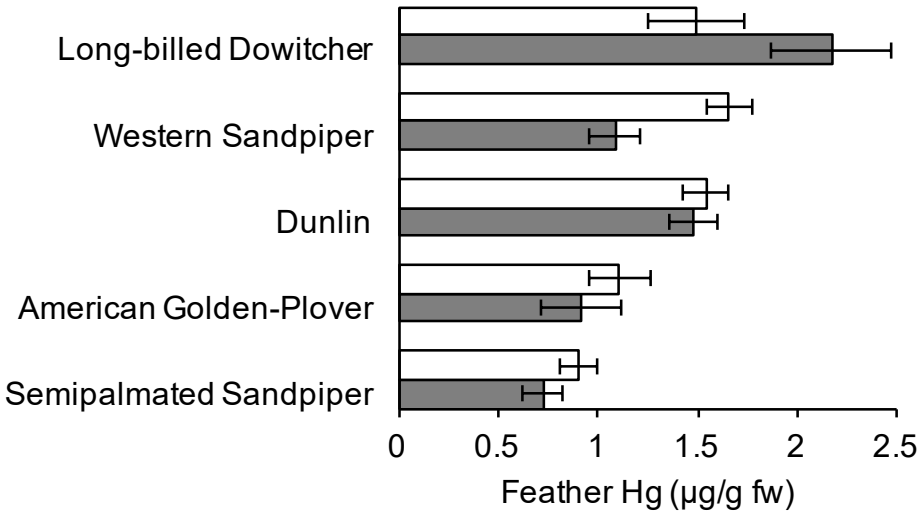


Figure 2.6. Comparisons of feather total Hg concentrations (least squares mean and standard error) between male (white bars) and female (grey bars) shorebirds sampled at Arctic-breeding study sites during 2012 and 2013. Years and study sites combined for all species.

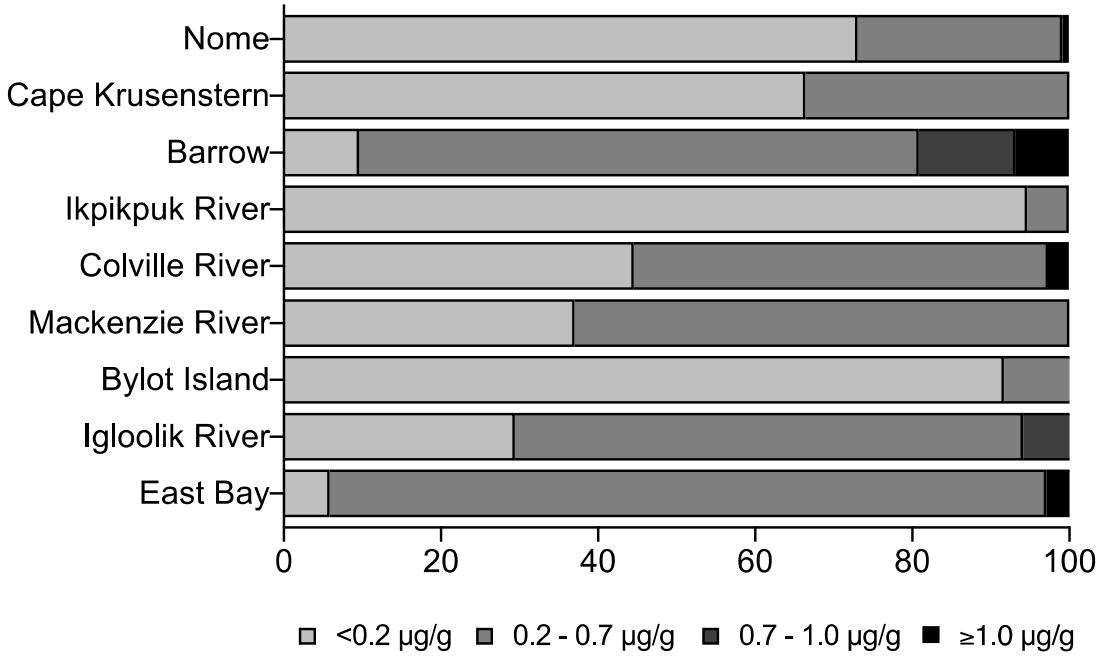


Figure 2.7. Comparison of the percentage of individual shorebirds at each study site with blood Hg concentrations falling within risk categories for adverse effects of Hg exposure. All species and years included. Risk categories: 1) <0.2 µg/g, background exposure levels; 2) 0.2 to <0.7 µg/g, low risk; 3) 0.7 to <1.0 µg/g, low to moderate risk; and 4) >1.0 µg/g, moderate risk (adapted from Jackson et al. 2011, Ackerman et al. 2016b).

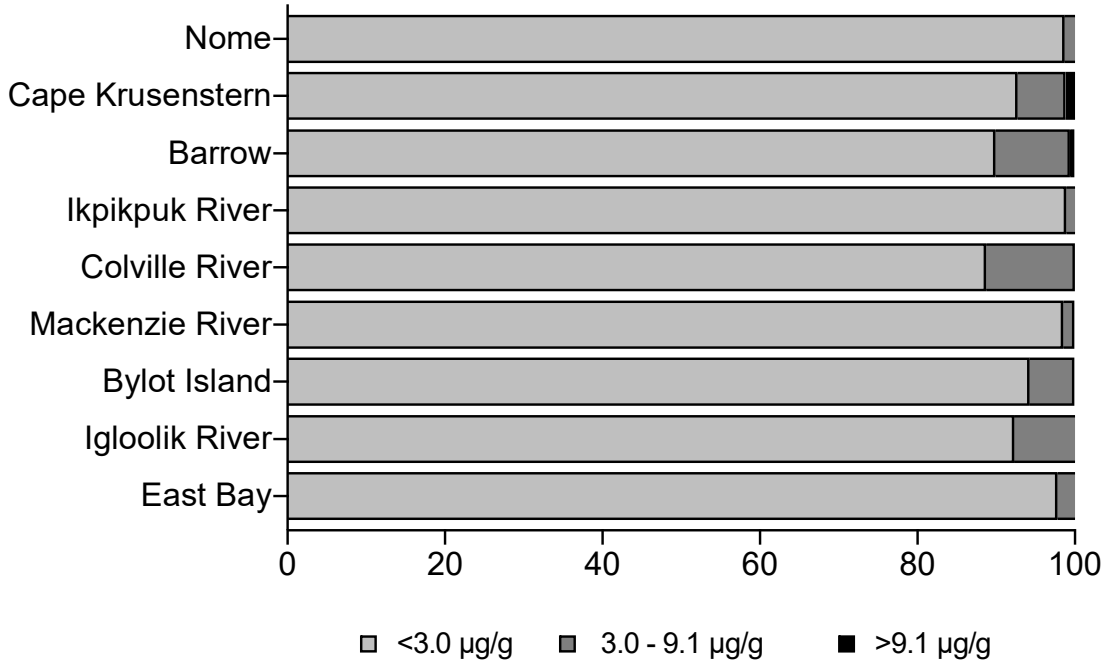


Figure 2.8. Comparison of the percentage of individual shorebirds at each study site with feather Hg concentrations falling within risk categories for adverse effects of Hg exposure. All species and years included. Risk categories based on flight feather concentrations: 1) <3.0 µg/g, low risk; 2) 3.0 to <9.1 µg/g, moderate risk; 3) >9.1 µg/g, high risk. For Dunlin only, risk categories based on body feather concentrations: 1) <2.4 µg/g, low risk; 2) 2.4 to <6.2 µg/g, moderate risk; 3) >6.2 µg/g, high risk (adapted from Jackson et al. 2011).

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Preface to Chapter 3

In chapter 2, we conducted a large-scale mercury (Hg) exposure assessment of birds sampled at remote study sites across the North American Arctic. This project proved successful for identifying regions and species that may be at the greatest risk for Hg exposure and determining factors influencing bird blood and feather Hg concentrations. These results highlight the great importance of future large-scale collaborations, however, the logistics and costs related to blood collection, storage, and shipping at such remote locations proved burdensome.

Chapter 3 describes the development and validation of a method to assess total Hg exposure in birds using dried blood spots (DBS). Emphasis was placed on the accuracy and precision of entire DBS and DBS punches for determining blood Hg concentrations in birds, as well as the stability of DBS THg concentrations when exposed to a variety of experimental time, temperature, and humidity treatments. The development of this method aims to help reduce logistic burdens and costs of collecting, storing, shipping, and analyzing field collected avian blood samples for conducting Hg exposure assessments, especially for those conducted at remote locations such as the Arctic as was experienced in chapter 2. To our knowledge, this is the first study to develop, validate, and apply a DBS-based method using direct Hg analysis to study Hg exposure in birds. This study greatly extends upon the limited studies that investigated the use DBS to determine Hg exposure in birds by digesting and analyzing samples using inductively coupled plasma mass spectrometry.

This chapter is co-authored by Dr. Niladri Basu with planned submission to *Environmental Pollution*.

Chapter 3

3 Dried Blood Spots for Estimating Mercury Exposure in Birds

3.1 Abstract

Mercury (Hg) is a pervasive environmental contaminant that can impair avian health, consequently, there is a need to gauge exposures. Bird blood provides a measure of recent dietary exposure to Hg, but blood collection and storage can be complex and costly. Dried blood spots (DBS) may help overcome challenges of whole blood analyses, therefore, this study aimed to develop and validate a novel method to assess Hg exposure in birds using DBS. First, we examined the accuracy and precision of using entire DBS and DBS punches to determine blood Hg concentrations for a model avian species, White Leghorn Chicken (*Gallus gallus domesticus*) dosed with methylmercury (MeHg) via egg injection. Chicken samples were then used to investigate Hg stability in DBS subjected to time, temperature, and humidity treatments. Lastly, we applied the method to DBS created using standard field methods from Zebra Finch (*Taeniopygia guttatato*) in the laboratory and American Golden-Plover (*Pluvalis dominica*) sampled in the field. All samples were analyzed for total Hg (THg) using direct Hg analysis. Accuracy was determined by comparing DBS concentrations with those of corresponding whole blood and reported as percent recovery. Results indicated that DBS provide an accurate measure of blood Hg concentrations for chickens. Recovery for entire DBS was 101.8 ± 5.4 %, however, analysis of DBS punches revealed lower recovery, ranging from 87.7 ± 4.0 to 92.4 ± 4.1 %.

These differences may be due to lower Hg concentrations of punches removed from the DBS interior (77.8 ± 5.5 % recovery) compared to those from the edge (102.6 ± 7.8 % recovery). There was little effect of time, temperature, and humidity storage treatments on Hg concentrations of entire chicken DBS, with mean DBS THg concentrations within ± 8 % of the whole blood for all treatments (n = 10 treatments). For Zebra Finch, DBS punches were more accurate (93.7 ± 9.7 %) compared to entire DBS (126.8 ± 19.4 %). While for American Golden-Plover, entire DBS resulted in the most accurate THg concentrations (111.5 ± 7.6 %) compared to DBS punches (edge: 115.4 ± 18.9 %, interior: 131.4 ± 16.1 %). Overall, results indicate that DBS analysis using a direct Hg analyzer can accurately evaluate Hg exposure in birds.

3.2 Introduction

Mercury (Hg) is a widespread contaminant that is mobilized in the environment mainly from anthropogenic activities (UNEP 2013). In birds and other wildlife, chronic exposure can cause sub-lethal effects, including impaired physiology, behavior, and reproductive success, ultimately having population level effects (Evers et al. 2008, Seewagen 2010, Scheuhammer et al. 2011). Given these potential impacts, large-scale efforts to reduce anthropogenic emissions, such as the United Nations Environmental Programme Minamata Convention on Mercury, are essential. Evaluating the effectiveness of this Convention (i.e., Article 22) will require the use of exposure science to assess and monitor Hg pollution across the landscape and over time (Evers et al. 2016, Gustin et al. 2016).

Birds are considered to be effective bioindicators since they are widespread and tend to have well-defined home ranges during the breeding season. Furthermore, birds have the potential to provide vital information about Hg contamination in remote or under represented regions.

Birds also assimilate and accumulate Hg rapidly in multiple tissues (Bearhop et al. 2000), including blood and feathers, both of which can be sampled with minimum risk to populations. Additionally, the majority of total Hg (THg) within bird blood and feathers is present as methylmercury (MeHg, Rimmer et al. 2005, Edmonds et al. 2010), a bioavailable organic form of Hg. Blood is particularly useful as a biomarker of recent Hg exposure, as studies on birds fed MeHg dosed diets demonstrate that Hg concentrations markedly increase within the first weeks of dosing (Bearhop et al. 2000, Fournier et al. 2002, Bennett et al. 2009). Researchers have recently started using large-scale datasets of blood or blood-equivalent THg concentrations of birds to determine Hg contamination and risk throughout North America (Evers et al. 2011, Jackson et al. 2015, Ackerman et al. 2016).

Blood sampling of both hatchling and adult birds generally does not result in any lasting effects on the sampled individual when conducted using established protocols (Sheldon et al. 2008). However, researchers should be properly trained and care should be taken to reduce handling time and the quantity of blood sampled (Fair et al. 2010). Blood sample volume should be limited to no more than 1 % of the birds' body weight, though this volume should be reduced when possible, especially during energetically demanding or stressful time periods (Fair et al. 2010). In small birds, this volume restriction can reduce the availability of samples for Hg analysis, since the limited blood sampled may be prioritized for other uses. Established blood collection methods can require heavy or bulky supplies such as blood storage tubes, coolers, and ice packs, which may be burdensome to transport to remote sampling locations. After collection, blood samples should be stored frozen with limited freeze-thaw cycles to reduce the possibility of Hg loss (Horvat and Byrne 1992, Liang et al. 2000, Varian-Ramos et al. 2011). This may require the availability of a freezer or cryoshipper during sampling and for the samples to be

transported or shipped frozen. For samples collected at remote locations, the equipment and shipping cost may be prohibitive or logistically unfeasible. In summary, while large-scale, multi-species Hg exposure assessments in birds are increasing, expanding these assessments to remote or resource-limited regions may be hindered by complex and costly blood collection and storage methods.

Developing a simple, accurate, and cost-effective technique for collecting bird blood in the field will allow for Hg exposure assessments at remote, resource-limited locations where data are currently lacking. Dried blood spots (DBS) are a method for collecting blood by applying it to specialized filter paper and allowing it to air dry. Dried blood spots were developed in the 1960s as a method to detect metabolic diseases in newborns (Guthrie and Susi 1963). They are still used today as part of the U.S. Centers for Disease Control Newborn Screening Quality Assurance Program, with standardized methods and materials for collecting and analyzing blood samples (Mei et al. 2001). In addition, more recent studies have used DBS to determine blood concentrations of toxic and essential elements in both humans (Chaudhuri et al. 2008, Basu et al. 2017) and birds (Shlosberg et al. 2012, Lehner et al. 2013). Avian blood sampling for DBS can be carried out according to standard blood collection methods, and can, therefore, be done safely without increasing bird handling time. Small blood volumes are used to create DBS, with each spot consisting of ≤ 60 μl of blood. Furthermore, DBS can be easily sub-sampled via punches. This may allow for accurate evaluation of blood Hg concentrations and for the remaining portion of the DBS to be archived and analyzed for additional measurements. Standardized filter paper used for DBS is small and light weight, and during collection, DBS samples do not need to be kept cold or stored frozen. This can reduce cost, weight, and the number of blood sampling supplies needed during field collection, as well as reduce shipping costs.

Dried blood spot use has been standardized for a variety of human health related analyses, and Shlosberg et al. (2011) have attempted to establish collection, storage, and analysis methods of avian DBS for a variety of toxicants, including Hg. However, Shlosberg et al. (2011) do not describe these methods in detail or provide information on the accuracy of these methods for determining blood Hg concentrations in birds. The overall objective of this study was to develop and validate a method to assess THg exposure in birds using DBS. There were 3 specific aims to this study: Aim 1) develop an accurate and precise method to determine blood Hg concentrations using entire DBS and DBS punches from a model avian species, White Leghorn Chicken (*Gallus gallus domesticus*); Aim 2) investigate the stability of DBS THg concentrations by exposing laboratory-created DBS from chickens to a variety of experimental time, temperature, and humidity treatments in a laboratory setting; and Aim 3) apply the developed method to DBS created with blood collected using standard field methods from Zebra Finch (*Taeniopygia guttatato*) dosed with MeHg in the laboratory and American Golden-Plover (*Pluvalis dominica*) sampled at an Arctic field site. Overall, this research intends to provide a method to help reduce logistic burdens and costs of collecting, storing, shipping, and analyzing field collected avian blood samples.

3.3 Methods

3.3.1 General Overview

A diagrammatic overview of the aims of this study is provided (Figure 3.1). In this section (3.3.1) we describe methodological details pertaining to the study species, sample collection, creating and sub-sampling DBS, and THg analysis. The subsequent sections in the Methods provide added specific details on each of the aims.

3.3.1.1 Study Species and Sample Collection

For Aims 1 and 2, blood samples were obtained from a model avian species, White Leghorn Chicken. Fertilized chicken eggs (Couvoir Simetin, Inc., Mirabel, QC, Canada) were dosed with MeHg by egg injection methods described in Rutkiewicz and Basu (2013). Specifically, 20 µl of methylmercury chloride (MeHgCl) dissolved in sterilized vegetable oil was injected into the air cell of each egg to result in a dose of 3.2 micrograms per gram (µg/g) egg, based on an average egg weight of 58 g. Eggs were then incubated at 37 °C and 65% humidity and were automatically turned every 45 minutes. At 14 days post-hatch, chickens were euthanatized by decapitation and whole blood samples were subsequently collected, mixed thoroughly and stored in trace metal grade K_2EDTA (di-potassium ethylenediaminetetraacetic acid)-coated vacutainers (BD, Mississauga, ON, Canada) at -20 °C until needed.

For Aim 3.2, nestling Zebra Finches, bred from an existing captive population, were dosed with MeHgCl dissolved in double deionized water from day 1 post-hatch until day 21 at Simon Fraser University Department of Biological Sciences (Morran 2016). Each day, nestlings were given 0.5 µl per g of body weight of a control (water only), a low dose (~60 µg/g), or a high dose (~150 µg/g) solution. Whole blood was collected at day 22 post-hatch in 2 heparinized capillary tubes via puncture of the brachial vein. The blood from one capillary tube per bird was transferred to individual 1.5 ml polypropylene tubes and stored at -20 °C until analyzed for THg concentrations. The second capillary tube was used to create DBS immediately after collection.

Additionally, for Aim 3.3, adult (after-hatch year) American Golden-Plover were captured (Bylot Island in Nunavut, Canada) during the 2015 breeding season, and whole blood samples were collected using standard protocols (Evers 2008). Blood was collected in heparinized capillary tubes (~50 µl of blood per tube) via puncture of the brachial vein using a

small gauge needle. Collected blood volumes did not exceed 1 % of the birds' body weight (Fair et al. 2010). Whole blood samples were collected in 1 capillary tube, sealed firmly on both ends with Critocaps (Leica Microsystems, Inc., Concord, ON, Canada), and placed in a labeled 6 mL plastic vacutainer (BD, Franklin Lakes, NJ, USA) to prevent breakage. During field collection, these samples were stored in a cooler with ice packs and stored in cold conditions throughout the breeding season. A second capillary tube was collected to create DBS in the field, immediately after collection. Whole blood samples were shipped to McGill University on ice in coolers and upon arrival stored frozen (-20 °C), while DBS were shipped and stored at ambient temperature until analysis.

All samples used for this research were collected under the proper animal care and sampling permits (chicken: McGill University Animal Use Protocol, N. Basu, 2014-7447; Zebra Finch: Simon Fraser University Animal Use Protocol Approval; American Golden-Plover: Université du Québec à Rimouski Animal Use Protocol Approval and Canadian Wildlife Service Permit).

3.3.1.2 Creating and Sub-sampling DBS

For all DBS in this study, Whatman 903 Protein Saver cards (GE Healthcare Services, Mississauga, ON, Canada; hereafter, filter paper) were used. For Aims 1 and 2, DBS were created in the laboratory using previously frozen whole blood collected from chickens dosed with MeHg via egg injection. Known volumes of whole blood were applied to the filter paper using a pipette, by holding the pipette tip slightly above the center of the outlined circle and slowly applying the blood. All cards were then air dried in the laboratory and stored in plastic storage bags at ambient temperature. Dried blood spots were sub-sampled using a 3 mm diameter punch (Harris Corporation, Melbourne, FL, USA), hereafter referred to as DBS punches. Unless

otherwise noted, all punches were removed from the interior of the DBS, with an effort made to avoid the DBS edge as described in Cernik (1974) and El-Hajjar et al. (2007) for analysis of other metals.

For Aim 3, DBS were created using a heparinized capillary tube filled with freshly collected whole blood. The capillary tube was held slightly above the center of an outlined circle, allowing blood drops to fall from the capillary tube onto the filter paper. If needed, the capillary tube was tapped gently on the filter paper. Zebra Finch and chicken DBS were dried and stored as described above for the chickens in Aims 1 and 2. American Golden-Plover DBS were air dried in the field, placed in labeled paper envelopes, and stored at ambient field conditions for the duration of the field season. Zebra Finch DBS were sub-sampled using a 6 mm diameter punch (Harris Corporation, Melbourne, FL, USA) and American Golden-Plover DBS were sub-sampled using a 3 mm diameter punch.

3.3.1.3 Mercury Analysis

Whole blood, entire DBS, and DBS punches were analyzed for THg concentrations with a Milestone DMA-80 (Milestone Inc., Shelton, Connecticut, USA) using the U.S. Environmental Protection Agency Method 7473 (U.S. EPA 2007) as we have detailed previously (Rutkiewicz and Basu 2013). Whole blood samples were vortexed thoroughly and pipetted (100 – 300 µl) into quartz sample boats, while entire DBS and punches were placed directly into quartz sample boats for analysis. A subset of whole blood samples (n = 17) were analyzed in duplicate to assess the precision of whole blood THg analysis. We determined limits of detection (LOD), which refer to values corresponding to the background signal of the instrument. Limits of detection were calculated as the mean value of blank boat samples plus 3x (theoretical method detection limit, TMDL) and 5x (practical method detection limit, PMDL) the standard deviation of the mean and

reported in ng of Hg. We also established the limit of quantification (LOQ) as the minimum concentration that can be reported with a high degree of confidence, based on THg analysis of blank filter paper. The LOQ was determined by analyzing 4, 3 mm blank filter paper punches removed from a subset of filter paper cards (n = 25). We used the estimated volume for 4 punches, determined below (section 3.3.4.1), to calculate THg concentrations for the blank filter paper punches. We calculated the LOQ as 10x the standard deviation of the mean and report this value in µg/g. Additionally, blank filter paper cut from a subset filter paper cards (n = 54) was analyzed and compared to the LODs to identify potential contamination of Hg in filter paper cards. Quality control (QC) samples were analyzed before and after every set of 10 samples and included multiple standard reference materials (SRM; DOLT-4 and DORM-4, National Research Council of Canada, and human blood QMEQAS10B-09 and QMEQAS09B-05, Institut National de Santé Publique du Québec) and Hg standard solution (Avantor, J.T. Baker Chemicals, Center Valley, PA, USA). Total Hg concentrations for all whole blood and DBS samples are reported in micrograms per gram (µg/g) wet weight (ww). Our analytical laboratory participates in the Northern Contaminants Program and Arctic Monitoring and Assessment Program inter-laboratory program that tests the performance of analytical laboratories. In Phase 10 of this program, which covers the period of the current study, our analytical laboratory's capabilities to measure MeHg and THg were ranked "excellent" with absolute z-scores less than 1.

3.3.2 Aim 1 - Method Development and Validation Using Chicken

3.3.2.1 Aim 1.1 - Blood Volume Estimates Using Frozen Blood

Under real-world sample collection conditions, the exact volume of blood in an entire DBS or a DBS punch is unknown. Without knowing the sample volume, we are unable to calculate a

concentration measurement. Aim 1.1 was designed to determine the relationship between DBS area and blood volume, in order to estimate the volume of unknown samples. We analyzed the area of DBS, created with known blood volumes, using ImageJ software, a free image processing program from the U.S. National Institutes of Health (https://imagej.nih.gov/ij/, Rasband 2016). We applied previously frozen chicken blood samples to the filter paper using a pipette as described earlier. The DBS were created from a pooled sample (n = 5) of chicken blood in 5 µl increments from 5 µl to 70 µl. Each DBS was photographed 3 times to determine variability in area measurements among photographs. The area of each DBS photograph was analyzed 3 times, with a minimum of 1 day between analyses, to determine inter-day variability in area measurements. We also analyzed the area of 3 mm and 6 mm DBS punches (n = 10) by photographing punches. All photographs included the presence of a standard metric ruler used to set the known unit of measurement during area analysis in ImageJ.

3.3.2.2 Aim 1.2 - Accuracy and Precision of Entire DBS and Punches

Aim 1.2 evaluated the accuracy and precision of using entire DBS and DBS punches to determine blood Hg concentrations in chickens. Both 60 µl and 25 µl DBS were created using blood samples from 5 individual chickens. Each 60 µl DBS was sub-sampled using a 3 mm diameter punch, resulting in 5 to 7 punches per 60 µl DBS. For each blood sample from an individual bird, we used a sampling scheme of 3 replicates each of 2, 4, and 8 punches, and an entire 25 µl DBS. Total Hg was measured in whole blood, entire DBS, and DBS punches for each individual bird (n = 5 individuals) to address Aim 1.2.

3.3.2.3 Aim 1.3 - Intra-DBS Variation

Aim 1.3 assessed the intra-DBS variability of Hg concentrations by comparing punches sampled from the DBS interior with those sampled from the edge. This was accomplished by creating 3

DBS (60 µl) for each of 5 individual chickens. Each DBS then had 3, 3 mm punches sub-sampled from the interior and also from the edge (Figure 3.2).

3.3.3 Aim 2 - Mercury Stability in Chicken DBS

We subjected entire DBS, prepared with blood from chicken dosed with MeHg via egg injection, to time, temperature, and humidity treatments (Table 3.1). The main treatment categories included: Aim 2.1) pathogen decontamination heat treatments; Aim 2.2) short-term (≤ 3 months) field collection and storage conditions; and Aim 2.3) long-term (1 year) laboratory storage conditions. For each treatment, 3 DBS (60 µl) were prepared from the whole blood of 5 individual chickens. Unless otherwise noted, all DBS were individually sealed in plastic sample bags. Aim 2.1 pathogen decontamination heat treatments were implemented by placing DBS, in individual sample weigh dishes, in a drying oven following 3 different time and temperature methods (Table 3.1). These 3 methods meet the United States Department of Agriculture importation requirements for avian materials from countries affected by H5N1 subtype of Highly Pathogenic Avian Influenza or Exotic Newcastle Disease (Paul 2005).

During field collection, DBS may be exposed to extreme or changing temperatures and humidity. Aim 2.2 emulated intermittent exposure to freezing field temperatures by storing DBS for a total of 3 months on a 2 week, alternating cycle of ambient temperature and -20 °C. DBS were also stored for 3 months at 30 °C with 80 % humidity in an egg incubator (Brinsea Products Inc., Titusville, FL. USA) to emulate field collection at tropical locations. DBS may be exposed to ultraviolet (UV) light when air-dried during field collection. In order to determine the effect of UV light, each DBS was set in a sample weigh dish and placed in a clean hood under a UV light bulb (200-280 nm at 4 watts) for 30 min (AirClean Systems, Creedmoor, NC, USA). For Aim 2.3 DBS were stored undisturbed for 1 year under designated temperature and humidity

treatments (Table 3.1). DBS stored under the low humidity treatment were placed in a desiccator.

3.3.4 Aim 3 - Method Application Using Additional Bird Species

3.3.4.1 Aim 3.1 - Blood Volume Estimates Using Freshly Collected Blood

Aim 3.1 was designed to determine the relationship between DBS area and blood volume for DBS created with a capillary tube using freshly collected blood. For this aim, blood was collected in the laboratory from live hatchling chickens in heparinized capillary tubes via puncture of the brachial vein, weighed, and immediately applied to filter paper as described previously (3.3.1.2). We determined the weight of each blood sample to use as a proxy for the known volume of each DBS. The DBS were then scanned alongside a standard metric ruler (CanoScan LiDE 90, Canon Canada Inc., Mississauga, ON, Canada) and the area was measured using ImageJ software.

3.3.4.2 Aim 3.2 - Laboratory-dosed Zebra Finch

Aim 3.2 evaluated the accuracy of using entire DBS and DBS punches to determine blood Hg concentrations with blood collected using standard field methods in a laboratory setting. Dried blood spots of unknown volume were created as described previously (3.3.1.2) and scanned for area analysis. Total Hg was measured for entire Zebra Finch DBS (n = 13 individuals) and DBS punches (n = 15 individuals), along with the corresponding whole blood samples.

3.3.4.3 Aim 3.3 – American Golden-Plover Sampled in the Field

Aim 3.3 evaluated the accuracy of using entire DBS and DBS punches to determine blood Hg concentrations with blood collected using standard field methods at an Arctic study site. Additionally, this aim investigated intra-DBS variation for DBS created under field settings. Dried blood spots of unknown volume were created as described previously (3.3.1.2) and

scanned for area analysis. To investigate intra-DBS variability of Hg concentrations, 2, 3 mm punches sampled from the DBS interior and 2 sampled from the edge were analyzed for each DBS (n = 12). In addition, the remainder (the entire spot after interior and edge punches were removed) of each DBS was analyzed. The amount of Hg in the interior and edge punches and the remainder were combined and used to determine the Hg concentration for the entire DBS. Corresponding whole blood samples were also analyzed for THg.

3.3.5 Statistical Analyses

In general, all results were reviewed visually and basic descriptive features of each study were recorded. Data were tested for normality using Shapiro-Wilk tests and non-parametric analyses were used as needed. For Aim 1.1, area analyses of photographed DBS, we calculated the relative standard deviation (% RSD) for among-day and among-photograph analyses to determine the precision of area analyses. We report the mean, standard deviation, and range of the calculated area for DBS samples created using incremental pipetted blood volumes from previously frozen chicken blood samples. We determined the relationship between DBS volume and area using simple linear regression for both previously frozen (Aim 1.1) and freshly collected (Aim 3.1) DBS samples. The resulting equation from the regression for freshly collected DBS was used to estimate the blood volume of entire DBS created using unknown blood volumes from Zebra Finch and American Golden-Plover (Aim 3.2 and 3.3). For both methods of applying blood to the filter paper (Aim 1.1 and 3.1) the average estimated punch volume for 3 mm and 6 mm punches were used to calculate Hg concentrations.

We report the mean, standard deviation, and range for whole blood THg concentrations for all bird species sampled. Additionally, we report the average precision for THg analysis of duplicate whole blood samples as relative percent difference (RPD %). Duplicate whole blood

concentrations were averaged for all remaining statistical analyses. We report the accuracy of DBS samples for chicken, American-Golden Plover, and Zebra Finch as percent recovery calculated as the ratio of the THg concentration for each DBS sample to that determined for whole blood. Chicken DBS samples (Aim 1 and 2) were analyzed in triplicate, and we report precision for DBS samples as the percent RSD for these samples. Triplicate DBS sample concentrations were averaged for all remaining statistical analyses. Descriptive statistics were used to summarize recovery for entire and sub-sampled DBS. Recovery was compared across sampling schemes and among treatments using analysis of variance (ANOVA) tests followed by Tukey's HSD post hoc tests, as appropriate. The relationship between whole blood and DBS sample Hg concentrations was investigated using Spearman's correlation. Mercury concentrations of DBS interior punches and edge punches were compared using paired t-tests. All analyses were performed using JMP 11 (SAS Institute 2013), and significance was determined if $P < 0.05$.

3.4 Results and Discussion

3.4.1 Quality Control for THg Analysis

Results obtained for QC samples analyzed throughout whole blood and DBS sample analysis indicated that the data were of acceptable quality (Supplemental Table 3.1) according to U.S. EPA Method 7473 performance criteria guidelines (U.S. EPA 2007). Average recoveries for human blood SRMs were 106.4 ± 10.5 % and 103.9 ± 16.9 %, with additional SRMs ranging from 93.6 ± 4.0 % to 109.7 ± 1.3 %. Analytical precision, calculated as mean percent RSD and RPD, was under 8 % for all SRM samples and duplicate whole blood samples (calculated as percent RPD), with the exception of Zebra Finch whole blood duplicate samples (18.8 ± 7.5 %

RPD, n = 4). Analytical precision for DBS samples are presented in their respective sections below but mean precision ranged from 2.1 ± 1.1 to 15.2 ± 3.5 %.

Mean LODs calculated from blank boat samples were 0.17 ± 0.10 ng, TMDL (mean value of blank boat samples plus 3x the standard deviation) and 0.24 ± 0.14 ng, PMDL (mean value of blank boat samples plus 5x the standard deviation). The LOQ was 0.012 µg/g (12.0 ng/ml), calculated as 10x the standard deviation of the mean for concentrations determined for 4, 3 mm blank filter paper punches (n = 25). This LOQ is similar to minimum blood THg concentrations seen for wild birds (Evers et al. 2005). Blank filter paper samples cut from a subset of filter paper cards were generally well below the TMDL, with mean levels of 0.055 ± 0.044 ng (n = 54 cards). Among these samples, we found a single filter paper card to have Hg levels slightly above detection (0.252 ng). Additionally, the blank filter paper punch samples used to calculate the LOQ were below TMDL, with mean levels of 0.029 ± 0.014 ng for 4, 3mm punches (n = 25 cards). While one blank filter paper sample did have higher than expected Hg levels, these results indicate that overall DBS filter paper cards do not have high background levels of THg.

The accuracy and precision of the SRMs analyzed for QC during this study were similar to those determined for previous analyses completed using this instrument and were within U.S. EPA Method 7473 performance criteria guidelines. However, there is currently no SRM for DBS with verified concentrations for Hg or other metals. As research using DBS to determine exposure to metals in humans, particularly for children and infants, increases, there is the possibility that an SRM for DBS metal analysis will be available for future use. In the meantime, researchers can create DBS using SRM blood to use as QC samples, as was done in a recent

study concerning MeHg exposure in humans (Basu et al. 2017). It is a limitation of the current study that we did not create and analyze DBS QC samples.

For birds, in particular, two previous studies have investigated the use of entire 50 µl DBS to determine Hg exposure by digesting and analyzing samples using inductively coupled plasma mass spectrometry (ICP-MS, Shlosberg et al. 2012, Lehner et al. 2013). Both studies created DBS samples using 3 different Lyphochek® whole blood SRMs for QC during sample analysis. They both found the SRM with the lowest Hg concentration (Lyphochek 1, Hg concentration not reported) to have variable and inaccurate results (reported recovery for Hg: 39.4 % (Lehner et al. 2013), and both Hg and lead: 40 - 240 % (Shlosberg et al. 2012) and this SRM was subsequently omitted. However, the DBS created using the other 2 SRM samples (Lyphochek 2: 51.9 µg/l and Lyphochek 3: 88.5 µg/l) provided good accuracy throughout sample analysis (mean recoveries ranged from 97 - 109 %, both studies). However, analysis of these DBS samples was more precise for Shlosberg et al. (2012) (8.1 and 4.3 % RSD) than for Lehner et al. (2013) (average error: 12.3 ± 13.3 % and 12.7 ± 12.0 %). The LOQ for both of these studies was determined based on analysis of blank filter paper (n = 10) and was calculated using a similar method as the current study. While sample analysis for the current study (which used direct Hg analysis) differed from these two studies, the LOQ (12.0 ng/ml) was similar, though slightly lower compared to the LOQs from these studies, 13.7 ng/ml (Lehner et al. 2013) and 20 ng/ml (Shlosberg et al. 2012). In addition to birds, a number of human studies have outlined methods to characterize Hg in DBS, and pertinent quality control aspects of those methods are summarized in Supplemental Table 1 found in Basu et al. (2017).

3.4.2 Aim 1 - Method Development and Validation Using Chicken

3.4.2.1 Aim 1.1 - Blood Volume Estimates Using Frozen Blood

A major challenge in analyzing DBS for THg is that the exact volume of blood in an entire DBS is often unknown under real-world sample collection conditions. Without such information, it is not possible to calculate the concentration of THg (or any other chemical) for a DBS sample. Under the assumption that blood spreads evenly across the filter paper during DBS preparation, punches of a known area have been used to estimate blood volume for Hg analyses of DBS (Chaudhuri et al. 2008, Nelson et al. 2016). However, the volume of blood within a punch is not standardized across the literature. Here we examine the relationship between the blood volume and area of DBS to help deepen understanding of this issue. Area calculations via images revealed low variability for both among-day area analyses (RSD = 1.2%) and among-photograph area analyses (RSD = 3.0%). Accordingly, we averaged the results and calculated that area measurements for pipetted blood samples range from 14.72 ± 2.01 mm^2 for a 5 µl DBS to 168.22 ± 7.94 mm^2 for a 70 µl DBS (Supplemental Table 3.2). There was a strong linear relationship between DBS volume and area ($F_{1,40}$ = 3168.7, P < 0.001, r^2 = 0.99, Figure 3.3), with a resulting equation of: volume (µl) = -1.60 + 0.42*area (mm^2). Peck et al. (2009) also found a strong linear relationship between area and volume by using a similar method for measuring the area of DBS created using 25, 50, and 100 µl of an EDTA whole blood sample. These results indicate that the ratio of area to volume is consistent across DBS volumes. Though, Mei et al. (2001) found a slight (13 %) increase in punch (6 mm) serum volume as DBS volume increased from 25 to 125 µl using whole blood spiked with ^{125}I-L-thyroxine.

We determined the average area of a 3 mm and a 6 mm punch to be 7.55 ± 0.09 mm^2 and 28.90 ± 0.58 mm^2, respectively, by analyzing the area of photographed punches (n = 10 per

punch size). We estimated volumes for these DBS punches, based on area analyses and applied blood volumes of previously frozen chicken blood using a pipette, to be 2.94 (95 % CI: 2.83 - 3.05) µl and 11.26 (95% CI: 10.84 - 11.69) µl. These calculated punch volumes are slightly lower than those used in previous studies investigating Hg concentrations of DBS for human blood. Chaudhuri et al. (2008) used a volume of 11.5 µl of blood to determine Hg concentrations for each ¼" (6.35 mm) DBS punch, and while they indicate that this volume was verified by multiple laboratories the verification method was not indicated. Nelson et al. (2016) used a volume of 3.1 µl of blood to determine Hg concentrations for 3 mm DBS punches, but they also do not describe how this volume was determined. However, this volume (3.1 µl) for a 3 mm punch was calculated in another study that used whole blood spiked with ^{125}I-L-thyroxine to determine punch volumes of DBS created with differing hematocrit (Adam et al. 2000). Adam et al. (2000) found the punch volume of 3.1 µl was specific to a hematocrit of 50.1 % and that both hematocrit and filter paper type influenced punch volume.

Further studies have also determined that DBS area and punch volume of human blood DBS created with Whatman 903 Protein Saver cards can be influenced by hematocrit (Denniff and Spooner 2010, Vu et al. 2011, Hall et al. 2015). The cited studies created DBS using human whole blood samples prepared with differing hematocrit (from 20 - 80 %, depending on the specific study) and evaluated area and punch volume. They determined that increasing blood hematocrit resulted in reduced DBS area and increased punch volume. The current study used a pooled chicken blood sample for investigating the relationship of DBS area and volume to reduce the effect of individual differences in hematocrit. While the expected hematocrit for chicken hatchlings (14 days post-hatch) is approximately 30 % (Glomski and Pica 2011), we did not investigate differences in hematocrit among chickens used in this study. Therefore, we were

unable to determine the effect of hematocrit on the ratio of area to volume for DBS created using chicken blood, though this should be explored in future studies.

3.4.2.2 Aim 1.2 - Accuracy and Precision of Entire DBS and Punches

Aim 1.2 was performed to determine if THg measurements taken in DBS and sub-sampled punches accurately reflect measurements taken from the corresponding whole blood. The mean whole blood THg concentration for chickens used in this study (Aim 1 and 2) was 1.05 ± 0.29 µg/g and concentrations ranged from 0.63 to 1.78 µg/g (n = 15 individuals). Mean precision for whole blood THg analysis was 2.4 ± 1.8 % (n = 7). Whole blood THg concentrations were correlated with measurements taken from DBS samples for all sampling schemes and replicates, however, the correlation coefficient was relatively weak (Spearman $\rho = 0.59$, P-value < 0.001, n = 60, Figure 3.4). The DBS THg concentrations for entire 25 µl spots were within ± 10 % of the whole blood THg concentrations with a mean accuracy of 101.8 ± 5.4 % (Table 3.2, Figure 3.5). For DBS punches (2, 4, and 8 punches), the THg concentrations were calculated using the estimated volume for a 3 mm punch obtained for previously frozen chicken blood (Aim 1.1, 2.94 µl per punch). Overall, the THg concentrations for the punches were within ± 15 % of the whole blood THg concentrations with a mean accuracy of 92.4 ± 4.1 %, 89.8 ± 2.8 %, and 87.7 ± 4.0 % for the 2, 4 and 8 punches respectively. The THg measurements for this aim were precise, with mean percent RSD for sample replicates less than 6 % for all sampling schemes. The accuracy of THg measures differed among sampling schemes ($F_{3,16} = 11.1$, $P < 0.001$), with DBS punches having a lower recovery than entire DBS.

We found that THg analysis of entire 25 µl DBS using direct Hg analysis precisely and accurately reflected THg concentrations measured in corresponding whole blood samples. Shlosberg et al. (2012) found most DBS samples analyzed for Griffon Vultures (*Gyps fulvus*)

were below the LOQ for Hg analysis (n = 22 of 25 birds) and did not compare DBS with corresponding whole blood samples. However, the study did report good method accuracy and precision for SRM samples, as outlined earlier. Lehner et al. (2013) compared Hg concentrations from duplicate Bald Eagle (*Haliaeetus leucocephalus*) DBS samples (n = 4 individuals), while they did not report percent RPD, a graph of the measurements showed the duplicates to be relatively precise. In the same study, the researchers compared DBS Hg concentrations to the corresponding whole blood samples for Golden Eagles (*Aquila chrysaetos*) and found strong linearity between DBS and whole blood concentrations ($r^2 = 0.9987$). However, the Hg concentrations were not widely dispersed across the range as most samples had low concentrations (<200 ng/ml) and 2 had relatively high values (~600 and 1600 ng/ml). In the current study, we found that DBS THg concentrations were correlated with those of whole blood, though this correlation was relatively weak. While environmentally relevant, THg concentrations for the chickens sampled in this study were not wide ranging (from 0.63 to 1.78 µg/g) compared to wild birds. For instance, blood Hg concentration of 0.05 to 8.63 µg/g have been seen for Common Loons (*Gavia immer*, Evers et al. 2005). Future studies investigating the use of DBS to determine Hg exposure in birds should include a wider range of Hg concentrations to confirm linearity and determine precision and accuracy for a more expansive range of concentrations.

Dried blood spot punches may provide an important benefit by allowing the remaining (or residual) DBS to be archived and analyzed for additional measurements. We aimed to determine if the number of punches analyzed influenced the accuracy and precision of THg concentrations by analyzing 2, 4, and 8 punches using direct Hg analysis. We found similar accuracy for 2, 4 and 8 punches, but that THg concentrations were more variable for 8 punches. This indicates that fewer punches may be ideal for THg analysis in birds and allow for more

sample to be archived for further analyses. However, concentrations for all punches were well above the LOQ, and it is possible that low concentration samples may benefit from the analysis of more punches. We found THg concentrations of all punches to be lower and less accurate than those for entire 25 µl DBS. It is possible that non-homogenous spread of Hg across DBS could result in the lower THg concentrations observed for punches. We investigated this possibility by comparing THg concentrations for punches removed from the DBS interior and from the edge (section 3.4.2.3).

3.4.2.3 Aim 1.3 - Intra-DBS Variation

The difference in accuracy of THg measures between punches and entire DBS observed in Aim 1.2 may be partly explained by variation in Hg content across a DBS. For Aim 1.3, we determined that THg concentrations of punches removed from the edge of DBS were greater than those taken from DBS interior ($t_{14} = -12.5$, $P < 0.001$, Figure 3.6). Specifically, the DBS punches removed from the edge (recovery: 102.6 ± 7.8 %, RSD: 7.3 ± 4.8 %) had more accurate blood Hg concentrations than those sampled from the DBS interior (recovery: 77.8 ± 5.5 %, RSD: 4.1 ± 1.6 %).

This is the first study to report non-homogenous spread of Hg across DBS, with greater THg concentrations observed for samples from the edge of the DBS. Similarly, El-Hajjar et al. (2007) found DBS created with lead spiked human blood had greater lead concentrations in samples that included the DBS perimeter, as opposed to interior punches or samples where the perimeter was excluded. Variability in metal concentrations across DBS may be due to a non-homogenous spread of red blood cells across the filter paper. Lenk et al. (2015) used color intensity analysis to investigate the distribution of red blood cells across DBS created from human blood. They investigated the 'coffee stain effect' in drying DBS, which occurs when solid

particles (red blood cells within blood) collect at the edge as the liquid within a sample evaporates. They determined that this effect explained the greater concentrations of red blood cells found at the edge of DBS. In blood, MeHg is preferentially bound to hemoglobin in the red blood cells (Ancora et al. 2002), and previous research on humans and birds has shown that Hg concentrations are dependent on hematocrit and hemoglobin concentrations (La Sala et al. 2011, Kim et al. 2014). Therefore, for avian DBS, a higher concentration of red blood cells at the edge of the DBS due to the coffee stain effect could explain the greater Hg concentrations for edge punches seen in this study.

Multiple factors may play a role in the extent of non-homogeneity seen in DBS. Lenk et al. (2015) determined that drying conditions, such as relative humidity and the position of the DBS card during drying affected the homogeneity of red blood cells within DBS. Other studies have found hematocrit also affects the homogeneity of DBS, with lower hematocrit resulting in reduced homogeneity (O'Mara et al. 2011, Cobb et al. 2013), however, these studies did not use Whatman 903 Protein Saver cards. Cobb et al. (2013) also determined that anticoagulant affected DBS homogeneity. Our results, combined with those of previous research, indicate that DBS punches could result in under- or over-estimation of blood THg concentrations. Since various factors can affect homogeneity in DBS, it may be difficult to determine how THg concentrations might be affected. Therefore, the analysis of entire DBS samples for THg concentrations may provide a more accurate estimate of Hg exposure in birds. In situations where the entire spot cannot be used for Hg analysis, it would be recommended to carefully document the punch location (e.g., through a photograph).

3.4.3 Aim 2 - Mercury Stability in Chicken DBS

Aim 2 used chickens to investigate the stability of THg concentrations in DBS by exposing laboratory created DBS to a variety of time, temperature, and humidity treatments in a laboratory setting. These treatments replicated common field collection and long-term storage conditions, as well as pathogen decontamination heat treatments. Across all treatments, THg concentrations for whole blood and DBS were significantly correlated (Spearman's $\rho = 0.88$, P-value <0.001, Figure 3.7), and the mean DBS THg concentrations were within ± 8 % of corresponding whole blood concentrations (Table 3.1, Figure 3.8). Precision was variable among treatments, with RSD ranging from 2.1 ± 1.1 % to 15.2 ± 3.5 %. Recovery did not differ among heat treatments ($F_{2,12} = 1.3$, P = 0.30) or among long-term storage treatments ($F_{4,20} = 0.11$, P = 0.98) for entire 60 µl chicken DBS.

We found the THg concentrations were generally stable under a range of short-term (3 months) simulated field conditions and long-term (1 year) basic laboratory storage conditions. Lehner et al. (2013) also saw little change in Hg concentrations for DBS in long-term storage under ultracold freezer conditions (-70 °C for 2 years), however, information on the types of samples, sample sizes, and precision of Hg concentrations is lacking. Additionally, Chaudhuri et al. (2008) investigated the stability of Hg concentrations in DBS punches created using reconstituted freeze-dried human blood reference material subjected to long-term storage conditions. Digested punches were analyzed for Hg concentrations using ICP-MS to determine initial Hg concentrations, then DBS samples were stored at either room temperature or in a refrigerator. Punches were removed from the DBS for Hg analysis 5 times over an 8.5-month period. The results of this small sample set indicated no loss of Hg over time, however, the results appeared variable and were biased with recovery >100 % for both storage treatments. The

current study compared THg concentrations for avian DBS among 5 common long-term laboratory storage treatments and found the accuracy similar across treatments. However, samples stored in a refrigerator had the best precision for THg concentrations, with the poorest precision seen in samples stored in ambient temperature conditions. Mercury concentrations in whole blood are also stable under long-term storage when frozen (Liang et al. 2000, Varian-Ramos et al. 2011), however, Hg loss has been seen for samples subjected to repeated freeze-thaw cycles (Horvat and Byrne 1992). Therefore, DBS may offer an advantage over the use of whole blood samples for determining Hg exposure in birds by offering Hg stability in a wide range of storage conditions.

Dried blood spots may also offer an advantage during field collection, storage, and shipping. We found Hg to be stable in both hot and humid conditions, as well as during freeze-thaw cycles. Additionally, we saw good Hg stability for DBS exposed to UV light. This is the first study to investigate the effect of short-term collection and storage conditions on Hg concentrations in DBS. These results indicate that DBS can be collected, stored, and eventually shipped without additional need to regulate transport humidity or temperature. Therefore, DBS offer the ability to reduce cost, weight, and the number of blood sampling supplies and equipment needed during field collection, as well as reduce shipping costs.

The current study also found THg concentrations remained constant when DBS were exposed to multiple pathogen decontamination heat treatments. Shlosberg et al. (2012) indicated that pathogen decontamination heat treatment (60 °C for 30 min.) did not result in lower analyte concentrations in DBS created with spiked blood, however, empirical data in support of this observation were not provided. Lehner et al. (2013) subjected Bald Eagle DBS (n = 5 individuals) to 3 treatments including control (no treatment), heat (60 °C for 30 min.), and

ethanol (dipped for < 5 s) treatments. Precision for THg concentrations among treatments was < 8 % (RSD) for all individuals. While the number of samples subjected to these treatments was low, these results also indicate that Hg concentrations are relatively stable in avian DBS subjected to pathogen decontamination treatments. Generally, pathogen decontamination heat treatment is not possible for whole blood samples used for Hg analysis, often restricting the importation and analysis of avian blood samples. Since researchers in many resource-limited countries or regions may be lacking access to Hg analysis laboratories, collaboration with the international research community for sample analysis is vital for understanding global Hg exposure in birds. Pathogen decontamination heat treatments of DBS can offer researchers from countries with the presence of the H5N1 subtype of Highly Pathogenic Avian Influenza or Exotic Newcastle Disease a simple method for treating avian blood samples prior to importation.

3.4.4 Aim 3 - Method Application Using Additional Bird Species

The final aim of this study was to apply the developed method to additional bird species, and to DBS created with blood collected using standard field methods. This was accomplished by creating DBS in the laboratory using unknown volumes of blood collected via capillary tube from MeHg dosed Zebra Finch (Aim 3.2). Additionally, DBS of unknown volumes were created in the field by sampling blood from breeding birds at an Arctic study site (Aim 3.3). For both Aims 3.2 and 3.3, whole blood was also collected and analyzed for comparison with the DBS.

3.4.4.1 Aim 3.1 - Blood Volume Estimates Using Freshly Collected Blood

Preliminary calculations indicated that blood volume estimates from Aim 1.1 were not accurate for DBS created with blood collected using standard field methods (Appendix Table 3.1). Therefore, Aim 3.1 was designed to determine the relationship between DBS area and blood

volume for DBS created with a capillary tube using freshly collected chicken blood. There was a strong linear relationship between DBS volume and area ($F_{1,21} = 1823.5$, $P < 0.001$, $r^2 = 0.99$, Figure 3.3). The regression equation determined from this analysis was: volume (ul) = -1.27 + 0.45*area (mm^2). Using the average area of a 3 mm and a 6 mm punch, the estimated volume for DBS punches based on area analyses and applied blood volumes using a capillary tube was determined to be 3.20 (95% CI: 3.13 - 3.28) µl and 12.21 (95% CI: 11.93 - 12.50) µl.

We also found a strong linear relationship between area and volume for DBS created using freshly collected chicken blood applied to filter paper using a capillary tube. These results further indicate that the ratio of area to volume is similar across DBS volumes, even for DBS created using different methods. However, we found that DBS created using freshly collected blood and capillary tubes resulted in a smaller area to volume ratio than those created by pipetting previously frozen chicken blood. Reconstituted freeze-dried blood (Seronorm whole blood reference material) has been shown to have different spreading characteristics compared to fresh whole blood (Cizdziel 2007). This is likely also true for previously frozen chicken blood, since the spread of blood across the filter paper may be less impeded by lysed red blood cells from previously frozen blood than by fully intact red blood cells from freshly collected blood. The reduced spread of blood for DBS created with using freshly collected chicken resulted in greater volume estimates for 3 mm and 6 mm punches. Preliminary calculations using blood volume estimates from Aim 1.1 to calculate THg concentrations for Zebra Finch and American Golden-Plover samples resulted in higher than expected THg concentrations and therefore high recovery (Appendix Table 3.1), indicating that the estimated blood volumes were too low. The smaller area to volume ratio and resulting greater punch volume estimates determined in this aim

appear to more accurately represent the blood volumes for both Zebra Finch and American Golden-Plover DBS.

3.4.4.2 Aim 3.2 - Laboratory-dosed Zebra Finch

For Zebra Finch, whole blood THg concentrations of MeHg dosed birds ranged from 0.21 to 1.06 µg/g with a mean value of 0.58 ± 0.28 µg/g. The non-exposed, control birds had very low blood THg concentrations (0.008 ± 0.009, range: 0.003 – 0.03 µg/g, n = 11). Mean precision for whole blood THg analysis was 18.8 ± 7.5 % (n = 4). For these birds, DBS were created by applying blood collected in the laboratory from live hatchlings immediately to filter paper. Entire DBS and DBS punches were analyzed for THg. Concentrations for entire DBS were calculated using the analyzed area for each DBS and the regression equation from Aim 3.1. For DBS punches, THg concentrations were calculated using the estimated volume for a 6 mm punch obtain from Aim 3.1 (12.21 µl per punch). We found THg concentrations for both punches and entire DBS to be 3 to 10 times greater than the corresponding whole blood concentrations for unexposed Zebra Finch, though THg concentrations were still low (0.02 – 0.06 µg/g). This is likely because the whole blood THg concentrations for these samples were very low, with concentrations less than the LOQ determined in this study (0.012 µg/g). However, interestingly we note that a single control bird had a whole blood concentration greater than the LOQ (0.03 µg/g) and this sample had a recovery of 109.6 % for an entire DBS. These low blood THg concentrations are expected for laboratory raised birds that have not been exposed to any Hg sources. Generally, birds sampled in the wild have some exposure to Hg, with blood THg concentrations < 0.20 µg/g considered as background levels of Hg exposure (Ackerman et al. 2016). Therefore, wild bird species with blood THg concentrations similar to those seen for unexposed Zebra Finches would likely be uncommon. We expect the majority of wild birds to

have blood THg concentrations greater than the LOQ determined for DBS in this study. However, these results indicate that DBS may considerably overestimate Hg exposure in birds with very low blood THg concentrations.

For dosed Zebra Finch used in this study, whole blood THg concentrations were significantly correlated with both entire DBS and DBS punch samples (whole blood and entire DBS: Spearman's $\rho = 0.92$, P-value < 0.001; whole blood and DBS punch: Spearman's $\rho = 0.91$, P-value < 0.001). DBS punches resulted in the most accurate blood THg concentrations (93.7 ± 9.7 %) compared to entire DBS (126.8 ± 19.4 %, Table 3.2). The DBS used in this aim were created in a laboratory using a model avian species, however, the intent was to simulate DBS sample collection of small songbirds using field collection methods. Therefore, small, unknown volumes of blood were collected using a capillary tube and applied directly to the DBS. All previous studies that investigated Hg concentrations in avian DBS have used DBS created using known blood volumes (Shlosberg et al. 2012, Lehner et al. 2013). We investigated the accuracy of analyzing both entire DBS and DBS punches for determining THg concentrations of DBS with unknown blood volumes. In general, we found that THg concentrations of entire DBS were higher than expected. Since DBS punches may underestimate THg concentrations, as seen in this study (3.3.2.3), and we found high recovery for entire DBS, there is a possibility that we are still underestimating volumes for DBS created using blood freshly collected with a capillary tube. Further research on determining accurate volume estimates for DBS created with unknown volumes may be warranted.

3.4.4.3 Aim 3.3 – American Golden-Plover Sampled in the Field

For American Golden-Plover, whole blood THg concentrations of ranged from 0.08 – 0.16 µg/g with a mean value of 0.12 ± 0.03. Mean precision for whole blood THg analysis was 4.4 ± 2.0 %

(n = 6). For these birds, DBS were created by applying blood collected in the field from live hatchlings immediately to filter paper. Again, concentrations for entire DBS were calculated using the analyzed area and the regression equation from Aim 3.1. For DBS punches, THg concentrations were calculated using the estimated volume for a 3 mm punch obtain from Aim 3.1 (3.20 µl per punch). Whole blood THg concentrations were significantly correlated for both entire DBS and DBS edge punch samples (whole blood and entire DBS: Spearman's $\rho = 0.92$, P-value < 0.001; whole blood and DBS edge punch: Spearman's $\rho = 0.87$, P-value < 0.001), but only marginally correlated for interior punches (Spearman's $\rho = 0.74$, P-value = 0.006). Like with the chicken study, THg concentrations of punches removed from the edge were greater than those taken from DBS interior ($t_{11} = -2.9$, P = 0.014, Figure 3.9) with interior punches resulting in the most accurate estimates of THg concentrations. For these samples, entire DBS resulted in the most accurate blood THg concentrations (111.5 ± 7.6 %) compared to DBS punches (edge: 115.4 ± 18.9 %, interior: 131.4 ± 16.1 %). However, overall, recovery ranged widely with some DBS samples having > 175 % recovery (Table 3.2).

This is the first study to our knowledge that has collected whole blood and created DBS using a capillary tube in the field to determine Hg exposure in birds. We were also able to confirm the non-homogenous spread of Hg across DBS seen for chicken samples also occurs in field collected, wild bird DBS samples. Total Hg concentrations for entire DBS were the most accurate, however, we found higher than expected concentrations for many of the interior punches, edge punches, and entire DBS samples. This further indicates that we may be underestimating volume estimates for DBS created using blood freshly collected with a capillary tube. Additionally, recovery for American Golden-Plover DBS samples ranged widely, indicating that the area to volume ratio (and therefore, the accuracy of volume estimates) may

vary among individuals. Hematocrit may play a role in this variability since hematocrit is known to affect blood spread in DBS and thus DBS area (Denniff and Spooner 2010, Wong and James 2014) differing hematocrit among species and individuals may lead to inaccurate DBS volume estimates. Future studies investigating differences in hematocrit among and within bird species and the effect on DBS may help to develop more accurate DBS volume estimates. As indicated by the previous aims in this study, creating DBS with known blood volumes is likely to provide more accurate and precise THg concentrations for avian DBS. Future research should investigate the development of easy to use methods of creating DBS of known blood volumes for wild birds under field conditions.

3.5 Conclusion

Overall, this study indicates that DBS, analyzed using a direct Hg analyzer, is a good method to evaluate Hg exposure in birds. We improve upon previous studies by addressing multiple factors related to the development and validation of a method to assess Hg exposure in birds using DBS. This is the first study to our knowledge to use direct Hg analysis to determine Hg concentrations in DBS. Direct Hg analysis offers multiple benefits for Hg analysis of DBS, including short analysis time and minimal sample preparation. We determined LODs for the instrument used in this study, as well as an LOQ by analyzing blank filter paper punches. Additionally, we evaluated the accuracy of DBS by comparing THg concentrations of DBS to those of corresponding whole blood, and provide measures of precision by analyzing replicate DBS samples. We compared the accuracy and precision of entire DBS with that of DBS punches and found better accuracy for entire DBS. We also determined that Hg was not spread evenly across DBS created with avian blood, with greater THg concentrations found at the DBS edge. In order

to calculate the concentration for DBS punches, we determined a linear relationship between DBS area and volume and used this relationship to estimate punch volumes.

We further investigated the stability of Hg in DBS by submitting them to a variety of time, temperature, and humidity treatments. We compared THg concentrations for avian DBS among 5 common long-term laboratory storage treatments and found Hg to be stable and the accuracy similar across treatments. Additionally, we determined that THg concentrations were stable when exposed to multiple pathogen decontamination heat treatments. This finding may offer researchers from countries with the presence of the H5N1 subtype of Highly Pathogenic Avian Influenza or Exotic Newcastle Disease a simple method for treating avian blood samples prior to importation to the United States for Hg analysis. This is the first study to investigate that effect of short-term collection and storage conditions on THg concentrations in DBS. These results indicate that DBS can be collected, stored, and eventually shipped without additional equipment for regulating the humidity or temperature of DBS. Therefore, DBS offer the ability to reduce cost, weight, and the number of blood sampling supplies and equipment needed during field collection, as well as reduce shipping costs.

Lastly, we applied the developed method to a model avian songbird species and to wild birds sampled in the field using DBS created with blood collected using standard field methods. This is the first study to our knowledge that has collected whole blood and create DBS using a capillary tube in the field to determine Hg exposure in birds. We determined that analyzing entire DBS of known volumes provided the most accurate and precise measures of Hg exposure in birds. Overall, this research validates DBS as a novel tool to assess Hg exposure in birds. In doing so, this work can help to reduce logistic burdens and costs of collecting, storing, shipping, and analyzing field collected avian blood samples.

3.6 Acknowledgments

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3.7 Tables and Figures

Table 3.1. Accuracy and precision of THg concentrations for 60 µl DBS prepared with chicken blood from all storage treatments. Accuracy represented as mean, standard deviation (SD), and range of recovery (%, as compared to whole blood THg concentrations, n = 5 per sampling scheme). Mean analytical precision, calculated as relative standard deviation (% RSD), and SD is presented (n = 3 per individual).

Treatment ID	Treatment	Duration	Temperature	Humidity	Recovery (SD)	Range	RSD (SD)
Heat 1	Heat*	20 min.	100 °C	Ambient	107.5 (5.6)	104.1 - 118.0	3.5 (1.7)
Heat 2	Heat*	30 min.	60 °C	Ambient	105.1 (3.5)	101.7 - 109.6	5.2 (3.3)
Heat 3	Heat*	180 min.	56 °C	Ambient	103.2 (1.6)	100.7 - 104.8	5.5 (4.8)
UV	Field conditions	30 min.	Ambient	Ambient	105.7 (5.7)	98.5 - 111.8	3.9 (1.6)
High Humidity	Field conditions	3 mos.	30 °C	80 %	94.2 (5.7)	88.2 - 102.1	4.3 (3.5)
Freeze thaw cycle	Field conditions	3 mos.	2 week alternate: Ambient / -20 °C	Ambient	95.3 (3.2)	91.4 – 99.2	2.1 (1.1)
Ambient	Long-term storage	1 yr.	Ambient	Ambient	97.1 (5.6)	89.2 - 103.2	15.2 (3.5)
Low Humidity	Long-term storage	1 yr.	Ambient	Low	95.6 (3.4)	92.7 - 101.2	10.1 (3.6)
Refrigerator	Long-term storage	1 yr.	4 °C	Ambient	96.8 (3.1)	92.3 - 100.7	6.2 (3.4)
Freezer	Long-term storage	1 yr.	-20 °C	Ambient	97.3 (4.6)	90.6 - 101.8	11.5 (5.1)

Ultracold Freezer	Long-term storage	1 yr.	-80 °C	Ambient	96.3 (5.3)	90.4 - 101.3	12.2 (3.7)

*Pathogenic heat treatment for Exotic Newcastle Disease and Avian Influenza

Table 3.2. Accuracy and precision of THg concentrations for chicken, Zebra Finch, and American Golden-Plover DBS punches and entire DBS. Accuracy represented as mean, standard deviation (SD), and range of recovery (%, as compared to whole blood THg concentrations) for chicken (n = 5 per sampling scheme), Zebra Finch (punches, n = 15 individuals; entire spot, n = 13 individuals), and American Golden-Plover (n = 12 individuals) DBS samples. Mean analytical precision, calculated as relative standard deviation (% RSD), and standard deviation (SD) is presented for chickens only (n = 3 per individual).

Species	Sample	Recovery (SD)	Range	RSD (SD)
Chicken	2 (3 mm punches)	92.4 (4.1)	84.6 – 98.4	3.5 (1.4)
	4 (3 mm punches)	89.8 (2.8)	85.5 – 93.2	2.7 (1.4)
	8 (3 mm punches)	87.7 (4.0)	83.3 – 91.3	5.6 (4.6)
	Entire Spot (25µl)	101.8 (5.4)	94.5 – 108.2	4.5 (2.3)
Zebra Finch	1 (6 mm punch)	93.7 (9.7)	74.6 - 110.4	NA
	Entire Spot	126.8 (19.4)	98.6 - 167.0	NA
American Golden-Plover	2 Interior (3 mm punches)	115.4 (18.9)	86.4 – 167.5	NA
	2 Edge (3 mm punches)	131.4 (16.1)	107.7 – 163.2	NA
	Entire Spot*	111.5 (7.6)	96.0 – 126.4	NA

*Entire spot THg concentrations calculated using the sum of interior and edge punches and the remainder of each DBS

Supplemental Table 3.1. Summary of THg quality control measurements for whole blood and DBS sample analysis, including mean accuracy and standard deviation (SD), calculated as the percent recovery compared to certified value, and mean analytical precision, calculated as relative standard deviation (RSD) or relative percent difference (RPD), for standard reference materials (human blood QMEQAS09B-05 and QMEQAS10B-09, Institut National de Santé Publique du Québec, and DORM-4 and DOLT-4, National Research Council of Canada) and Hg standard.

Standard Reference Material	Number of Days	Number of Samples	% Accuracy (SD)	% Precision (SD)
QMEQAS09B-05	10	35	106.4 (10.5)	6.6 (4.8)
QMEQAS10B-09	5	14	103.9 (16.9)	7.6 (3.5)
DORM-4	5	22	93.6 (4.0)	3.4 (2.1)
DOLT-4	3	10	108.3 (3.0)	4.1 (0.7)
Hg Standard	4	27	109.7 (1.3)	3.4 (2.3)

Supplemental Table 3.2. The relationship between known volumes of previously frozen chicken blood applied to filter paper with a pipette and the resulting area determined using Image J software. The mean, standard deviation (SD), and range of area measurements are shown (n = 3 DBS per test volume).

Volume (µl)	Area (mm^2)	SD	Range
5	14.72	2.01	12.70 - 16.72
10	28.04	1.51	26.42 - 29.41
15	40.89	2.40	39.28 - 43.65
20	52.94	2.11	50.87 - 55.08
25	64.25	2.73	61.10 - 65.92
30	78.19	0.95	77.42 - 79.25
35	87.32	0.56	86.68 - 87.66
40	96.05	1.96	93.93 - 97.79
45	109.41	1.23	108.03 - 110.40
50	129.73	5.90	123.62 - 135.39
55	147.07	2.71	144.62 - 149.98
60	145.86	9.03	138.73 - 156.02
65	152.44	1.32	151.30 - 153.89
70	168.22	7.94	159.07 - 173.33

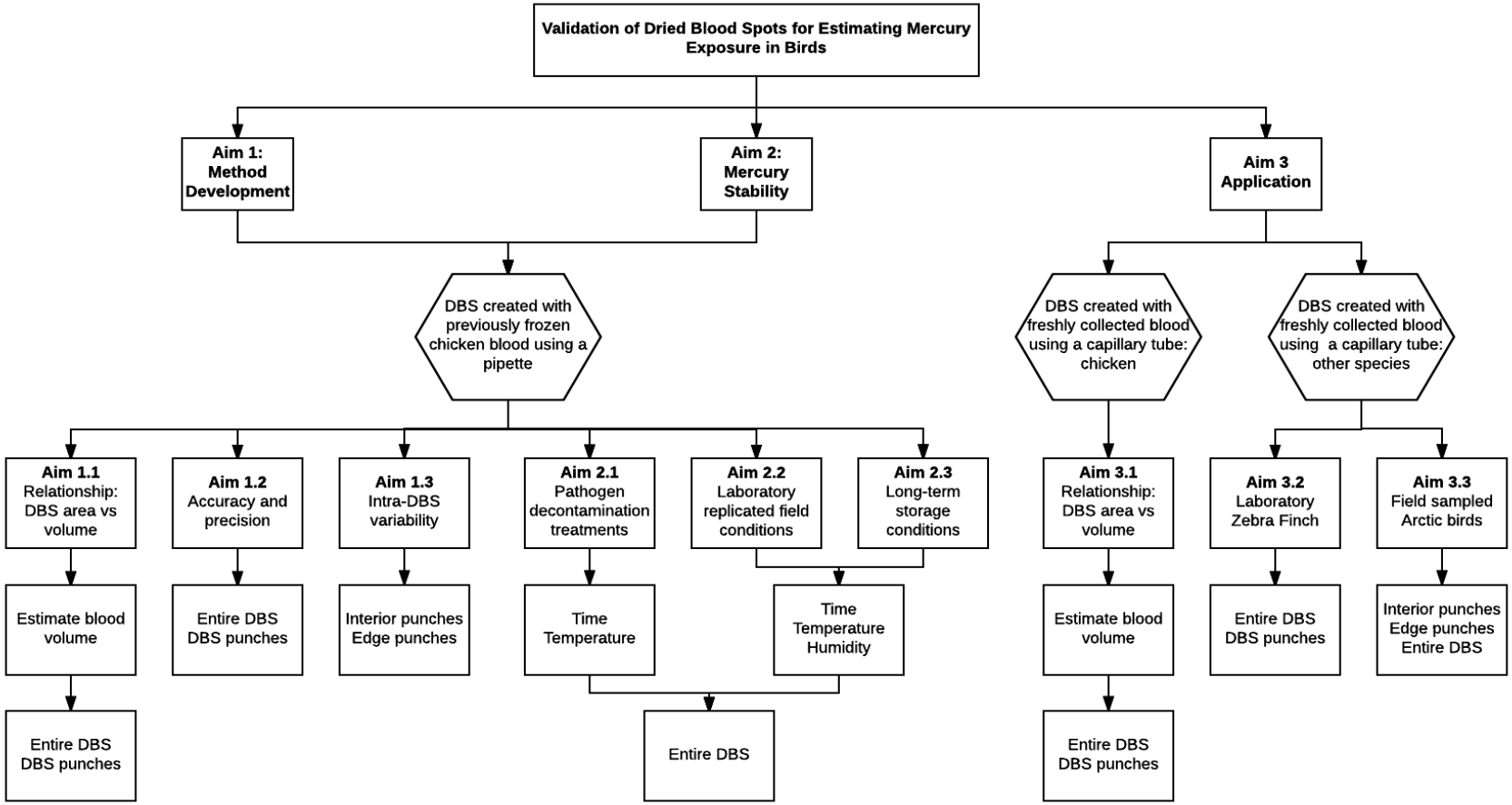

Figure 3.1. A diagrammatic overview of the study aims.

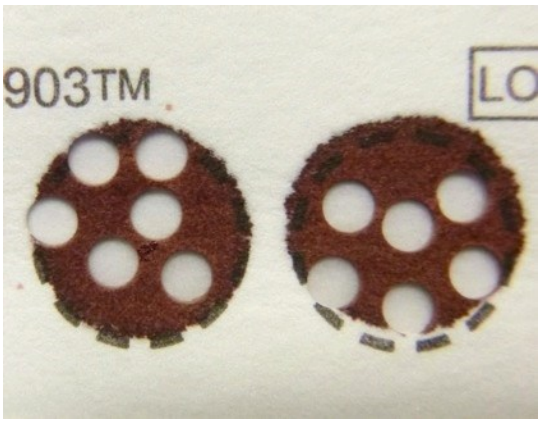

Figure 3.2. Image of 60 µl DBS with 3 mm punches removed from the interior and the edge.

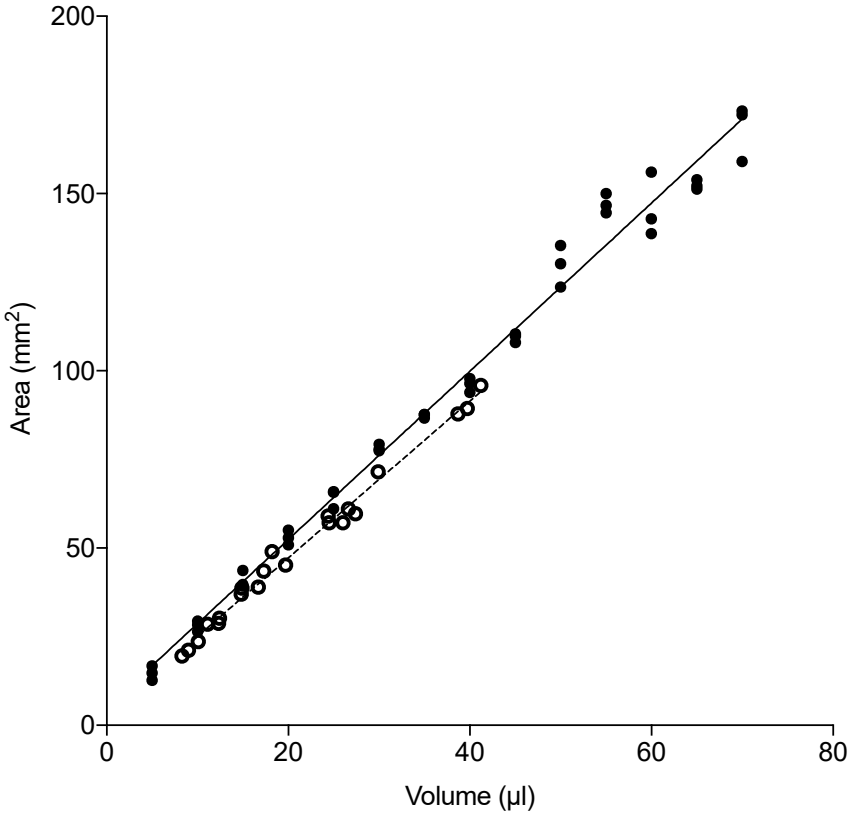

Figure 3.3. The relationship between chicken DBS area (mean, n = 3 DBS per volume) determined using ImageJ vs known, measured blood volumes applied using a pipette (solid line) and between DBS area determined using ImageJ vs known blood volume (based on weight) collected from live chickens and applied using a capillary tube (dashed line).

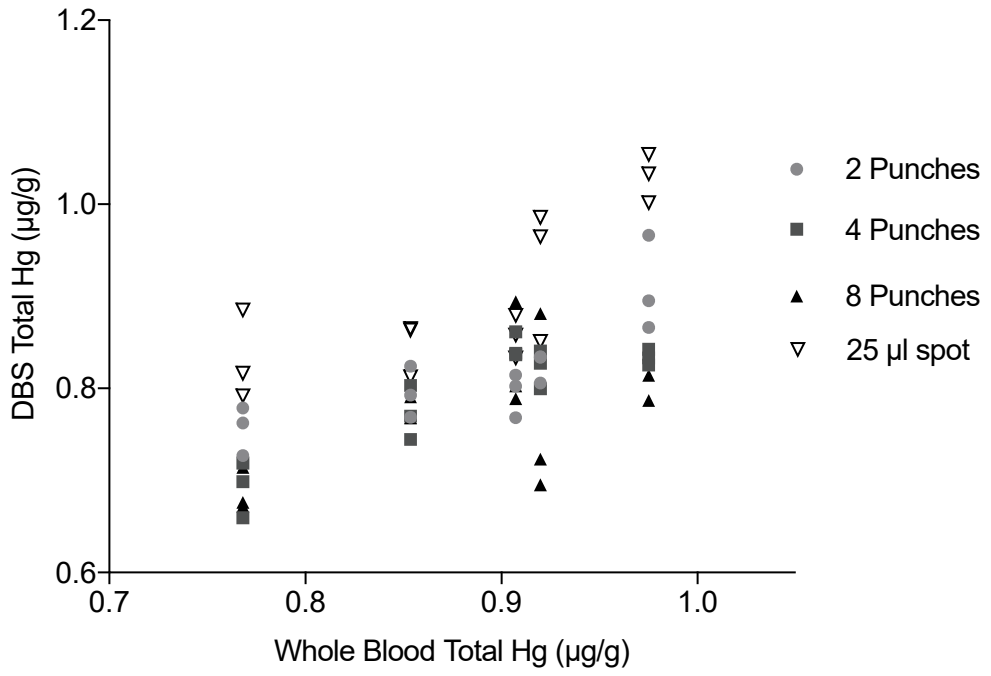

Figure 3.4. The relationship between whole blood THg concentrations and THg concentrations for chicken DBS (2, 4, and 8) punches and entire 25 µl DBS (Spearman $\rho = 0.59$, P-value < 0.001).

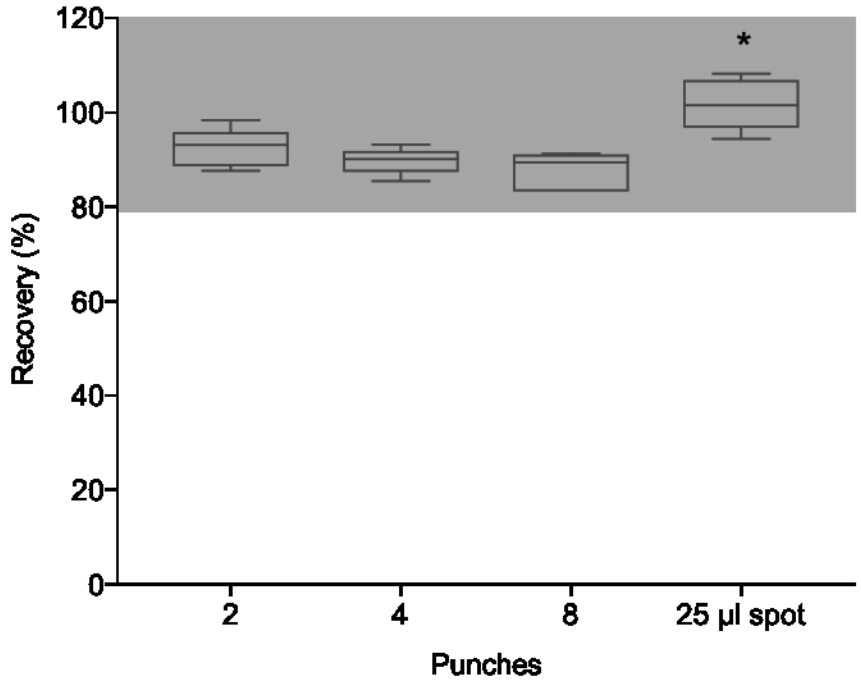

Figure 3.5. Comparison of the accuracy of chicken DBS samples for 2, 4, 8 punches, and an entire 25 µl DBS (n = 5 per sampling scheme). Accuracy represented as recovery (%, compared to whole blood Hg concentrations). Box plot indicates median with the center line and the 1st and 3rd quartiles with the box outline, whiskers represent 95 % confidence intervals. The shaded region represents acceptable accuracy (± 20 % of 100 %) based on U.S. EPA Method 7473 performance criteria guidelines. *denotes sample differed ($P < 0.05$).

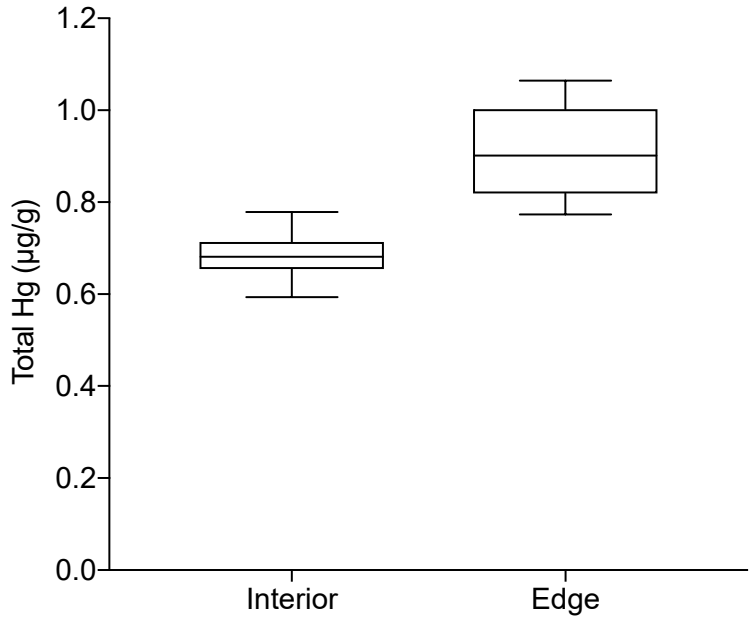

Figure 3.6. Comparison of Hg concentrations (µg/g) for punches taken from the edge vs the interior of 60 µl chicken DBS (n = 15 per location). Box plot indicates median with the center line and the 1st and 3rd quartiles with the box outline, whiskers represent 95 % confidence intervals. Samples differed by location ($P < 0.001$).

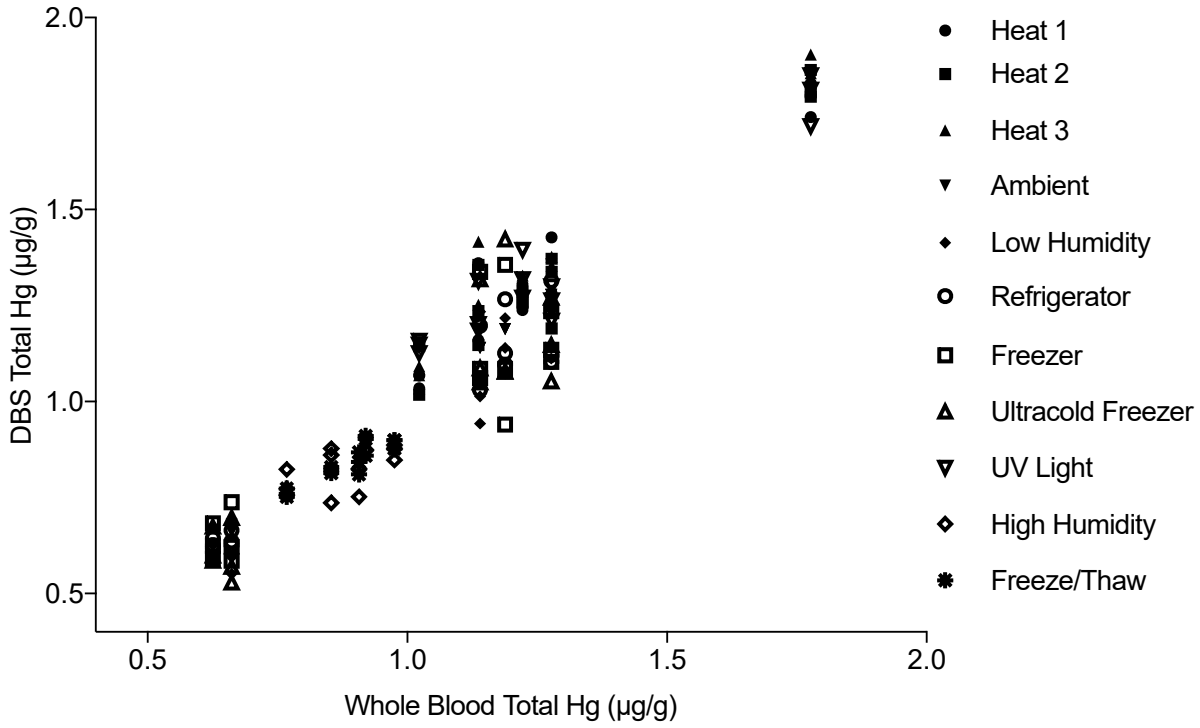

Figure 3.7. The relationship between whole blood Hg concentrations and Hg concentrations for 60 µl chicken DBS samples subjected to time, temperature, and humidity treatments (Spearman's $\rho = 0.88$, P-value <0.001).

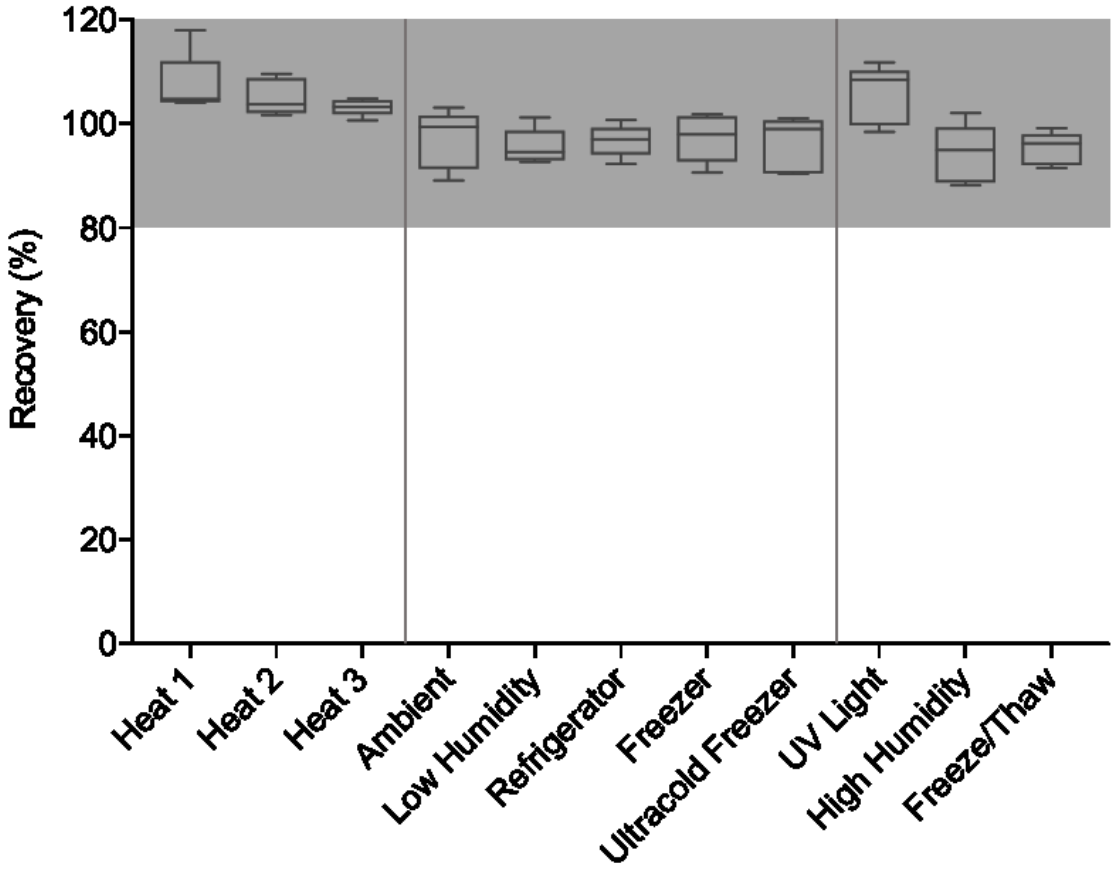

Figure 3.8. Comparison of the accuracy (recovery %, as compared to whole blood Hg concentrations) for 60 µl DBS samples subjected to time, temperature, and humidity treatments (n = 5 per treatment). Box plot indicates median with the center line and the 1st and 3rd quartiles with the box outline, whiskers represent 95 % confidence intervals. The shaded region represents acceptable accuracy (± 20 % of 100 %) based on U.S. EPA Method 7473 performance criteria guidelines.

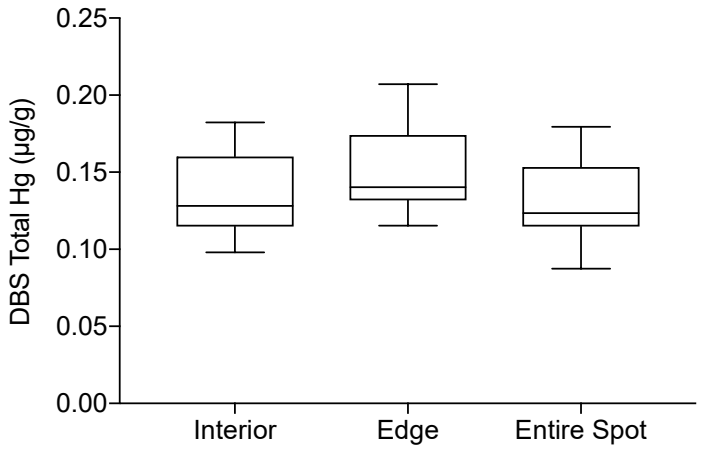

Figure 3.9. Comparisons of Hg concentrations (µg/g) for entire DBS and DBS punches taken from the edge and the interior of field collected American Golden-Plover DBS (n = 12 per location). Box plot indicates median with the center line and the 1st and 3rd quartiles with the box outline, whiskers represent 95 % confidence intervals.

3.8 Appendix: Using Blood Volume Estimates from Frozen Blood for Method Application Using Additional Bird Species

For Zebra Finch and American Golden-Plover DBS created using unknown blood volumes, we initially calculated THg concentrations for entire DBS and punches using the regression equation and punch volumes determined in Aim 1.1 (volume (µl) = -1.60 + 0.42*area (mm^2); 3 mm punch: 2.94 µl; and 6 mm punch: 11.26 µl). However, these preliminary calculations resulted in higher than expected THg concentrations and therefore high recovery (Appendix Table 3.1), indicating that these estimated volumes were too low.

Appendix Table 3.1. Accuracy and precision of THg concentrations for Zebra Finch and American Golden-Plover DBS punches and entire DBS. Accuracy represented as mean, standard deviation (SD), and range of recovery (%, as compared to whole blood THg concentrations) for Zebra Finch (punches, n = 15 individuals; entire spot, n = 13 individuals), and American Golden-Plover (n = 12 individuals) DBS samples. Concentrations calculated using volumes estimated from Aim 1.1.

Species	Sample	Recovery (SD)	Range
Zebra Finch	1 (6 mm punch)	101.6 (10.5)	80.9 - 119.7
	Entire Spot	137.5 (20.9)	107.0 - 180.7
American Golden-Plover	2 Interior (3 mm punches)	125.6 (20.5)	94.0 - 182.3
	2 Edge (3 mm punches)	143.0 (17.6)	117.2 - 177.6
	Entire Spot*	120.5 (8.2)	103.6 - 136.6

*Entire spot THg concentrations calculated using the sum of interior and edge punches and the remainder of each DBS

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Preface to Chapter 4

In chapter 2, we conducted a large-scale field-base mercury (Hg) exposure assessment to improve the current understanding of Hg toxicokinetics in birds. From this chapter, we found that blood and feather Hg concentrations were greatly influenced by species. While we also determined that multiple extrinsic and intrinsic factors influenced blood and feather Hg concentrations, these factors did not entirely account for the observed differences among species. Biochemical factors may also influence Hg toxicokinetics in birds, however, we were unable to examine biochemical factors or incorporate important developmental time points in chapter 2.

Chapter 4 investigated key biochemical processes, Hg speciation and subcellular distribution of Hg, in tissues of embryos and hatchlings birds. This chapter describes a laboratory-based study which examines Hg speciation within avian tissues (blood, brain, and liver) and explores the subcellular distribution of Hg in livers of embryonic and hatchling birds. Emphasis was placed on better understanding how Hg speciation and subcellular distribution change over developmental time points and differ by species. We used MeHg egg injection of a model avian species, White Leghorn Chicken (*Gallus gallus domesticus*), and compared these results with those from maternally-deposited methylmercury (MeHg) in wild embryonic Ring-billed Gulls (*Larus delawarensis*) to further improve the current understanding of Hg toxicokinetics in birds.

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Chapter 4

4 Mercury Speciation and Subcellular Distribution in Experimentally-dosed and Wild Birds

4.1 Abstract

Many bird species are exposed to methylmercury (MeHg) at levels shown to cause sub-lethal effects, however, MeHg sensitivity and assimilation can vary among species and developmental stages. Differences in MeHg toxicokinetics may influence the variability observed in MeHg assimilation and risk. We investigated two biochemical processes of Hg toxicokinetics in birds: 1) mercury (Hg) speciation in blood, brain, and liver; and 2) Hg subcellular distribution in liver. We used MeHg egg injection of White Leghorn Chicken (*Gallus gallus domesticus*), sampled at 3 early-developmental stages, and embryonic Ring-billed Gulls (*Larus delawarensis*) exposed to maternally-deposited MeHg. The percent MeHg in blood, brain, and liver ranged from 93.8 ± 12.9 to 121.3 ± 12.9 %, indicating little MeHg demethylation. A liver subcellular partitioning procedure was used to determine how total Hg was distributed between potentially sensitive and detoxified compartments. The distributions of total Hg among subcellular fractions were similar among chicken time points, and between embryonic chicken and Ring-billed Gulls. A greater proportion of total Hg was associated with metal-sensitive fractions than detoxified fractions. Within the sensitive compartment, total Hg was found predominately in heat-denatured proteins (range = 42.3 to 46.1 %). A low rate of MeHg demethylation and a high proportion of Hg in

metal-sensitive subcellular fractions further indicate that embryonic and hatchling time points are Hg-sensitive developmental stages.

Keywords: Mercury; Subcellular fractions, Toxicokinetics, Birds, Liver

4.2 Introduction

Mercury (Hg) is a ubiquitous environmental contaminant that poses a significant threat to the health of humans, wildlife, and ecosystems [1]. Anthropogenic emissions, such as those from mining and the release of Hg as a by-product of burning coal and other fossil-fuels, are major sources of Hg mobilization throughout the global environment [1]. Mercury deposited across the landscape is converted into its more toxic and biologically available form, methylmercury (MeHg), by bacterial processes under anoxic conditions [2]. Birds are known to readily assimilate and accumulate dietary MeHg [3,4], and chronic exposure has been shown to impair physiology, behavior, and reproductive success [5,6]. Furthermore, recent studies indicate birds across North America are exposed to Hg at levels that may pose significant health risks [7–10].

Mercury exposure assessments in birds are a vital and regularly-used tool to determine health risks of Hg to bird populations [8]. Birds have also been used as bioindicator species to provide current levels for Hg risk in a variety of systems, including marine habitats, coastal, estuarine, and freshwater wetlands, and upland forests [7,11,12]. As Hg emissions continue to rise globally [1], efforts to reduce these emissions, such as the Minamata Convention on Mercury, are essential. The Minamata Convention (i.e., Articles 19 and 22) requires Parties to commit towards collaborative efforts to monitor Hg concentrations in biotic media including birds [13,14] to assess whether reductions in Hg emissions result in decreased exposures.

Bird blood and feathers are frequently used as Hg biomarkers as these tissues are easily sampled and analyzed for Hg concentrations with minimum risk to populations (i.e. live, non-destructive sampling). Bird liver and other internal tissues such as kidney, brain, and muscle (by destructive sampling) have also commonly been used as Hg biomarkers [15–17]. However, considerable variability in Hg concentrations of these biomarker tissues has been observed among and within species and individuals [7,18,19]. Additionally, in wild birds, the relationship between Hg biomarker concentrations and adverse effects from Hg exposure can vary among species [5,20,21]. Laboratory studies have shown that Hg sensitivities can vary among bird species. For example, Heinz et al. [21] used MeHg egg injections to rank 23 bird species into categories of low, medium, and high sensitivity based on dose–response curves and median lethal concentrations (LC50), with a 4-fold difference in dose between the low and high sensitivity groups. Unfortunately, field and laboratory studies to determine adverse effect levels are difficult and costly to conduct. Therefore, inter-species Hg sensitivities and how these sensitivities relate to biomarker tissue concentrations remain unknown for most wild bird species. Differences in Hg toxicokinetics among bird species and individuals may play a role in the variability in biomarker tissue concentrations and the associated health risk.

Recent studies have investigated how Hg distributes in tissues of embryonic, hatchling, and adult birds [22–24], though less is known about how biochemical processes, such as demethylation, influence Hg toxicokinetics. Mercury speciation studies on avian liver tissue have shown the ratio of MeHg to total Hg (THg) is consistently less than 1 [25–28], indicating that MeHg demethylation (the extent to which MeHg is converted to inorganic Hg in vivo) occurs within the organism, and that this likely occurs in the liver. These studies also indicate that MeHg demethylation ability differs among species, by sex and age, and with Hg exposure.

Methylmercury demethylation in the liver may influence both biomarker tissue concentrations and ultimately Hg risk. For instance, research indicates that both blood and feather Hg concentrations may be influenced by the ability to demethylate MeHg in the liver. Henny et al. [26] found a strong correlation between MeHg concentrations in liver and blood for both adult and fledgling birds of multiple species, while liver inorganic Hg was not correlated with blood MeHg concentration. Additionally, a reduction of the proportion of Hg excreted into feathers has been found for species that have a greater hepatic MeHg demethylation ability [29], which is considered to be an important detoxification scheme. Research suggests that conversion of organic Hg (OHg) to inorganic Hg (IoHg) in the liver, and its subsequent sequestration, may prevent OHg exposure in the cardiovascular and nervous systems, which may be particularly sensitive to OHg [6,26]. In this context, species or individuals with higher ability to demethylate MeHg may be at lower risk of negative health effects from MeHg exposure.

In addition to MeHg demethylation within whole tissues, other biochemical processes, such as intracellular binding of Hg, may play an important role in influencing Hg toxicokinetics and risk in birds. Recent studies have used subcellular partitioning procedures to determine how metals are distributed among subcellular fractions isolated from the tissues of aquatic organisms [30–34]. These procedures, which often make use of differential centrifugation, allow for the distinction between metal-binding to potentially sensitive target molecules (enzymes and organelles) and metal accumulation in detoxified fractions such as heat-stable proteins and metal-rich granules [35]. A greater proportion of Hg bound to detoxified fractions may indicate a reduced risk of Hg binding to physiologically sensitive organelles and molecules. Whereas Hg bound to metal-sensitive fractions may indicate an increased health risk, due to negative effects on organelles or inactivation of intracellular molecules.

Previous studies have focused on the subcellular distribution of Hg in fish liver [33,34,36], other fish tissues [36,37], invertebrates [38], and marine mammal liver [31]. However, only one species of bird, a marine foraging, piscivorous species (Black-footed Albatross, *Diomedea nigripes*, n = 5), has been investigated for subcellular distribution of Hg [31,39]. Species differences in the distribution of Hg among subcellular fractions have been noted for invertebrates [38] and marine mammals [31]. Onsanit and Wang [37] reported that subcellular Hg distribution in a single fish species differed with size, indicating that larger fish were better at detoxifying Hg than smaller fish. Additionally, Peng et al. [36] determined that subcellular MeHg distribution in fish muscle, gills, liver, and intestines differed over time following Hg exposure. Further research on how Hg is distributed within the tissues of bird species and over developmental time points is warranted to better understand Hg toxicokinetics and risk in birds.

In birds, biochemical processes such as MeHg demethylation ability and subcellular distribution are poorly understood but may influence biomarker tissue concentrations and species sensitivities to Hg exposure. In the present study, we used MeHg egg injection of a model avian species, White Leghorn Chicken (*Gallus gallus domesticus*), to determine Hg speciation within tissues (blood, brain, and liver) and to explore the subcellular distribution of THg in livers of embryos and hatchlings. We compared these results with those from maternally-deposited MeHg in wild embryonic Ring-billed Gulls (*Larus delawarensis*). We aimed to better understand how Hg speciation and subcellular distribution change over developmental time points and differ by species. This study focuses on commonly used biomarker tissues and tissues integral to MeHg detoxification and mobility (blood and liver), as well as tissues sensitive to the adverse effects of MeHg (liver and brain). We expect that a better understanding of these biochemical processes

will improve avian Hg exposure assessments and help identify species and developmental stages at the greatest risk for negative health effects of Hg exposure.

4.3 Methods

4.3.1 Study Organisms

Fertilized chicken eggs were obtained from Couvoir Simetin, Inc., hatchery (Mirabel, QC, Canada). Eggs were dosed with MeHg by egg injection methods described in Rutkiewicz and Basu [24], using a dosing solution of methylmercury chloride (MeHgCl) dissolved in sterilized vegetable oil. Most eggs were injected with 15 µl of MeHgCl dosing solution to result in a dose of 1.6 micrograms per gram (µg/g) of egg based on an average egg weight of 56.2 ± 4.0 g (n = 51). A small subset of eggs was injected with 15 µl of sterilized vegetable oil as a vehicle control (n = 13). After injection, eggs were incubated at 37 °C and 65 % humidity and were automatically turned every 45 minutes. To investigate Hg speciation and subcellular distribution over time, we sampled tissues at 3 time points: embryonic day 19 (ED 19), post-hatch day 1 (PH 1), and post-hatch day 7 (PH 7). The average incubation period for chicken is approximately 21 days, therefore, ED 19 embryos were sampled just prior to hatch. Embryos were removed from eggs and terminated by decapitation and hatchlings were euthanatized by decapitation. For all time points, entire cerebrum and liver tissues were dissected, placed in metals-free 1.5 ml polypropylene tubes, and frozen immediately on dry ice. Cerebrum and liver tissue samples were stored at -80 °C until processing and analysis. For time points PH 1 and PH 7, whole blood was collected upon decapitation and placed in a trace metal grade K_2EDTA (di-potassium ethylenediaminetetraacetic acid) coated vacutainer (BD, Mississauga, ON, Canada). Blood was mixed thoroughly to prevent coagulation and stored frozen at -20 °C until analysis.

Ring-billed Gull eggs were collected on 3 May 2016 from a single nesting colony located near Beauharnois, QC, Canada (45.316052, -73.906218 degrees decimal). Eggs were collected from single egg nests only, to ensure collection occurred prior to the onset of incubation (n = 20). All eggs were transported to McGill University and incubated at 37 °C and 65 % humidity within 3 hours of collection. Eggs were automatically turned every 45 minutes. These eggs were only exposed to maternally-deposited MeHg. At the first sign of pipping, embryos were removed from eggs and terminated by decapitation. Entire cerebrum and liver tissues were dissected, placed in metals-free 1.5 ml polypropylene tubes, and frozen immediately on dry ice. Cerebrum and liver tissue samples were stored at -80 °C until processing and analysis.

All samples used for this research were obtained under the proper animal care and research permits. For chickens, a McGill University Animal Use Protocol Approval (N. Basu, 2015-7661) was obtained for this research. Ring-billed Gull eggs were collected under an Environment Canada, Canadian Wildlife Service scientific research permit (J. Head, SC-63).

4.3.2 Mercury Speciation

4.3.2.1 Total Mercury Analysis

For all species and time points, entire cerebrum samples and liver subsamples were freeze-dried (SP Industries, Inc., Warminster, PA, USA) for ~40 h, and then manually homogenized prior to whole tissue THg analyses. Total Hg concentrations of blood samples and freeze-dried liver and brain tissues were determined using direct Hg analysis (DMA-80, Milestone Inc., Shelton, CT, USA and NIC MA-3000, Nippon Instruments North America, College Station, TX, USA) following United States Environmental Protection Agency (US EPA) method 7473 [40] as we have detailed previously [24]. In brief, whole blood samples (~20 – 30 µl) were weighed (wet

weight, ww) in ceramic sample boats and freeze-dried tissue was weighed (~5 – 20 mg, dry weight, dw) into nickel or ceramic sample boats for analysis. Quality control (QC) samples including standard reference materials (SRM; DORM-4 fish protein, National Research Council of Canada; human hair #13, National Institute for Environmental Studies, Japan; human blood QMEQAS10B-09, Institut National de Santé Publique du Québec), sample replicates, and empty boat blanks were run every 10 to 20 samples. Total Hg concentrations for brain and liver tissues are reported in µg/g dw and blood samples are reported in µg/g ww.

4.3.2.2 Methylmercury Analysis

Dried tissue samples were analyzed for MeHg with a Tekran 2700 (Tekran Instruments Corporation, Toronto, Ontario, Canada) using purge and trap, gas chromatography separation, and cold vapor atomic fluorescence spectroscopy detection following US EPA 1630 [41]. In brief, freeze dried brain and liver samples (~15 mg) and blood samples (30 µl) were digested using 8 ml of 25 % KOH in methanol heated to ~140 °C on a hot plate for 4 hours. Cooled digests were filled to 30 ml with methanol and stored at -20 °C until analysis. For analysis, 30 µl of sample digest (300 µl for Ring-billed Gull liver samples) were added to ultrapure water (1000-fold dilution), adjusted to pH 4.5 using acetate buffer, and ethylated using 1 % $NaBEt_4$. Standard reference material (DOLT-5 fish liver, National Research Council of Canada and DORM-4) were digested using the same procedure and analyzed concurrently. Quality control samples including the SRMs, reagent blanks, and initial and ongoing precision and recovery (IPR and OPR) samples were analyzed every 12-20 samples. Reagent blanks were prepared with KOH and methanol without the addition of any sample and 30 µl of the digest were used for analysis. Methylmercury concentrations for whole tissues are reported as above.

4.3.3 Subcellular Partitioning

Liver tissues were subjected to a differential centrifugation procedure to separate them into 6 operationally defined subcellular fractions, including: 1) mitochondria, 2) microsomes and lysosomes, 3) cytosolic heat-stable proteins such as metallothionein, 4) cytosolic heat-denatured proteins, 5) debris and nuclei, and 6) sodium hydroxide (NaOH)-resistant granules (Figure 4.1) [32,42]. Liver subsamples from individual birds were pooled (~50 mg wet weight per individual) with 4 individuals per composite, resulting in 3 composites per species/time point. Each composite was processed in triplicate for PH 1 and PH 7 chickens and in duplicate for Ring-bill Gulls. Due to the small quantity of ED 19 chicken liver tissue available, only one composite sample was processed in duplicate. All liver samples were weighed and manually sliced with a surgical steel razor blade. Each composite was homogenized on ice at low speed (620 rpm) in 1.5 mL of a 10 mM Tris (pH 7.4) and 250 mM sucrose solution using a drill press (Mastercraft Canada, Toronto, ON, Canada) and a Potter-Elvehjem teflon pestle with a glass tube (Sigma Aldrich, Oakville, ON, Canada). For all species and time points, composites were homogenized for 5 s followed by a 30 s break. This process was carried out a total of 5 times. After homogenization, the homogenate was transferred to an acid-washed 2 ml polypropylene microcentrifuge tube, with similar tubes used throughout the procedure. The homogenate was vortexed briefly and 100 µl were removed and used to determine composite THg concentrations for mass-balance calculations.

The remaining homogenate (1400 µl) was centrifuged at 800 *g* for 15 min at 4 °C. The resulting supernatant (S1) was removed and set aside on ice for further separations. The resulting pellet (P1) was suspended in 500 µl of ultrapure water and heated at 100 °C for 2 min. It was then digested with 500 µl of 1 M NaOH (98 %, Bioshop, Canada Inc., Burlington, ON, Canada)

at 65 °C for 60 min. The digest was centrifuged at 10,000 *g* for 10 min at ambient temperature (20 °C). The resulting supernatant (S2) was removed as the debris and nuclei fraction and the remaining pellet (P2) reserved as the NaOH-resistant granule fraction. The supernatant (S1, from the original homogenization step) was centrifuged at 10,000 *g* for 30 min at 4 °C to separate the mitochondrial fraction (pellet, P3). The resulting supernatant (S3) was centrifuged at 100,000 *g* for 60 min at 4 °C to separate the microsomes and lysosomes fraction (P4) and the cytosolic fractions (S4). The S4 fraction was heated at 80 °C for 10 min, placed on ice for 60 min, and centrifuged at 50,000 *g* for 10 min at 4 °C to separate the heat-stable proteins fraction (S5) from the heat-denatured proteins fraction (P5). An Eppendorf microcentrifuge (Eppendorf AG, Hamburg, Germany) was used for speeds ≤10,000 *g*, while an Optima LE-80K Ultracentrifuge with a type 50.2 Ti rotor (Beckman Coulter Canada, LP., Mississauga, ON, Canada) was used for higher speed centrifugations. This centrifuge required a minimum of 2 ml of sample per vial, therefore, additional homogenization buffer was added to each sample prior to each high-speed centrifugation step. The efficacy of the homogenization step and differential centrifugation procedure was determined by measuring marker enzymes (lactate dehydrogenase, LDH, and cytochrome c oxidase) specific to particular fractions or organelles (Appendix Figure 4.1) [42]. Following separations, the homogenates and subcellular fractions were stored for <36 hours at 4 °C until THg analysis.

Total Hg of liver homogenates and subcellular fractions was determined using direct Hg analysis (NIC MA-3000) [40]. For homogenates and most of the fractions, the entire sample was used for analysis. Given the relatively large sample volumes (1.5 – 2 ml, due to the addition of either ultrapure water, NaOH, or homogenization buffer) for the heat-stable proteins and the debris and nuclei fractions, a known proportion was analyzed and the THg for the entire fraction

was calculated based on the known total volume of the fractions. Quality control samples including SRMs (DORM-4, hair #13) and empty boat blanks were run every 20 samples. The THg concentration for each subcellular fraction within a composite was calculated by dividing the THg burden (μg) of the fraction by the dry weight (g, dw) of the liver composite. Dry weight was estimated for each composite using an average moisture content (67.8 %) determined from a subset of whole liver samples. The proportion of THg in each subcellular fraction was determined relative to the total Hg burden and reported as % THg of the homogenate.

4.3.4 Statistical Analyses

We report the mean, standard deviation, and range of THg and MeHg concentrations for blood, brain, and liver tissues of PH1 and PH 7 chickens and brain and liver tissues of ED 19 chickens and of Ring-billed Gull embryos. We report the ratio of MeHg to THg X 100, as a percentage (percent MeHg) for all these tissues. Data were tested for normality using Shapiro-Wilk tests and non-parametric analyses were used as needed. The relationship among tissue THg concentrations was investigated using Pearson's correlation. We also investigated the relationship between percent MeHg and THg concentrations for chicken blood, brain, and liver tissues (time points combined) and Ring-billed Gull liver tissue using Spearman's correlation. For chickens, we compared THg concentrations of each tissue among time points and among tissues within each time point using t-tests and analysis of variance (ANOVA) tests followed by Tukey's HSD post hoc tests, as appropriate. Additionally, we compared THg concentrations of brain and liver tissues of embryonic Ring-billed Gulls using a t-test.

We verified THg for the subcellular fractions for each composite by performing a mass balance calculation using the 100 μl sample removed immediately following the first homogenization step as a reference value. We report mean and standard deviation of the

concentration of THg (µg/g dw) in subcellular fractions of composite liver samples from ED 19, PH 1, and PH 7 chickens and from embryonic Ring-billed Gulls. Additionally, we report the mean and standard deviation of the proportion of THg (%) in each subcellular fraction relative to the total Hg burden for composite liver samples. We report the average precision for THg analyses of replicate subcellular fractions, calculated as relative standard deviation (RSD %) for PH 1 and PH 7 chickens and relative percent difference (RPD %) for Ring-billed Gulls and ED 19 chicken. Replicate composite samples were averaged for all remaining statistical analyses. We investigated if the THg concentrations of subcellular fractions differed among time points for chickens using ANOVA tests followed by Tukey's HSD post hoc tests, as appropriate, and between embryonic chickens and Ring-billed Gulls using t-tests. Additionally, we investigated if the proportion of THg for subcellular fractions differed among time points for chickens and between embryonic chickens and Ring-billed Gulls using Wilcoxon signed-rank tests. All statistical analyses were performed using JMP 11 [43] and significance was determined if $P < 0.05$.

4.4 Results

4.4.1 Mercury Analysis

4.4.1.1 Quality Assurance / Quality Control (QA / QC)

Our analytical laboratory participates in the Northern Contaminants Program and Arctic Monitoring and Assessment Program inter-laboratory program that tests the performance of analytical laboratories. In Phase 10 of this program, which covers the period of the current study, our analytical laboratory's capabilities to measure MeHg and THg were ranked "excellent" with absolute z-scores less than 1. Results obtained for QC samples analyzed throughout THg and

MeHg sample analysis indicated that the data were of acceptable quality (Supplemental Table 4.1Supplemental Table 4.2) according to US EPA guidelines. For THg analysis, two direct Hg analyzers were used. Accuracy and precision were similar between the analyzers, therefore these values were combined, however, limits of detection differed between instruments and were calculated separately. Average recoveries for SRMs ranged from 96.0 ± 1.8 % for DORM-4 to 101.9 ± 2.1 % for Hair. Analytical precision, calculated as mean percent RSD and RPD, was under 4.0 % for SRMs and was much lower for duplicate tissue samples (1.2 ± 1.7 %, calculated as percent RPD). Limits of detection were calculated as the mean value of blank boat samples plus 3X (theoretical method detection limit, TMDL) and 5X (practical method detection limit, PMDL) the standard deviation of the mean and are reported as nanograms (ng) of Hg. For the DMA-80, mean detection limits were (TMDL) 0.35 ± 0.23 and (PMDL) 0.46 ± 0.35 ng (n = 3 days). For the NIC MA-3000 mean detection limits were much lower, (TMDL) 0.04 ± 0.03 and (PMDL) 0.07 ± 0.05 ng (n = 11 days). All whole tissue and subcellular fraction samples were above detection limits except for Ring-billed Gull NaOH-resistant granule fractions (measured on the NIC MA-3000), even though these samples were under detection, we used the analyzed concentrations for all subsequent evaluations.

Methylmercury analysis showed the accuracy of IPRs and OPRs were 99.1 ± 8.2 % and 106.1 ± 2.0 %, respectively, with analytical precision respectively less than 10.0 %. Analytical precision for duplicate tissue samples was 9.5 ± 9.7 %, calculated as RPD. The accuracy of DORM-4 was 118.9 ± 21.8 %, with analytical precision 23.6 % RSD. For DOLT-5 the results were variable, with an accuracy of 95.9 ± 41.7, but a high analytical precision of 43.5 % RSD. However, for all chicken samples, DORM-4 and DOLT-5 samples were digested and analyzed concurrently. For Ring-billed Gull liver samples, a single DOLT-5 sample was digested and

analyzed, with an accuracy of 109.3 %. Limits of detection were calculated as the mean value of reagent blank KOH samples plus 3X (theoretical method detection limit, TMDL) and 5X (practical method detection limit, PMDL) the standard deviation of the mean and are reported as ng/ml of Hg. Concentrations for blank KOH sample MeHg concentrations were variable, resulting in higher than expected limits of detection, with a mean of (TMDL) 49.6 and (PMDL) 72.0 ng/ml (n = 7). All chicken tissue samples were above detection limits. Some Ring-billed Gull samples were below the mean PMDL, however, the blank KOH sample digested and analyzed with these samples had a very low concentration (0.05 ng/ml).

4.4.2 Mercury Speciation

4.4.2.1 Total Hg in Bulk Tissues

Total Hg concentrations in chicken brains ranged from 1.72 to 8.20 µg/g dw, livers ranged from 1.77 to 11.75 µg/g dw, and blood (PH 1 and PH 7 only) ranged from 0.69 to 1.51 µg/g ww (Table 4.1). Embryonic Ring-billed Gull brain and liver tissues had much lower THg concentrations ranging from 0.06 to 0.15 µg/g dw and 0.08 to 0.17 µg/g dw, respectively. Within time points, brain and liver THg concentrations did not differ ($t_{22} = 0.5$, $P = 0.634$) for ED 19 chickens. For PH 1 chickens, THg concentrations were lower in blood than for both brain and liver tissues ($F_{2,33} = 39.7$, $P < 0.001$). Total Hg concentrations for PH 7 chickens differed among all tissues sampled ($F_{2,33} = 35.6$, $P < 0.001$), with the greatest concentrations in liver tissue. For Ring-billed Gulls, brain THg concentrations were lower than those of liver ($t_{21} = 2.1$, $P = 0.047$, Table 4.1). For chickens, THg concentrations of blood, brain, and liver tissue differed by time point (blood: $t_{14} = -5.3$, $P < 0.001$; brain: $F_{2,33} = 5.1$, $P = 0.012$; liver: $F_{2,33} = 6.8$, $P = 0.003$).

Tissue THg concentrations were correlated for Ring-billed Gulls and for each chicken time point (Supplemental Table 4.3).

4.4.2.2 Methylmercury in Bulk Tissues

The majority of Hg in hatchling and embryonic chicken blood, brain, and liver tissues was in the form of MeHg, with the mean percentage of MeHg ranging from 99.6 ± 6.8 to 121.3 ± 12.9 % (Table 4.1). Ring-billed Gull liver tissues had a lower mean percentage of MeHg (93.8 ± 12.9). We found no relationship between percent MeHg and THg concentrations for chicken blood (r_s = 0.19, P-value = 0.377) or Ring-billed Gull liver tissue (r_s = -0.02, P-value = 0.948). However, the percent MeHg decreased as THg concentrations increased for chicken brain (r_s = -0.52, P-value = 0.001) and liver (r_s = -0.66, P-value < 0.001).

4.4.3 Subcellular Partitioning

The analysis of marker enzymes (LDH and CCO) indicated high efficacy for our subcellular partitioning protocol (Appendix, 4.8). For all chicken time points and embryonic Ring-bill Gulls, the mass balance results were excellent, with respective recoveries of 96.0 ± 5.9 % and 105.0 ± 6.8 %. Mean precision for replicate composite samples was <26% for all subcellular fractions, with the exception of NaOH-resistant granules (Supplemental Table 4.4).

Total Hg concentrations of the composites ranged from 2.24 to 5.03 µg/g dw for chickens and 0.07 to 0.08 µg/g dw for Ring-billed Gulls. For both PH 1 and PH 7 chickens and Ring-billed Gulls, mean THg concentrations were greatest in heat-denatured proteins, followed by debris and nuclei, mitochondria, heat-stable proteins, microsomes and lysosomes, and NaOH-resistant granules having the lowest THg concentrations. Subcellular fractions for embryonic chickens showed a similar pattern in THg concentrations, though they had a greater THg

concentration for the microsomes and lysosomes fraction than for the heat-stable proteins fraction. For chickens, THg concentrations were lower in embryonic chickens than in hatchlings for mitochondria ($F_{2,6} = 5.6$, $P = 0.043$), microsomes and lysosomes ($F_{2,6} = 45.7$, $P < 0.001$), heat-stable proteins ($F_{2,6} = 32.1$, $P < 0.001$), and heat-denatured proteins ($F_{2,6} = 17.5$, $P = 0.003$, Figure 4.2). Overall, Ring-billed Gulls had much lower liver THg concentrations than the embryonic chickens, therefore, this was also the case for all subcellular fractions, except granules (Figure 4.3).

Both species and all time points had the greatest proportion of THg in the heat-denatured proteins fraction, with mean proportions ranging from 42.3 to 46.1 % (Supplemental Table 4.5, Figure 4.4). For both PH 7 and PH 1 chickens and Ring-billed Gulls, the fraction with the next greatest proportion of THg was debris and nuclei, followed by mitochondria, heat-stable proteins, microsomes and lysosomes, with NaOH-resistant granules having a very low proportion of THg. Subcellular fractions for embryonic chickens showed a similar pattern, though they had a greater proportion of THg found in the microsomes and lysosomes fraction than in the heat-stable proteins fraction. The proportion of THg in subcellular fractions differed little among time points for chicken liver composites, except for embryos having a significantly lower proportion of THg associated with heat-stable proteins compared to hatchlings ($P = 0.027$, Figure 4.2). The THg proportion for subcellular fractions also differed slightly between embryonic chickens and Ring-billed Gulls, with the gulls having marginally greater THg in the heat-stable proteins fraction ($P = 0.050$) and marginally lower THg in the debris fraction ($P = 0.050$, Figure 4.3).

4.5 Discussion

This research aimed to better understand Hg toxicokinetics in order to improve avian Hg exposure assessments and help identify species at the greatest risk for negative health effects of Hg exposure. We investigated Hg speciation by measuring THg and MeHg concentrations in blood, brain, and liver tissues of chicken dosed with MeHg via egg injection and in liver of embryonic Ring-billed Gulls exposed to maternally-deposited MeHg. Additionally, we investigated subcellular distribution of THg in livers of embryos and hatchlings. By comparing these processes among developmental time points in chickens and between species, we can evaluate how these processes vary across species, developmental time points, or with increasing Hg exposure.

4.5.1 Mercury Speciation

4.5.1.1 Total Hg in Bulk Tissues

Total Hg results for chicken brain and liver tissues, following MeHg egg injection, were similar to those found by Rutkiewicz and Basu [24], which used the same egg injection methods. Comparably, we found greater, but not significantly different, THg concentrations in liver compared to brain tissue for both ED 19 and PH 1 chickens. We found THg concentrations were significantly greater in liver than brain tissue for PH 7 chickens and for Ring-billed Gull embryos. These results add to an already strong set of laboratory and field studies demonstrating that the liver is important for Hg sequestration in embryonic, juvenile, and adult birds [22,24,26]. Again, similar to Rutkiewicz and Basu [24], we found THg in liver and brain tissue increased in chickens from ED 19 to PH 1, possibly due to yolk absorption. Previous studies have found hatchling birds have greater THg concentrations in growing feathers than in internal tissues

[19,22,24], indicating that Hg sequestration in growing feathers is an important detoxifying method for hatchling birds. We noted a decrease in blood THg concentrations between PH 1 and PH 7 time points, a period when chickens are growing juvenile flight feathers. We did not see a concurrent decrease in tissue THg concentrations, indicating that blood Hg was incorporated into growing feathers, but Hg was not mobilized from liver and brain tissues during this time.

Previous field studies have shown strong correlations in THg concentrations among internal tissues for multiple species of juvenile and adult birds [19,44,45]. We found THg concentrations to be strongly correlated for all chicken tissues across all three time points. Additionally, we saw a similarly strong correlation between Ring-billed Gull liver and brain THg concentrations. In the present study, we observed that the relationship among tissue THg concentrations for chickens dosed with MeHg via egg injection was similar to that seen in wild birds. In addition, these results indicate that non-lethal blood sampling can be a good predictor of internal THg burden. Mercury is known to be neurotoxic, and the strong correlation among brain THg concentrations and those of blood and liver allow these more commonly sampled biomarker tissues to be used to indicate neurological risk in embryos and hatchlings.

4.5.1.2 Methylmercury in Bulk Tissues

This study found that the majority of Hg in hatchling and embryonic chicken blood, brain, and liver tissues was in the form of MeHg. For blood, this result was expected as previous studies on wild birds also indicate that the majority of Hg is in the form of MeHg [46,47]. However, little is known about whether this proportion varies across species, developmental time points, or with increasing Hg exposure. We found this proportion was similar between PH1 and PH 7 chickens and saw no relationship with increasing THg concentrations. In contrast, previous Hg speciation studies on avian liver tissue have shown the percent MeHg in liver tissue to be consistently less

than 100 %, indicating hepatic MeHg demethylation occurs in both birds dosed with MeHg via egg injection [24] and in wild birds [25,27,28]. These limited studies indicate that liver MeHg demethylation ability in birds differs among species, by sex and age, and with Hg exposure. For brain tissues, previous studies indicate that MeHg demethylation occurs in wild birds and differs by species [44,45]. However, other studies have found no indication of MeHg demethylation in avian brain tissues [22,26]. In the current study, there was little indication of MeHg demethylation in liver or brain tissues for all time points for chickens dosed with MeHg via egg injection. However, an inverse relationship between the percent MeHg and THg concentrations for both tissues was observed. Due to the small amount of tissue available from each bird, we were unable to investigate MeHg concentrations for Ring-billed Gull brain tissue. We found the mean percent MeHg for Ring-billed Gull liver tissue to be less than 100 %. Additionally, we did not observe a relationship between percent MeHg and THg for Ring-billed Gull liver tissue. This indicates that there may be some slight differences in MeHg demethylation ability between species, however, differences in exposure concentration and method of exposure may also play a role. The Ring-billed Gulls were exposed to maternally-deposited Hg, which has been shown to be predominately MeHg in wild birds [48], therefore, the two species in the present study were exposed to MeHg. They were sampled ≤ 1 week after hatching, allowing only a short period of time for MeHg demethylation to occur. Future studies are needed to investigate Hg speciation across later life stages.

A previous study by Rutkiewicz and Basu [24] also investigated MeHg demethylation in liver and brain for ED 19 chicken embryos dosed with MeHg via egg injection. They found lower percent OHg in both brain (mean: 82%, n = 14) and liver (mean: 70%, n = 17) tissue than in the current study. These 2 studies differed in important ways, first, Rutkiewicz and Basu [24]

used micro-scale extraction and direct Hg analysis for OHg analysis, while the current study used alkaline digestion followed by cold vapor atomic fluorescence spectroscopy detection, which is a more sensitive method of analysis, and allows for the direct quantification of MeHg. Secondly, embryos in the Rutkiewicz and Basu [24] study were dosed with higher MeHg concentrations. Notably, we observed a negative relationship between the percent MeHg and THg concentrations for brain and liver tissue for all time points. This may indicate that increased THg concentrations result in greater demethylation of MeHg. However, Rutkiewicz and Basu [24] did not observe this relationship for brain tissue and saw only a marginally negative relationship for liver tissue.

4.5.2 Subcellular Partitioning

We investigated the subcellular distribution of Hg in bird liver by adapting subcellular partitioning procedures previously used to determine the subcellular distribution of Hg and other metals in fish liver and invertebrates [32,34,42,49]. We used enzymatic assays to optimize and verify our partitioning procedure [42], and found our protocol was highly effective at separating liver cells into the appropriate operationally-defined fractions. Additionally, mass balance results showed that our procedure resulted in excellent agreement between THg concentrations for subcellular fractions and their respective liver homogenates. Nevertheless, caution should be applied to the resulting Hg concentrations in the operationally defined fractions. For instance, as the homogenization process disrupts membranes and cellular compartments, there is the possibility for intracellular reactions to occur that could change the distribution of Hg within the cells [50]. Further, metals may be released from the heat-denatured proteins fraction during the heat denaturation process [35]. Caution should also be applied when grouping these fractions into metal-sensitive (mitochondria, microsomes and lysosomes, heat-denatured proteins) and

detoxified (HSP and NaOH-resistant granules) compartments. For instance, within the microsomes and lysosomes fraction, there may be both metal-sensitive sites, such as those related to protein synthesis and transport (microsomes), as well as detoxifying sites related to storage and elimination (lysosomes) [35]. Nevertheless, subcellular partitioning procedures can provide useful information about the potential toxic effects of non-essential metals, such as Hg. In this context, our results indicate that the partitioning procedure for the determination of THg in avian liver subcellular fractions can be used to provide important information on MeHg toxicokinetics and risk in birds.

Total Hg concentrations for most subcellular fractions were lower in embryonic chickens than in hatchlings, as expected due to the lower THg concentrations seen for whole liver tissue. Similarly, Ring-billed Gulls had much lower THg concentrations than the embryonic chickens for all subcellular fractions, except granules. Barst et al. [34] investigated Hg subcellular distribution in the livers of Arctic char (*Salvelinus alpinus*) with significantly different Hg exposures, yet noted that THg was distributed similarly among subcellular fractions of fish with both low and high Hg exposure. This is consistent with our observations that THg was distributed similarly among fractions isolated from chicken and Ring-billed Gulls despite significant differences in Hg concentrations in both bulk tissue and subcellular fractions. This research indicates that Hg subcellular distribution is not associated with increasing Hg concentrations for embryonic and hatchling birds. However, a previous study investigating liver subcellular distribution in northern fur seal (*Callorhinus ursinus*), Dall's porpoise (*Phocoenoides dalli*), and Black-footed Albatross saw that Hg was preferentially distributed in the nuclear, lysosomal, and mitochondrial fraction as liver THg concentrations increased for all species combined [31]. We further elaborate on the findings from this study below.

We found only a small proportion of THg within the detoxified fractions, indicating that Hg detoxification was minimal at the subcellular level for both species and all time points. In contrast, we found the greatest proportion of THg within the metal-sensitive fractions with consistent measurements for both species and all time points. For all samples, cytosolic heat-denatured proteins had the greatest proportion of THg, followed by the debris and nuclei and mitochondria fractions. Mercury accumulation in the heat-denatured proteins fraction, containing enzymes, could lead to negative health effects. Evidence for this comes from field and laboratory studies on birds, which demonstrate that exposure to MeHg may lead to decreased activities of antioxidant enzymes [51–53]. Previous research on fish indicates that MeHg exposure can cause structural abnormalities and inhibit respiration in the mitochondria of muscle tissue [54], however, the effect of MeHg on mitochondria of birds is unknown. We saw little evidence of Hg detoxification in liver tissue for both species and all time points, with low proportions of THg in both the NaOH-resistant granule and HSP fractions. While grouping subcellular fractions into metal-sensitive and detoxified fractions is likely an oversimplification, these results add to the body of evidence that indicates embryonic and hatchling time points are developmental stages in birds that are sensitive to Hg exposure. Chickens have been categorized as having a medium sensitivity to Hg, according to an egg injection study [21]. While Hg sensitivity of Ring-billed Gulls has not been classified, this research indicates that they have a similar ability to detoxify Hg at the subcellular level as chickens.

Subcellular distribution of Hg and other metals in bird liver tissue has been investigated for one other species, the Black-footed Albatross, sampled in the wild [31]. In this study, researchers used a different subcellular partitioning procedure resulting in 3 subcellular fractions: a nuclear, lysosomal, and mitochondrial fraction, a microsomal fraction, and a cytosolic fraction. The

differing procedures make it difficult to compare these two studies. Additionally, Ikemoto et al. [31] offered little evidence to validate the efficacy of their partitioning procedure. Even so, results indicate differences in subcellular distribution among bird species. Ikemoto et al. [31] did not partition the cytosolic fraction into HSP and heat-denatured proteins, and they found a low proportion of Hg in the entire cytosolic fraction. For chickens and Ring-billed Gulls, we found combined cytosolic fractions (HSP and heat-denatured proteins) had ≥50 % of the THg, whereas Ikemoto et al. [31] found >75 % of the THg in the nuclear, lysosomal, and mitochondrial fraction for Black-footed Albatross. In addition to differences in study methods, some major differences among the study species may explain the disparities in results between the two studies. Black-footed Albatrosses are long-lived, large bodied piscivorous seabirds. They had very high THg liver concentrations (range: 36 – 150 μg/g ww), with high MeHg demethylation ability, only 6.6 % of THg in the liver was OHg. Further, the Black-footed Albatrosses in this study were mostly adult birds (n = 4 adults, 1 juvenile). Further research on the subcellular distribution of Hg in adult bird liver, as well as the distribution of Hg species is needed to better understand how these affect Hg distribution within avian liver cells.

The current study investigates subcellular distribution in liver tissue during a period of feather growth in hatchling chickens. Feather growth is a Hg depuration process for birds, and internal tissue Hg burdens are known to be mobilized to feathers during growth [55–57]. Peng et al. [36] subjected rabbitfish (*Siganus canaliculatus*) to a MeHg depuration process and saw that liver subcellular fractions eliminated MeHg in varying amounts. We saw no changes in the subcellular distribution of THg for chicken between PH1 and PH 7, even though this was a period of feather growth. These results further indicate that Hg was not mobilized from the liver into growing feathers over this time period. In order to better understand how Hg risk changes

over time, further studies are needed to determine if liver subcellular fractions differentially eliminate Hg during depuration events such as feather growth.

This is the first study to investigate subcellular distribution of Hg in the liver of a model avian species exposed to MeHg via egg injection. Additionally, we compared the results with those from a wild avian species exposed to maternally-deposited MeHg. The THg proportion for liver subcellular fractions differed marginally between chickens and Ring-billed Gulls sampled at similar embryonic time points. Previous studies using MeHg egg injection of chickens to investigate avian Hg toxicokinetics and adverse health effects and sensitivity to Hg exposure [21,24,58] indicate this exposure method is useful for studying toxicity in Hg exposed birds. Heinz et al. (2009) cautions that MeHg exposure via egg injection may be more embryotoxic than maternally-deposited MeHg. However, the current study offers evidence that, within liver cells, MeHg egg injection of chicken mimics maternally-deposited MeHg exposure.

4.5.3 Conclusion

The present study investigated Hg toxicokinetics using MeHg egg injection of a model avian species, White Leghorn Chicken, and wild embryonic Ring-billed Gulls exposed to maternally-deposited MeHg. This work adds to previous MeHg egg injection studies on chicken by focusing on MeHg demethylation in important biomarker tissues (blood and liver) as well as tissues sensitive to the adverse effects of MeHg (liver and brain). Additionally, this is the first study, to our knowledge, that has used MeHg egg injection to investigate subcellular distribution of Hg in bird liver. We investigated how Hg speciation and subcellular distribution changed over early life developmental time points and differed by species. We adapted and verified a partitioning procedure for separating liver tissue into operationally defined subcellular fractions. We found that subcellular distribution of Hg did not differ between birds exposed to MeHg via egg

injection and birds exposed to maternally-deposited MeHg. This research further indicates that MeHg egg injection of a model avian species, the White Leghorn Chicken, can offer useful information on MeHg toxicokinetics in birds. This study adds to a growing body of research indicating that embryonic and hatchling time points are sensitive developmental stages at risk for negative health effects of Hg exposure.

4.6 Acknowledgments

The authors thank Jenny Eng and Gordana Martincevic for their technical assistance, Maikel Rosabal and Antoine Caron for help with subcellular optimization, Matthew Alloy for help with egg injections, and Krittika Mittal for tissue dissections. Funding for this research was provided by Fulbright Canada (Perkins), NSERC CREATE EI (Barst), and the Canada Research Chair program (Basu).

4.7 Tables and Figures

Table 4.1. Comparisons of THg and MeHg concentrations (mean, standard deviation (SD), and range) and the percent MeHg relative to THg for blood (µg/g ww), brain and liver tissues (µg/g dw) from chickens sampled at embryonic day 19 (ED 19) and post-hatch day 1 (PH 1) and 7 (PH 7), and embryonic (EMB) Ring-billed Gulls (n = 12 individuals per species and time point). Tissues sharing a letter do not differ for THg concentration (P > 0.05). Capital letters indicate comparisons among tissues within time points, lower case letters indicate comparisons among time points for each tissue (chickens only).

Species	Time Point	Tissue	THg (SD)	THg Range	MeHg (SD)	MeHg Range	% MeHg (SD)
Chicken	ED 19	Brain	3.88 (1.41) $^{A\ a}$	1.72 - 6.42	3.80 (1.21)	1.83 - 5.86	99.6 (6.8)
		Liver	4.18 (1.64) $^{A\ a}$	1.77 - 7.64	4.41 (1.44)	2.03 - 7.19	108.0 (8.2)
	PH 1	Blood	2.07 (0.62) $^{A\ a}$	1.21 - 3.31	2.47 (0.66)	1.47 - 3.70	121.3 (12.9)
		Brain	5.58 (1.39) $^{B\ b}$	3.22 - 8.20	5.65 (1.15)	4.37 - 7.99	103.5 (16.1)
		Liver	6.83 (1.80) $^{B\ b}$	3.90 - 10.70	6.87 (1.60)	4.56 - 9.29	101.8 (11.2)
	PH 7	Blood	1.06 (0.24) $^{A\ b}$	0.69 - 1.51	1.20 (0.30)	0.75 - 1.76	113.5 (4.8)
		Brain	4.52 (1.13) $^{B\ ab}$	2.68 - 6.23	4.58 (0.93)	3.17 - 6.26	103.1 (9.9)
		Liver	7.12 (2.83) $^{C\ b}$	3.43 - 11.75	6.83 (2.27)	3.60 - 10.10	99.4 (15.1)
Ring-billed Gull	EMB	Brain	0.09 (0.02) ^{A}	0.06 - 0.15	NA	NA	NA
		Liver	0.11 (0.03) ^{B}	0.08 - 0.17	0.11 (0.03)	0.06 - 0.15	93.8 (12.9)

Supplemental Table 4.1. Summary of THg quality control measurements for whole tissue and subcellular fractions analysis, including mean accuracy and standard deviation (SD), calculated as the percent recovery compared to certified value, and mean analytical precision, calculated as relative standard deviation (RSD) or relative percent difference (RPD), for standard reference materials (DORM-4 fish protein, National Research Council of Canada, human hair (#13), Japanese National Institute for Environmental Studies, and human blood QMEQAS10B-09, Institut National de Santé Publique du Québec).

Standard Reference Material	Number of Days	Number of Samples	% Accuracy (SD)	% Precision (SD)
DORM-4	14	48	96.0 (1.8)	2.8 (2.6)
Hair #13	10	21	101.9 (2.1)	1.0 (0.7)
QMEQAS10B-09	1	5	97.7 (3.1)	3.1

Supplemental Table 4.2. Summary of MeHg quality control measurements for whole tissue samples, including mean accuracy and standard deviation (SD), calculated as the percent recovery compared to certified or expected value, and mean analytical precision, calculated as relative standard deviation (RSD) or relative percent difference (RPD), for initial and ongoing precision and recovery (IPR and OPR) samples, standard reference materials (DOLT-5 fish liver and DORM-4 fish protein, National Research Council of Canada), and matrix spikes for DORM-4 and liver and brain tissues.

Sample	Number of Days	Number of Samples	% Accuracy (SD)	% Precision (SD)
IPR	4	4	99.1 (8.2)	8.3
OPR	5	26	106.1 (2.0)	4.5 (2.2)
DORM-4	3	6	118.9 (21.8)	23.6 (10.3)
DOLT-5	3	4	95.9 (41.7)	43.5
Matrix Spike	2	7	126.7 (7.0)	19.0 (4.0)

Supplemental Table 4.3. The relationship among tissues sampled for THg concentrations. Test statistics (Pearson's Coefficient and P-value) for correlations between tissue THg concentration of embryonic day 19 (ED 19) and post-hatch day 1 (PH 1) and 7 (PH 7) chickens and embryonic (EMB) Ring-billed Gulls (n = 12 per time point and species).

Species	Time Point	Tissues		Pearson's Coefficient	P-value
Chicken	ED 19	Brain	Liver	0.95	<0.001
	PH 1	Blood	Brain	0.87	<0.001
		Blood	Liver	0.90	<0.001
		Brain	Liver	0.97	<0.001
	PH 7	Blood	Brain	0.91	<0.001
		Blood	Liver	0.80	0.002
		Brain	Liver	0.78	0.003
Ring-billed Gull	EMB	Brain	Liver	0.89	<0.001

Supplemental Table 4.4. Precision of THg in subcellular fractions for replicate composite liver samples (mean and standard deviation, SD) for chickens sampled at embryonic day 19 (ED 19) and post-hatch day 1 (PH 1) and 7 (PH 7), and embryonic (EMB) Ring-billed Gulls. Subcellular fraction abbreviations: Mito = mitochondria, M+L = microsomes and lysosomes, HSP = heat-stable proteins, HDP = heat-denatured proteins, Debris = debris and nuclei, Gran = NaOH-resistant granules. Precision calculated as relative standard deviation (RSD %) for PH 1 and PH 7 chickens (n = 3 composites, 3 replicates per composite) and relative percent difference (RPD %) for Ring-billed Gulls (n = 3 composites, 2 replicates per composite) and ED 19 chicken (n = 1 composite, 2 replicates per composite).

Species	Time Point	Fraction	RSD or RPD (%)	SD
	ED 19	Mito	20.3	
		M+L	18.8	
		HSP	14.0	
		HDP	8.0	
		Debris	25.9	
		Gran	8.2	
Chicken	PH 1	Mito	3.2	2.7
		M+L	18.5	16.2
		HSP	8.5	7.1
		HDP	1.6	0.5
		Debris	7.7	3.7
		Gran	16.5	8.0
	PH 7	Mito	14.2	9.1

		M+L	16.0	11.7
		HSP	18.2	4.3
		HDP	3.7	2.1
		Debris	9.6	5.3
		Gran	43.2	2.7
Ring-billed Gull	EMB	Mito	7.6	6.8
		M+L	14.9	10.2
		HSP	6.5	14.0
		HDP	3.0	4.2
		Debris	17.7	21.6
		Gran	51.2	108.2

Supplemental Table 4.5. Comparisons of concentration (µg/g dw) and proportion (%) of THg in subcellular fractions (mean and standard deviation, SD) for chicken livers sampled at embryonic day 19 (ED 19) and post-hatch day 1 (PH 1) and 7 (PH 7), and embryonic (EMB) Ring-billed Gull liver (n = 3 composite samples per species and time point). Subcellular fraction abbreviations: MIT = mitochondria, M+L = microsomes and lysosomes, HSP = heat-stable proteins, HDP = heat-denatured proteins, DE = debris and nuclei, GR = NaOH-resistant granules Tissues sharing a letter do not differ (P > 0.05). Capital letters indicate comparisons among time points for each subcellular fraction of chicken livers, lower case letters indicate comparisons between embryonic chickens and Ring-billed Gulls, % THg did not differ.

Species	Time Point	Fraction	THg, µg/g dw (SD)		% THg (SD)	
Chicken	ED 19	MIT	0.42 (0.03)	B a	16.5 (0.8)	A
		M+L	0.24 (0.03)	B a	9.4 (1.8)	A
		HSP	0.19 (0.01)	B a	7.3 (0.8)	B
		HDP	1.09 (0.10)	B a	42.3 (2.0)	A
		DE	0.63 (0.18)	A a	24.2 (4.4)	A
		GR	0.01 (0.01)	A a	0.4 (0.2)	A
	PH 1	MIT	0.71 (0.12)	A	15.2 (1.3)	A
		M+L	0.46 (0.04)	A	10.1 (0.2)	A
		HSP	0.49 (0.08)	A	11.3 (0.7)	A
		HDP	2.15 (0.33)	A	46.1 (2.6)	A
		DE	0.79 (0.08)	A	17.1 (2.6)	A

		GR	0.02 (0.01)	A	0.3 (0.1)	A
	PH 7	MIT	0.74 (0.18)	A	17.5 (2.5)	A
		M+L	0.40 (0.01)	A	9.6 (0.8)	A
		HSP	0.41 (0.03)	A	9.9 (0.3)	A
		HDP	1.83 (0.19)	A	44.2 (1.9)	A
		DE	0.77 (0.08)	A	18.5 (0.9)	A
		GR	0.01 (0.00)	A	0.2 (0.0)	A
Ring-billed Gull	EMB	MIT	0.073 (0.005)	b	17.5 (1.3)	
		M+L	0.013 (0.001)	b	7.6 (0.7)	
		HSP	0.006 (0.001)	b	14.2 (1.0)	
		HDP	0.010 (0.001)	b	42.7 (1.6)	
		DE	0.031 (0.001)	b	17.6 (2.2)	
		GR	0.013 (0.002)	a	0.4 (0.4)	

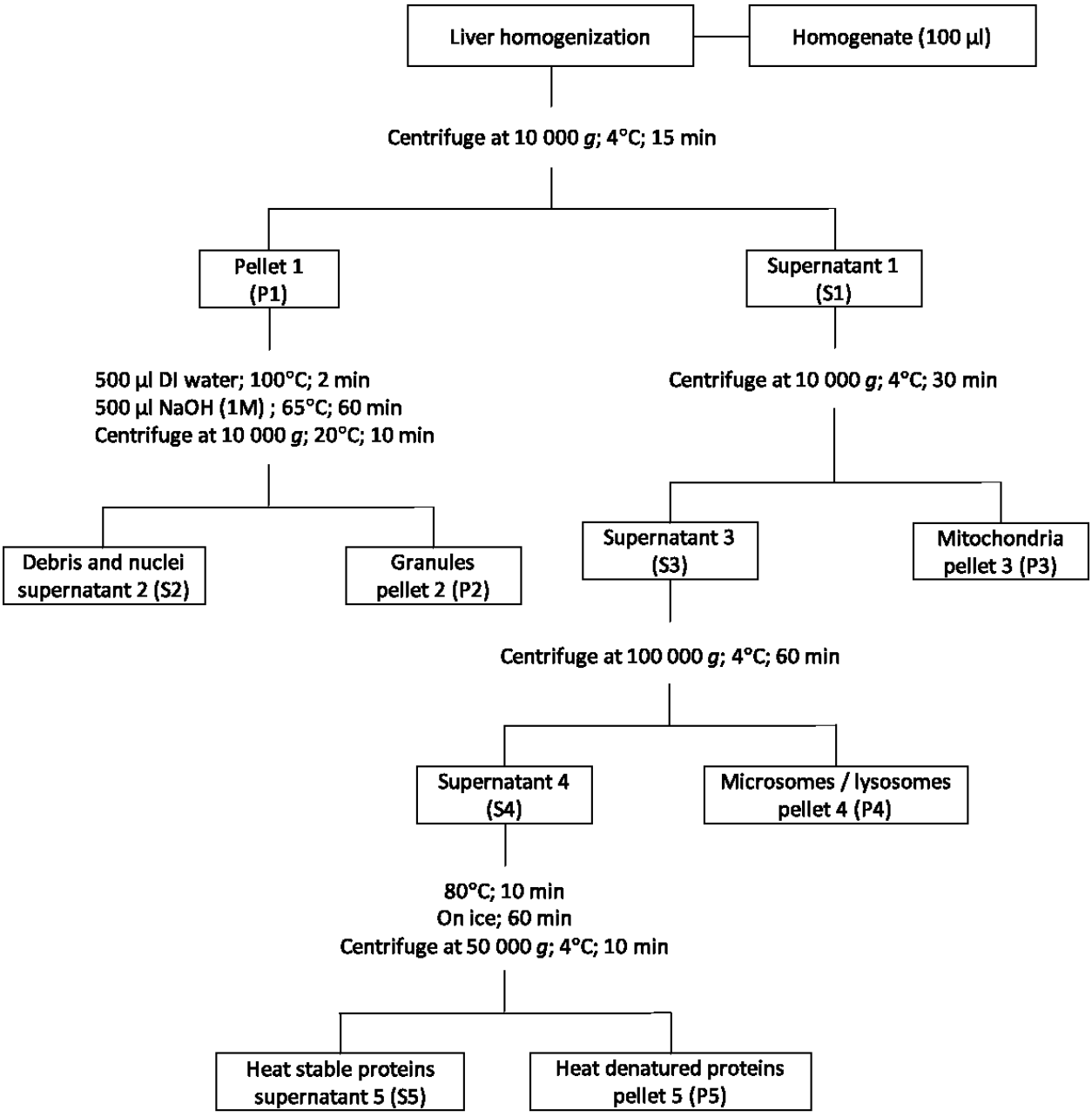


Figure 4.1. Schematic illustration of the differential centrifugation procedure used to separate avian liver tissues into defined subcellular fractions (Modified from Giguère et al. [32] and Rosabal et al. [42]).

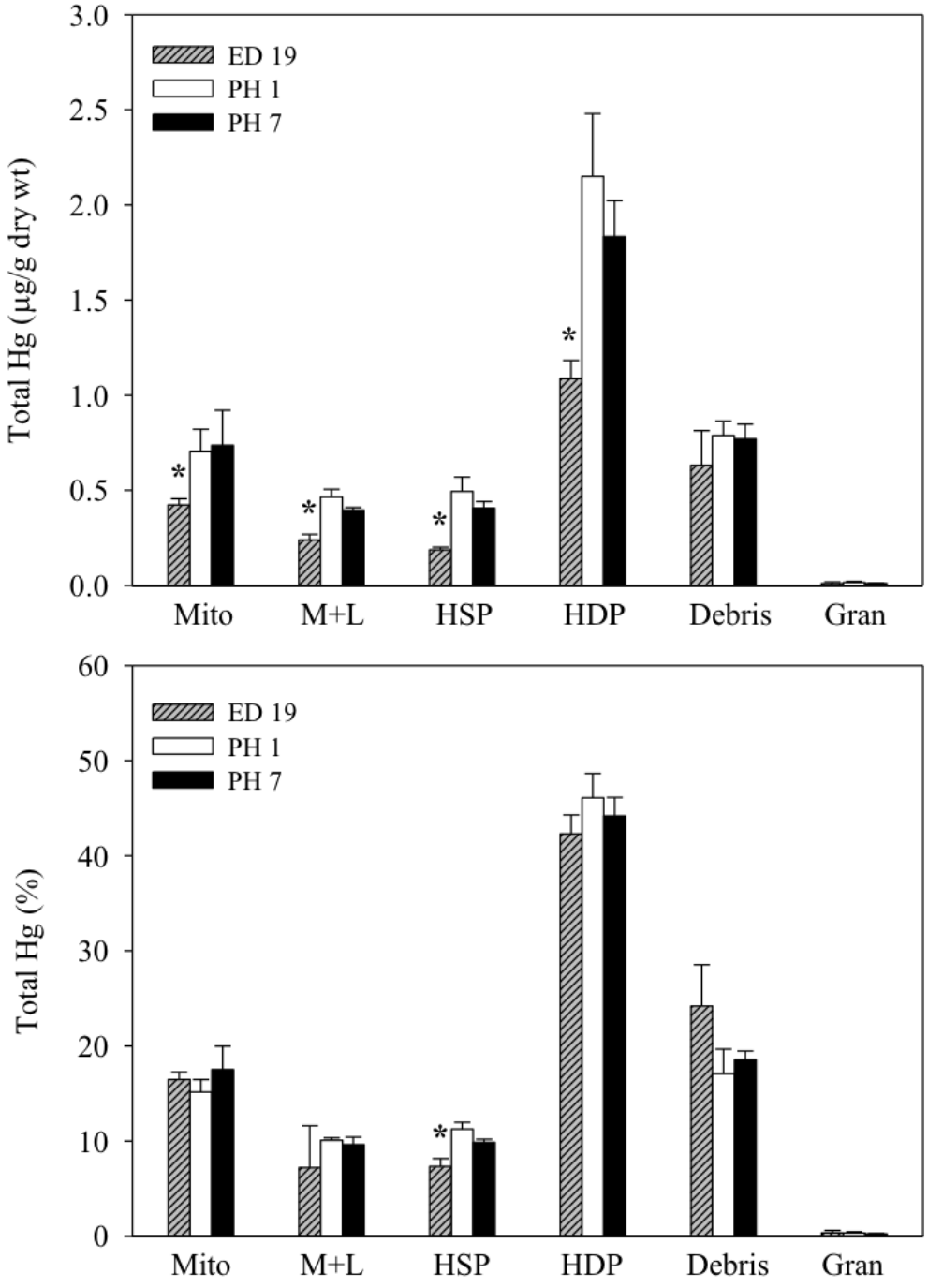

ED 19
PH 1
PH 7
Total Hg (µg/g dry wt)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Mito
M+L
HSP
HDP
Debris
Gran
Total Hg (%)
60
50
40
30
20
10
0

Figure 4.2. Comparisons of the concentration (top, µg/g dw) and the proportion (bottom, %) of THg (mean and standard deviation) in subcellular fractions for chicken livers sampled at embryonic day 19 (ED 19) and post-hatch day 1 (PH 1) and 7 (PH 7). Significant differences for fraction THg among time points denoted by * (ANOVA, top; Wilcoxon signed-rank, bottom; P-value < 0.05). Subcellular fraction abbreviations: Mito = mitochondria, M+L = microsomes and lysosomes, HSP = heat-stable proteins, HDP = heat-denatured proteins, Debris = debris and nuclei, Gran = NaOH-resistant granules (n = 3 composite samples per time point).

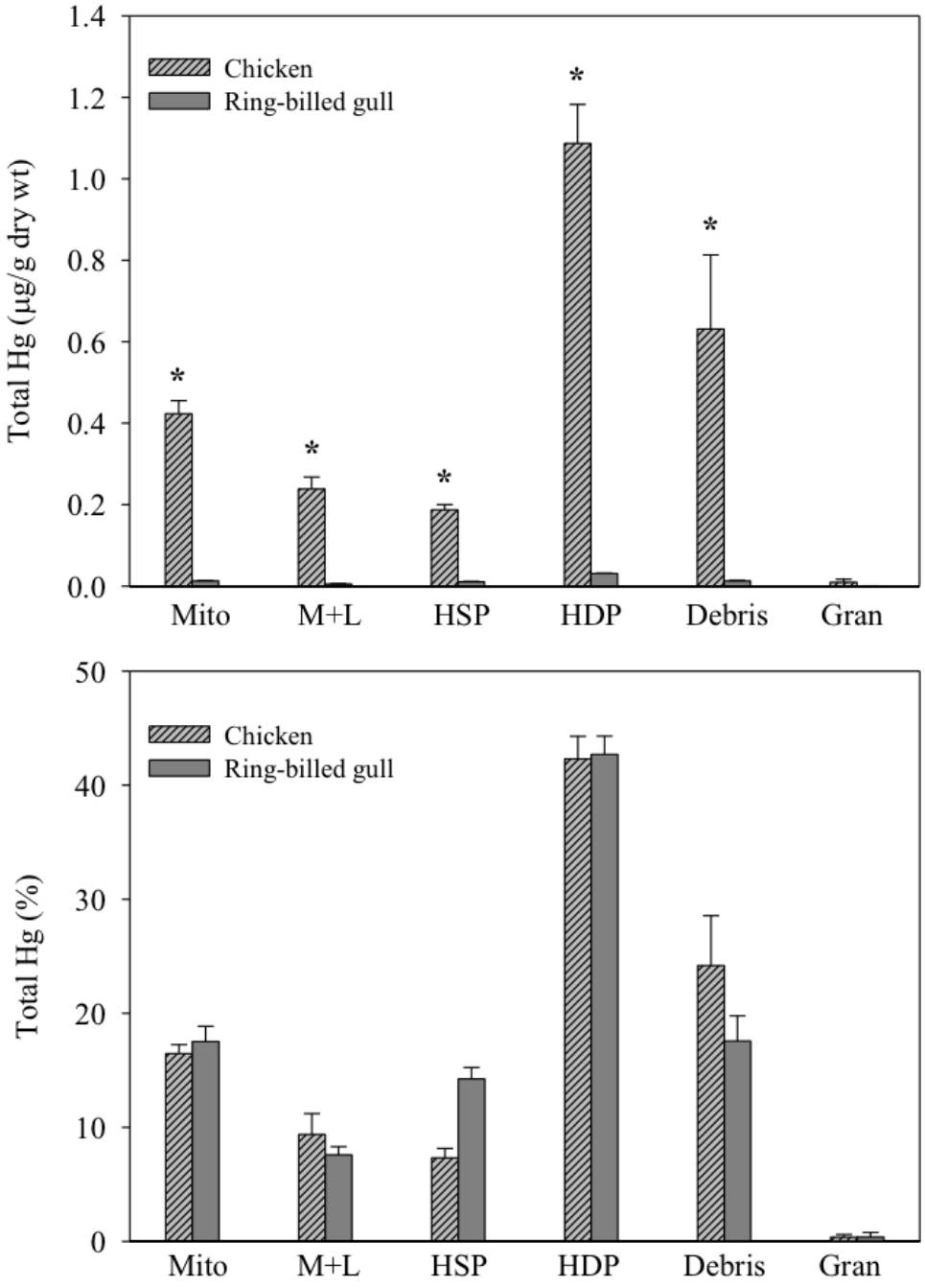

Chicken
Ring-billed gull
Total Hg (µg/g dry wt)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Mito
M+L
HSP
HDP
Debris
Gran
Chicken
Ring-billed gull
Total Hg (%)
50
40
30
20
10
0
Mito
M+L
HSP
HDP
Debris
Gran

Figure 4.3. Comparisons of the concentration (top, µg/g dw) and the proportion (bottom, %) of THg (mean and standard deviation) for embryonic chicken and Ring-billed Gull livers. Significant differences for fraction THg between chicken and Ring-billed Gull denoted by * (t-test, top; Wilcoxon signed-rank tests, bottom; P-value < 0.05). Subcellular fraction abbreviations: Mito = mitochondria, M+L = microsomes and lysosomes, HSP = heat-stable proteins, HDP = heat-denatured proteins, Debris = debris and nuclei, Gran = NaOH-resistant granules (n = 3 composite samples per species).

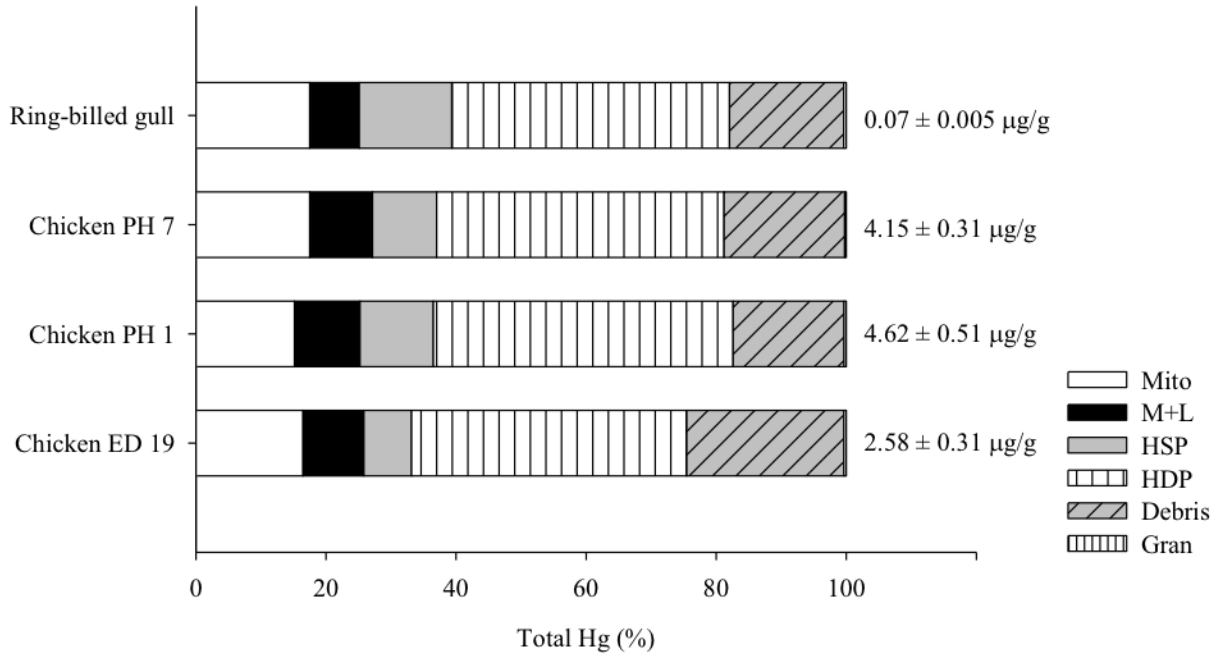


Figure 4.4. The mean proportion of THg in subcellular fractions for embryonic Ring-billed Gull and embryonic day 19 (ED 19) and post-hatch day 1 (PH 1) and 7 (PH 7) chicken livers, 100 % is equal to the sum of all 6 fractions. The mean total Hg concentration (± SD) per composite (n = 3 composite samples per species and time point) is noted to the right of each bar. Subcellular fraction abbreviations: Mito = mitochondria, M+L = microsomes and lysosomes, HSP = heat-stable proteins, HDP = heat-denatured proteins, Debris = debris and nuclei, Gran = NaOH-resistant granules (n = 3 composite samples per time point or species).

4.8 Appendix: Assessment of the Subcellular Partitioning Procedure Using Marker Enzymes

4.8.1 Introduction

Enzymes known to be specific to particular organelles or subcellular fractions can be used as markers to assess whether tissue homogenization was effective at disrupting cell membranes, while maintaining the integrity of organelles. Furthermore, marker enzymes may be used to judge the overall efficacy of the subcellular partitioning procedure [42].

4.8.2 Methods

For the optimization procedure, approximately 200 mg of liver sample, from 3 individual chickens, was weighed and manually sliced with a surgical steel razor blade. Each sample (n = 3) was homogenized on ice as described in the subcellular partitioning method section (4.3.3) in a 25 mM Tris; 250 mM sucrose; 1 mM EDTA sodium phosphate buffer (pH 7.4). The differential centrifugation procedure illustrated in Figure 4.1 was used to isolate four operationally-defined fractions: mitochondria (P3); microsomes and lysosomes (P4); cytosol (S3) and debris and nuclei (P1). Prior to enzymatic analysis, the weight of each individual fraction was determined for calculation of enzymatic activities.

The marker enzymes lactate dehydrogenase (LDH), specific for the cytosol, and cytochrome c oxidase (CCO), found in the inner mitochondrial membrane, were chosen to assess the performance of the partitioning procedure. All spectrophotometric measurements, used to determine the enzymatic activity, were performed using 96-well plates (Corning Costar, flat bottom, Corning, Inc., Corning, NY, USA) and a Synergy HT spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). Enzymatic activities were expressed in units (U) per µl

of diluted sample. One U represents the amount of enzyme necessary to transform 1 μmol of substrate per minute.

Prior to the enzyme measurements, both the debris and nuclei and mitochondria pellets (P1 and P3) were re-suspended separately in Triton-X solution (1% in 25 mM Tris buffer, 250 mM sucrose, 1 mM EDTA, pH 7.4) in a 1:1 ratio (mass of pellet to volume of Triton-X solution); the microsomes and lysosomes pellet (P4) was re-suspended in the same solution in a 1:4 ratio in order to disrupt any intact cell membranes within the isolated fraction. Fractions were allowed to rest on ice for 30 min and were then vortexed thoroughly until complete re-suspension of the pellets.

4.8.2.1 Lactate Dehydrogenase Assay

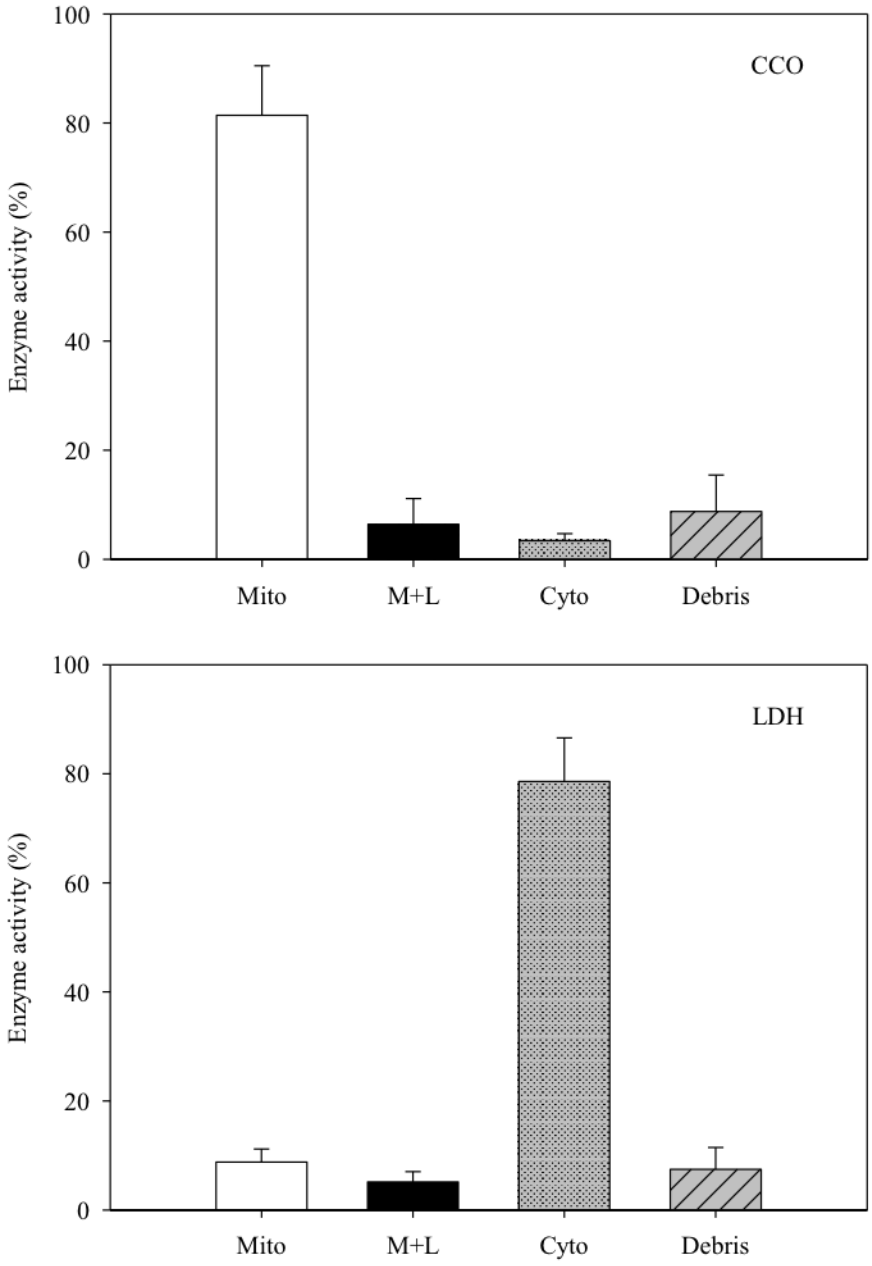
The LDH assay measures the decrease in absorbance resulting from the reduction of pyruvate to lactate, coupled with the oxidation of β-Nicotinamide adenine dinucleotide, reduced (NADH) to β-Nicotinamide adenine dinucleotide (NAD) and H+, catalyzed by LDH. For the purpose of the assay, each fraction was diluted to a final dilution of 1:50 in 0.1 M potassium phosphate buffer (pH 7.0). Diluted samples (10 μl) were added to each well in triplicate and 170 μl of a NADH solution (0.16 mM in 0.1 M phosphate buffer pH 7.0) was added to the wells. The mixture was incubated for 2 min before the decrease in absorbance at 340 nm was monitored for 6 min to determine the control rate. The reaction was started by adding 20 μl of pyruvate solution (10 mM in 0.1 M potassium phosphate buffer pH 7.0) and the decrease in absorbance at 340 nm was monitored immediately for 10 min. The LDH activity was determined using the linear rate of decreasing absorbance over 3 minutes, after subtracting the control rate. The resulting rate, in absorbance units per minute, represents the oxidation of NADH with an extinction coefficient of 6.22 mM-1 cm-1.

4.8.2.2 Cytochrome C Oxidase Assay

This CCO assay measures the decrease in absorbance generated by the oxidation of ferricytochrome c by CCO, based on a protocol described by Spinazzi et al. [59] with a few modifications. For this assay, the mitochondria fraction and microsomes and lysosomes fraction were respectively diluted to a final dilution of 1:4 and 1:8 in 0.1 M phosphate buffer (pH 7.0). No further dilutions were performed on the cytosol and the debris and nuclei fractions. The substrate in this assay was a 1 mM cytochrome c solution prepared in 20 mM potassium phosphate buffer (pH 7.0). Before use, the cytochrome c solution was reduced by adding a few crystals of sodium dithionite. To test the effectiveness of the reduction, 196 µl of 20 mM phosphate buffer (pH 7.0) and 4 µl of the 1 mM cytochrome c solution were added to 3 wells and the absorbance was measured at 550nm and 565 nm. An absorbance ratio (550 nm:565 nm greater than 6 was used as an indication of an effective cytochrome c reduction). For the CCO measurements, 5 µl of the diluted samples were added to the wells in triplicate. To each well, 183 µl of 50 mM potassium phosphate buffer (pH 7.0) was added, followed by 12 µl of the 1 mM reduced cytochrome c solution. As control samples, 200 µl of a solution of potassium hexacyanoferrate(III) ($K_3Fe(CN)_6$) (0.33% m/v in 50 mM phosphate buffer pH 7.0) was added to three wells. The change in absorbance at 550 nm was monitored for 20 min following a 5 s shaking step. The CCO activity was determined using a linear rate of decreasing absorbance over 3 minutes, from which the control rate was subtracted. The resulting rate, in absorbance units per minute, represents the oxidation of ferricytochrome c with an extinction coefficient of 18.5 $mM^{-1}cm^{-1}$.

4.8.3 Results

For each marker enzyme, the percentage of the enzymatic activity in each fraction was expressed as a ratio of the activity in each defined fraction divided by the sum of the activity of all fractions, multiplied by 100. The LDH assay demonstrated that ~79 % ± 8.0 of the LDH activity was found in the cytosol and only ~7 % ± 4.0 of the activity was found in the debris and nuclei fraction (Appendix Figure 4.1). These results indicate that the homogenization procedure was effective in disrupting the cell membranes. The CCO assay showed that ~81 % ± 9.1 of the CCO activity was found in the mitochondria fraction (Appendix Figure 4.1), indicating that the mitochondria pelleting was efficient. Collectively, these results confirm efficient homogenization of the tissue and indicate excellent separation of the subcellular fractions.



Appendix Figure 4.1. Distribution of activities of marker enzymes in subcellular fractions isolated from chicken livers. Cytochrome c oxidase (CCO) activity is presented in the upper panel. Lactate dehydrogenase (LDH) is presented in the lower panel. Activities are presented as percentages. Subcellular fraction abbreviations: Mito = mitochondria, M+L = microsomes and lysosomes, Cyto = cytosol, and Debris = debris and nuclei (n = 3 individuals).

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Chapter 5

5 Summary and Conclusions

Mercury (Hg) is a ubiquitous environmental contaminant that poses a significant health hazard to humans, wildlife, and ecosystems. Efforts to reduce anthropogenic Hg emissions, such as the United Nations Environmental Programme Minamata Convention on Mercury, are vital to the protection of human and ecosystem health. The Minamata Convention (i.e., Articles 19 and 22) requires Parties to commit towards collaborative efforts to monitor Hg concentrations in biota to assess whether reductions in Hg emissions result in decreased exposures. Mercury exposure assessments of bioindicator species, such as birds, are critical to monitoring Hg in the environment and understanding how reductions in Hg emissions relate to the bioavailability of Hg within ecosystems and food webs.

This doctoral thesis was developed based on a need to better understand how Hg concentrations of important biomarker tissues, specifically avian blood and feathers, for non-piscivorous species relate to the bioavailability of Hg within the environment. In this thesis, I aimed to improve Hg exposure assessments by conducting both field studies using wild birds and laboratory studies using methylmercury (MeHg) dosing of model avian species. The overall objective of this work was to increase understanding of extrinsic and intrinsic factors that influence blood and feather Hg concentrations in non-piscivorous bird species. Additionally, I aimed to develop novel methods which can be used by future researchers, such as a subcellular partitioning procedure for avian liver tissue and a dried blood spot (DBS) sampling method. This DBS sampling method to determine blood Hg concentrations in birds aims to reduce logistic

burdens and costs related to Hg exposure assessments and is intended to encourage future large-scale collaborations like the one conducted for this thesis.

This thesis fills an important knowledge gap in Arctic Hg exposure research by conducting a Hg exposure assessment on a group of terrestrial avian invertivores for which information on Hg exposure has been lacking. Additionally, this is the first study to our knowledge to use direct Hg analysis to determine Hg concentrations in DBS. This study greatly extends upon previous studies on the use of DBS in birds by investigating non-homogenous spread of Hg across DBS and the effect of short-term collection and storage conditions on the stability of Hg in DBS. This is also the first study to our knowledge that has collected whole blood and created DBS using a capillary tube in the field to determine Hg exposure in birds. We also adapted and verified a partitioning procedure for separating avian liver tissue into operationally defined subcellular fractions with high effectiveness. To our knowledge, this is the first study that has used MeHg egg injection to investigate subcellular distribution of Hg in bird liver and compared the results with those from a wild avian species exposed to maternally-deposited MeHg.

5.1 Summary and Discussion of Results

In chapter 2, we utilized a large-scale collaborative network to investigate Hg exposure in multiple shorebird species across the North American Arctic. We analyzed 2,478 blood and feather samples collected from 12 breeding shorebird species during 2012 and 2013. Sampling locations included five sites across Alaska and four sites across Canada. This broad study fills an important knowledge gap in Arctic Hg exposure research by determining current Hg concentrations for terrestrial avian invertivores sampled across the North American Arctic.

Additionally, it provides baseline measurements that can aid in evaluating changes in Hg emissions and terrestrial Arctic deposition over time. Previous studies have found that foraging guild and ecoregion are important factors influencing blood and feather Hg concentrations. This expansive Hg exposure assessment for birds within the same foraging guild (invertivore) and ecoregion (Arctic tundra) allowed for the investigation of additional factors influencing Hg concentrations without having to account for differences in ecoregion and foraging guild.

We found blood Hg concentrations for Arctic-breeding shorebirds were influenced by species and breeding site but were not influenced by year. While blood Hg concentrations were influenced by the moisture classification of preferred foraging habitat at some study sites, no clear trend was determined. The differences in blood Hg concentrations among species found in this study suggest that including multiple species from the same foraging guild and ecoregion in Hg exposure assessments may better elucidate regions of high Hg contamination. We found blood Hg concentrations were also influenced, to a lesser extent, by factors such as sex, body mass, and Hg turnover (as reflected by capture day). Feather Hg concentrations were influenced by species, sex, and year, but were not influenced by breeding site. We determined that overall, Arctic-breeding shorebirds were at low risk of adverse effects of Hg exposure. However, Hg exposure for some Arctic-breeding shorebirds may exceed concentrations that could result in impaired health and birds sampled at the Barrow study were at the greatest risk for adverse effects of Hg exposure. These findings indicate that understanding the factors that influence blood and feather Hg concentrations can improve the use of birds as bioindicators of environmental Hg contamination. The success of this study is a great example of how Hg exposure research can be incorporated into ongoing avian projects.

In chapter 3, we developed and validated a novel method to assess blood Hg concentrations in birds using dried blood spots (DBS). Blood is considered to provide a good biomarker of recent dietary exposure to Hg, therefore, may be an ideal biomarker tissue to sample when using birds as bioindicators for Hg contamination. However, blood collection and storage can be complex and costly, and equipment and shipping costs can be prohibitive or logistically unfeasible in some remote or resource-limited regions. Dried blood spot use has been standardized for a variety of human health related analyses, however collection, storage, and analysis methods have not been established for Hg assessment of bird blood. Previous studies have analyzed DBS for Hg concentrations using inductively coupled plasma mass spectrometry (ICP-MS). We found that direct Hg analysis offered many benefits over ICP-MS for Hg analysis of DBS, including short analysis time and minimal sample preparation. We compared the accuracy and precision of entire DBS with that of DBS punches and found better accuracy for entire DBS. This was likely because Hg was not spread evenly across DBS created with avian blood, with greater Hg concentrations found at the DBS edge.

We investigated the stability of Hg in DBS by submitting them to treatments that replicated common field collection and long-term storage conditions, as well as pathogen decontamination heat treatments. We found Hg to be stable across treatments, indicating that DBS may offer an advantage over the use of whole blood samples for determining Hg exposure in birds by offering Hg stability in a much wider range of conditions. This is the first study to investigate that effect of short-term collection and storage conditions on Hg concentrations in DBS. indicate that DBS can be collected, stored, and eventually shipped without additional supplies or equipment to regulate humidity or temperature. This work indicates that DBS can

help to reduce logistic burdens and costs of collecting, storing, shipping, and analyzing field collected avian blood samples.

In chapter 4, we investigated two key biochemical processes of Hg toxicokinetics in birds: 1) Hg speciation in blood, brain, and liver; and 2) Hg subcellular distribution in liver. These two processes are not well understood in birds but may influence biomarker tissue concentrations and species sensitivities to Hg exposure. We used MeHg egg injection of White Leghorn Chicken (*Gallus gallus domesticus*), sampled at 3 early-developmental stages, and embryonic Ring-billed Gulls (*Larus delawarensis*) exposed to maternally-deposited MeHg. This work adds to previous studies on MeHg demethylation by investigating important biomarker tissues (blood and liver) and tissues sensitive to the adverse effects of MeHg (liver and brain). It also adds to previous MeHg demethylation studies by comparing Hg speciation between a model avian species exposed to MeHg via egg injection and a wild species exposed to maternally-deposited MeHg. The percent MeHg in blood, brain, and liver ranged from 93.8 ± 12.9 to 121.3 ± 12.9 %, indicating little MeHg demethylation among time points or between species.

We adapted and verified a partitioning procedure for separating avian liver tissue into operationally defined subcellular fractions with high effectiveness. This is the first study, to our knowledge, that has used MeHg egg injection to investigate subcellular distribution of Hg in bird liver. We found that subcellular distribution of Hg did not differ between birds exposed to MeHg via egg injection and birds exposed to maternally-deposited MeHg. This research further indicates that MeHg egg injection of a model avian species, the White Leghorn Chicken, can offer useful information on MeHg toxicokinetics in birds. A low rate of MeHg demethylation and a high proportion of Hg in metal-sensitive subcellular fractions for embryonic and hatchling time points found in this study further indicated that these are Hg-sensitive developmental stages.

5.2 Conclusion

This doctoral thesis combined both field studies and laboratory studies to improve understanding of Hg toxicokinetics in non-piscivorous bird species, to ultimately improve Hg exposure assessments. This work advances the understanding of the extrinsic and intrinsic factors that influence Hg concentrations of biomarker tissues in both wild birds and a model avian species exposed to MeHg via egg injection. Additionally, this thesis provides the development of novel methods which can be used by future researchers, including the use of DBS to assess blood Hg concentrations in birds and a subcellular partitioning procedure for avian liver tissue. The novel sampling method of DBS can aid in reducing logistic burdens and costs of collecting, storing, shipping, and analyzing field collected samples. Additionally, the differences in blood Hg concentrations among species found in this study suggest that including multiple species from the same foraging guild and ecoregion in Hg exposure assessments may better elucidate regions of high Hg contamination.