DISSERTATION

SPATIAL TRENDS OF TOTAL MERCURY (THg) EXPOSURE, AND THE ROLE OF INTESTINAL HELMINTHS ON ITS DISTRIBUTION WITHIN PISCIVOROUS MAMMALIAN HOSTS

Submitted by

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ABSTRACT

SPATIAL TRENDS OF TOTAL MERCURY (THg) EXPOSURE, AND THE ROLE OF INTESTINAL HELMINTHS ON ITS DISTRIBUTION WITHIN PISCIVOROUS MAMMALIAN HOSTS

This research project is unique in that it has explored the interface of three broad disciplines: ecology, toxicology, and parasitology. The primary objectives were to determine the role of gastrointestinal (GI) helminths in total mercury (THg) distribution within piscivorous mammalian hosts, and explore the complex interactions that exist within the host GI tract. The project was designed to address these objectives in two pinniped populations: Alaskan ice seals (*Phoca largha* and *Phoca hispida*), and California sea lions (*Zalophus californianus*). Initially, Alaskan gray wolves (*Canis lupus*) were selected as a reference species for this project, as these animals represent a mammalian definitive host, occupying a top trophic position in a terrestrial food web; nevertheless, preliminary findings demonstrated a subset of these wolves to be subsisting, at least in part, on prey sources of marine origin. Therefore, the project was expanded to include the Alaskan gray wolves as an additional "piscivorous" host for study.

At necropsy, host tissues and GI tracts were collected. During GI tract processing, intestinal helminths were removed, weighed, and either saved for identification, or frozen for further analyses. Host tissues (e.g. liver, kidney, cardiac muscle, skeletal muscle), GI lumen contents, and parasites were then analyzed for THg concentrations and stable isotope values (C, N, and S).

In Alaskan gray wolves, THg concentrations, and $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ isotope values, provided four separate measures supporting the contention that Alaskan gray wolves, with access to marine resources, are relying on piscivory or exploitation of other organisms of marine origin. THg uptake was demonstrated to occur in these animals, and the toxicant-parasite interactions that exist within the GI tract may ultimately affect the host-toxicant interface. The interactions described depend not only on type of parasite and specific toxicant, but also on the complex ecological-like interactions within the host's body. In pinnipeds, parasites were shown to effectively bioaccumulate and/or biomagnify mercury (Hg) within the host GI tract. Within-parasite THg concentrations were not necessarily associated with concentrations in host lumen contents, or host liver and kidney. These data demonstrated that THg distribution in the host is affected by the presence of parasites; consequently, bioavailability of this toxicant to the host may also be affected. A design has been proposed for building an agent-based model, to further explore the interactions described in these studies. This framework will provide a foundation for future work focused on the ecotoxicoparasitology of other related systems.

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LIST OF ABBREVIATIONS

AK	Alaska
C	carbon
CA	California
CC	colon contents
df	degrees of freedom
DH	definitive host
dSI	distal small intestine
dSI-L	distal small intestine lumen contents
dw	dry weight
GI	gastrointestinal
GMU	Game Management Unit
Hg	mercury
IH	intermediate host
LI	large intestine
MeHg	methyl mercury
N	nitrogen
ppb	parts per billion
pSI	proximal small intestine
pSI-L	proximal small intestine lumen contents

S	sulfur
SC	stomach contents
SD	standard deviation
SE	standard error
SI	small intestine
THg	total mercury
TMMC	The Marine Mammal Center
ug/kg	micrograms per kilogram
ww	wet weight
‰	per mil
±	plus or minus
$\delta^{13}C$	stable isotope for carbon
$\delta^{15}N$	stable isotope for nitrogen

INTRODUCTION

Parasites have been proposed as effective indicators of marine pollution (Mackenzie et al., 1995; Jankovská et al., 2011; Sures, 2006; Sures, 2008; Nachev & Sures, 2009). The loss of parasites in a system can occur when short-lived, free-living stages that are sensitive to toxicants are intrinsic to the life cycle (Pietrock and Marcogliese 2003); alternatively, their loss in a polluted system can occur when indirect life cycles are utilized, incorporating intermediate hosts that are sensitive to harsh environmental conditions (MacKenzie et al. 1995; Marcogliese 2005; Pietrock et al. 2008).

Certain intestinal helminths, including cestodes and acanthocephalans, have been shown to be capable of sequestering heavy metals at concentrations higher than those detected in host tissues and the environment (Galli et al., 1998). These parasites derive their nutrients directly from the gastrointestinal lumen of the definitive host in which they live, and it is through this process that essential and non-essential elements may be acquired. Parasites, therefore, can effectively provide an estimate of exposure of their host to metal pollution within the host's natural range (Sures et al., 1997). Sures et al. (1997) found that three species of acanthocephalans contained relatively high concentrations of heavy metals (Pb and Cd), relative to host muscle tissue. Mean concentrations of Pb and Cd in *Pomphoryhnchus laevis* from intestines of chub (*Leuciscus cephalus*) were 2,700 and 400 times higher than in the muscle of the host (Sures et al., 1994), and 11,000 and 27,000 times higher than concentrations in the water,

respectively (Sures & Taraschewski, 1995). Uptake of non-essential elements has also been quantified in the nematodes of numerous hosts (Sures et al., 2002; Barus et al., 2001), and in the cestodes of ruminants (Jankovská et al., 2010). Despite a growing number of studies relating to toxicant-parasite interactions, the effects of non-essential elements on the host-parasite system remain poorly understood (Golinska & Bany, 2000).

Little is known regarding the effects of environmental mercury (Hg) on biological systems. Hg is a non-essential element that exists in the environment due to both natural and anthropogenic inputs. Increasing concentrations of Hg in the environment have led to questions regarding the condition of terrestrial, freshwater, and marine ecosystems. Natural sources of Hg include volcanoes, soil particles, and forest fires. Elevated concentrations of this non-essential element are also thought to have arisen, in part, due to rapid population growth, increased urbanization, expansion of industrial activities (e.g. high-temperature processes including the burning of fossil fuels, and the roasting and smelting of ores), and other anthropogenic causes (Biney et al., 1994). The ubiquitous nature of Hg and other elements in the environment is widely recognized, and their presence in arctic ecosystems impacts the local freshwater and marine food supply (Jewett & Duffy, 2007).

Mercury can undergo a process known as methylation (Gilmour et al., 2011; Porcella, 1994). Methylation can take place in the presence of sulfate reducing bacteria, or can occur as the result of abiotic mechanisms, such as intense heat and pressure. In its methylated form, Hg may biomagnify in aquatic systems and move within the food web through dietary transfer. This is thought to be one of the primary pathways for Hg uptake in carnivorous mammals. Most of the total Hg (THg) in fish is monomethylmercury

(MeHg) (Bloom, 1992), and consumption of this neurotoxic form of Hg may pose an increased risk to upper-trophic-level organisms; in fact, MeHg concentrations have been shown to be as high as 80-98% in the muscle of a fish host (Harmelin-Vivien et al., 2009). Hg is primarily compartmentalized in red blood cells, most likely binding to the sulfhydryl groups of hemoglobin and other proteins (Rothstein, 1981). The slow rate of excretion and metabolism, therefore, also contribute to increased concentrations of Hg transferred from prey to predator (Braune et al., 2005).

Defining the host-parasite-toxicant relationship is necessary in order to understand the chemical-ecology of the host alimentary tract, and ultimately, host toxicant exposure (that is actually absorbed into circulation) and health. Toxicants can enter circulation and distribute to various parts of the body, and/or can be eliminated. Hg bioaccumulates in many host tissues, and biomagnification may occur at successive trophic levels. Biotransformation may also occur, thereby affecting the amount of bioavailable Hg present in a system. The role of parasites in toxicant distribution and dynamics within the host are critical to understanding host-toxicant-parasite interactions, and the various ways in which the host responds to stressors in an ever-changing environment.

In addition to affecting wildlife populations, exposure to environmental sources of Hg may affect the health of the most susceptible human sub-populations, since both marine mammals and fish are important resources for subsistence users in the Arctic. Anthropogenic contaminants have been a growing concern in arctic regions in the last 30 years, due to the increasing concentrations of toxic elements in certain arctic biota and humans (Fisk et al., 2005). In the North, the presence of Hg in food, leading to chronic

exposure, has become both an economic and political issue (Jewett & Duffy, 2007). Certain forms of Hg are known to cross the blood brain barrier (Weil et al., 2005), which poses important health risks to exposed populations. With the increased knowledge of the health hazards of Hg, monitoring of spatial and temporal changes of this non-essential metal in the environment is of utmost importance (Jewett & Duffy, 2007). A better understanding of the interaction of parasitism and heavy metal pollution on both aquatic-ecosystem- and human-health will be instrumental in guiding policy, management, and regulatory decisions in the future (O'Shea et al., 1999).

Stable isotopes have been increasingly recognized for their importance in feeding-ecology studies of carnivores (Dehn et al., 2006; Brookens et al., 2007; Brookens et al., 2008, Semmens et al., 2009). Stable isotopes are different forms of the same element, and differ in their atomic mass based on the number of neutrons they possess. Nitrogen stable isotope ratios of prey are reflected in tissues of the consumer, with enrichment occurring at each trophic level (Kelly, 2000). In general, ^{15}N is enriched relative to ^{14}N at approximately 3-5 $^{0}/_{00}$ per trophic level (Hoekstra et al., 2002) due to selective elimination of the lighter isotope over its heavier form. The ratio of $^{13}C/^{12}C$, while not as useful as an indicator of trophic position, can elucidate trophic interactions by quantifying relative contributions of various carbon sources (France & Peters, 1997). Likewise, while stable sulfur isotope ratios ($^{34}S/^{32}S$) do not change dramatically with increasing trophic level, $\delta^{34}S$ signatures can be helpful in distinguishing marine and freshwater prey sources (Peterson & Fry, 1987).

Determination of the toxicant-parasite interactions in a piscivorous mammalian host will elucidate effects on wild populations, as there remains great uncertainty about

specific effects of contaminants in marine mammals, to what effect these contaminants occur in wild marine mammals, and what impact they are having on marine mammal population dynamics (O'Shea et al., 1999). Marine mammals possess the capacity to accumulate toxic substances at relatively high concentrations, and their role as top predators in marine food webs indicates that they have the potential to be exposed to high levels of substances that accumulate in lower trophic levels. There is also little information available on C, N and S stable isotope ratios in the tissues of marine mammals (Dehn et al., 2006).

There are relatively few investigations examining the interaction between ecotoxicology and parasitology in freshwater ecosystems (e.g. Marcogliese et al., 2005; Minguez et al., 2009), and research that explores the overlap of these two fields in marine systems has also been limited. Exploring whether parasites play a protective role in populations of animals may help define risk of exposure to Hg in wildlife species. Parasitic diseases are thought to play a prominent role in structuring the population dynamics of aquatic communities, and needs to be more widely recognized by ecotoxicologists (Morley, 2010). Increasing evidence suggests that parasites have the potential to uniquely alter food-web topology in terms of chain length, connectance and robustness (Lafferty et al., 2008). Given that virtually all vertebrates are hosts for parasites, the investigation of the combined effects of contamination and parasitism is important in the framework of aquatic bioindication procedures (Minguez et al., 2009). Combined stressors (e. g. infectious diseases and pollutants) may be synergistically detrimental to affected populations (Morley, 2010); conversely, they may play an integral, or even advantageous role in the balance of interactions within a complex

system. The primary goal of this project was, therefore, to determine the ecotoxicoparasitological interactions in piscivore hosts, as a means of assessing the role of gastrointestinal macroparasites in Hg distribution. The ability of helminths to bioaccumulate high concentrations of heavy metals, their life cycle, and their place in multiple food webs make them a particularly interesting parasite group to study, especially since their life cycles are largely determined by ecological factors (Baer, 1971). Finally, because acanthocephalans may decrease toxicant levels within their hosts, it is possible that these parasites, as well as other helminths, may have a positive influence on the health of their hosts by reducing absorbed toxicants below toxic thresholds (Sures, 2006).

CHAPTER 1

Spatial trends of total mercury (THg) and C, N, and S stable isotopes in Alaskan gray wolves (*Canis lupus*)

Certain forms of Hg bioaccumulate in organisms, and have the potential to biomagnify within food webs. Alaskan gray wolves (*Canis lupus*) acquire mercury (Hg) primarily through dietary means; therefore, determining the geographic distribution of Hg among Alaskan wolves from interior and coastal regions, and how exposure is linked to their feeding habits, offers important insight into the feeding ecology of these animals. In this study, stable isotopes and THg were measured on wolf liver and muscle from 162 wolves collected by the Alaska Department of Fish and Game between 2006 and 2009. Hepatic THg concentrations ranged from 5.5 ug/kg wet weight (ww) to 7,260.7 ug/kg ww. Renal THg concentrations ranged from 8.3 ug/kg ww to 11,173 ug/kg ww. THg concentrations in the liver of coastal wolves (n=28) were significantly higher than those of wolves from interior Alaska (n=117) ($p = <0.0001$), with median concentrations of 1,159.4 and 28.4 ug/kg ww, respectively. Stable isotope values (C, N, and S) showed that coastal wolves in this study are subsisting on marine prey sources representing several trophic levels. In summary, THg concentrations in the wolves from this study varied significantly based on GMU and presumed coastal proximity. Wolves from coastal GMUs had significantly higher THg concentrations in all tissues examined, compared to wolves from interior

units, and many of the wolves from coastal units had liver and kidney THg concentrations exceeding 1 ug/g.

KEY WORDS: bioaccumulation, mercury, *Canis lupus*, stable isotopes, feeding ecology

1. Introduction

A variety of stressors exist in the environment that may ultimately affect the functioning of biological organisms (Lafferty & Holt, 2003). The increasing concentrations of both essential and non-essential metals in the environment, due to both anthropogenic and natural causes, may lead to profound effects at numerous levels of biological organization. One element of particular concern is mercury (Hg), and exposure to this non-essential metal is problematic due to its propensity to become methylated (MeHg), and biomagnify in food webs. High exposure to this toxicant can threaten the health of individuals, especially the fetus or neonate, and consumers that occupy high trophic positions (Chumchal et al., 2011). Because certain forms of Hg bioaccumulate in fish muscle tissue, animals feeding on fish are likely to acquire Hg through dietary exposure. The diet of Alaskan gray wolves (*Canis lupus*) has been shown to be subsidized by marine organisms, including fish (Adams et al., 2010), thereby making this an ideal mammalian organism for studying Hg concentrations in top predator wildlife populations.

Stable isotopes have been used in feeding ecology studies to elucidate food web structure and energy pathways in an ecosystem. Nitrogen stable isotope values can be used to describe trophic interactions (Dehn et al., 2006). The stable isotope ratios of

consumer tissues reflect diet in a predictable manner, and represent assimilation rather than ingestion (Inger et al., 2006). Specifically, enrichment of ^{15}N, occurs at 3.4 ± 1.1 ‰, per trophic level, and this increase in the heavier isotope, relative to its lighter form, is independent of habitat (Newsome et al., 2010; Minagwa & Wada, 1984). Nitrogen stable isotopes have been extensively used to quantify marine-based dietary sources to a variety of mammalian consumers, including wolves (Adams et al., 2010), polar bears (Dehn et al., 2006), and mink (Lake et al., 2007). This relates to the fact that nitrogen in the ocean is also slightly enriched in ^{15}N, relative to the atmosphere (Benson & Parker, 1961; Miyaki & Wada, 1967).

Marked isotopic differences exist between marine and terrestrial biomes, therefore, sulfur and carbon stable isotopes have also been used to determine whether a population is utilizing marine or terrestrial resources, (Inger et al., 2006). In marine plants, $\delta^{13}C$ values have been found to be considerably higher, relative to those in terrestrial plants (Inger et al., 2006). Sulfur isotope values also differ greatly in marine and terrestrial sources of prey, and allow for further distinction of marine and non-marine sources of protein. Evaluating THg concentrations in Alaskan gray wolves, in conjunction with using stable isotope ratios of C, N and S to understand their feeding ecology, will provide important insight into the ecotoxicological interactions within these animals, and help in better understanding spatial trends of THg in Alaska and how dietary exposure is perpetuated through feeding habits. The purpose of this study is to determine the geographic distribution of THg, as reflected by concentrations in wolves with or without coastal access, and to use stable isotopes to show how dietary sources in these two groups of wolves contribute to overall THg exposure.

2. Materials and Methods

2.1 Sample collection

Tissues or carcasses of 162 Alaskan gray wolves were collected by the Alaska Department of Fish and Game between 2006 and 2009, representing 13 Game Management Units (GMU) across the state of Alaska (Fig. 1). GMUs were subdivided into those that directly bordered coastline (coastal), and those that had no coastline (interior). At necropsy, sex and age class were determined for each animal. Wolves were divided into two age classes, pup (<12 months) and non-pup (≥12 months), as previously described (Gipson et al., 2000). Samples including liver (n=145), kidney (n=145), skeletal muscle (n=60), and cardiac muscle (n=16) were collected in individual Whirlpac™ bags and frozen immediately at -20 ºC. Samples were transferred to the University of Alaska Fairbanks and stored at -80 ºC until analysis could take place.

2.2 Stable isotope analyses

In preparation for stable isotope analysis, samples were freeze-dried and ground to a fine powder using a mortar and pestle, followed by further homogenization using a ball mill (mini-bead beater, BioSpec). Approximately 1.5-2.0 mg of dried, homogenized liver and muscle tissue were loaded into 5 x 9 mm tin capsules for C and N isotope analyses; 5.5 mg of sample was amended with 2.0 mg of V_2O_5 for S stable isotope analysis.

All samples were analyzed for stable isotopes by continuous-flow isotope-ratio mass spectrometry using an elemental analyzer (Carlo Erba NC1500 or Thermo Flash 2000) interfaced to a mass spectrometer (Micromass Optima or Thermo-Finnigan Delta

Plus XP), as described by Fry et al. (1992). Isotope values are reported in delta (δ) notation:

$$\delta X = (R_{sample} / R_{standard}) - 1$$

where X represents ^{13}C, ^{15}N, or ^{34}S in parts per thousand (‰) deviation relative to a standard (monitoring) gas and R_{sample} and $R_{standard}$ represent the ratio of $^{13}C/^{12}C$, $^{15}N/^{14}N$, or $^{34}S/^{32}S$ for sample and standard, respectively. Isotopic data were normalized to V-PDB, Air, and V-CDT using the primary standards USGS 40 (-26.24‰ and -4.52‰ for $\delta^{13}C$ and $d^{15}N$ respectively), USGS 41 (37.76‰ and 47.57‰ for $\delta^{13}C$ and $\delta^{15}N$ respectively), NBS127 (21.1‰ for $\delta^{34}S$), and IAEA-SO6 (-34.05‰ for $\delta^{34}S$). Analytical error was assessed by replicate measures of primary standards (<0.2‰ for all three isotopes across all analytical sequences) and quality control was assessed using several secondary standards analyzed several times within individual analytical sequences (<0.3 ‰). Accuracy was assessed using primary standards as unknowns, and was within 0.2‰ for all three isotopes. Sample reproducibility was generally better than 0.2‰ for all three isotopes.

2.3 Total mercury (THg) analysis

Samples were thawed to room temperature and sub-sampled (70-150 mg) using stainless steel forceps and scissors. Instruments were washed with ultrapure water and dried between each sample. THg concentrations are reported on a wet weight (ww) basis in ug/kg. Samples were analyzed with a direct mercury analyzer (DMA) on a Milestone DMA-80 instrument (Butala et al., 2006; EPA 600-R-04-012). The method detection limit for THg determination was 0.005ng/g, ww.

Quality assurance and quality control (QA/QC) samples included instrument and method blanks, standard reference materials (SRMs), check standards, and sample duplicates. All samples were run in duplicate and re-analyzed if the percent difference between samples was >10%. The SRM utilized was DORM-3 fish protein homogenate (National Resource Council Canada; 0.382 +/- 0.060 ng Hg/g). Percent recovery for check standards (5, 20, and 100 ng aqueous Hg) was >90%. Analysis of the standard reference material was within 10% of the certified value for Hg.

2.4 Statistical analyses

Data distributions were assessed for normality using graphical techniques including histograms and box-and-whisker plots, supplemented by quantile-quantile plots (Henderson, 2006). Based on these assessments, data were determined to be non-normal. Chi-square analyses were used to evaluate effects between the dichotomous variables, age class, sex, and location (interior vs. coastal). The Mann-Whitney U test (alpha=0.05) was used to evaluate differences in liver, kidney, and muscle THg concentrations based on location (interior vs. coastal GMUs), sex, and age class (Belle, et al., 2004). Isotope values were compared based on location (interior vs. coastal GMUs). Differences in which $p<0.05$ were considered to be significant. Means are presented for reference only since medians were used in the non-parameteric statistical testing. Statistical analyses were performed using StatCrunch5.0 statistical software (Integrated Analytics LLC, Pearson Education, 2007-2009). Using multiple linear regression, THg concentrations in host muscle and liver were evaluated as two separated outcomes based on age class, gender, location (coastal vs. interior), and host stable isotope values (C, N, and S). A forward stepwise approach was applied with both the final model and the model

including significant interaction terms presented, and bivariate relationships were determined for all pair-wise comparisons (Dohoo et al., 2009).

3. Results

Thirty wolves were from coastal GMUs and 132 were from interior GMUs (Figure 1). Sex and age data were available for 161/162 and 158/162 wolves, respectively. Among interior wolves, 20 males were < 12 months and 40 males were > 12 months; there were 25 females < 12 months and 44 females ≥ 12 months. Among wolves from coastal GMUs, 4 males were <12 months and 11 males belonged to the older age class. There were 2 coastal females < 12 months and 12 that were ≥12 months. There was no significant association between age class and sex, age class and location (interior vs. coastal), or location and sex.

Overall, median THg values were significantly higher in liver and kidney relative to muscle (Table 1). Wolves from coastal GMUs had significantly higher THg concentrations in all tissues than wolves from interior GMUs (Table 2) ($p \leq 0.05$); however, even the highest liver and kidney concentrations from interior wolves were ≤50% of the maximum values seen in the coastal wolves. Concentrations in females were 30.9 ug/kg (5.5 to 7,206 ug/kg), and 36.9 ug/kg for males (5.7 to 7,260 ug/kg). There was no significant difference in median liver or kidney THg concentrations based on sex. However, liver and kidney median THg concentrations were significantly higher in animals older than 12 months, compared to pups (<12 months) (Table 3).

Stable isotope (C, N and S) analysis was conducted on 95 liver samples and 59 muscle samples (Figure 2, 3). The mean and range of $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ values were -24.1 (-19.95 to -26.76), 8.2 (6.0 to 15.89), and 6.7 (-1.64 to 17.12), respectively. Median

values for all stable isotopes ($p<0.01$) were found to be significantly higher in coastal animals. Interior wolves had both low liver THg concentrations as well as low $\delta^{34}S$ values (Figure 4a). Within coastal wolves, those with higher $\delta^{34}S$ values displayed a wide range of THg concentrations in liver (Figure 4b).

Chi-square analyses were used to determine whether a significant association exists between gender, location, and age class, and no significant differences were seen; therefore, interaction terms between these dichotomous variables were not considered in the model. Interaction was evaluated between $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$. The interaction between $\delta^{15}N$ and $\delta^{34}S$ was found to be significant for muscle tissue, so a model including the effect of this interaction on muscle THg, relative to the other variables, was included for reference. The regression analyses included the variables age, gender, location, $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ in the forward-stepping model. $\delta^{15}N$ was the only variable found to significantly affect muscle THg concentrations in the host (Table 4). As per its regression coefficient, for every one-unit change in $\delta^{15}N$, muscle THg increases an average of 37.96 ug/kg. In contrast, all three isotopic values had a significant effect on the THg concentrations seen in host liver (Tables 5-9). As per their regression coefficients, for every one unit change in $\delta^{13}C$, liver THg decreases approximately 248.82 ug/kg, and for every unit increase in $\delta^{15}N$ and $\delta^{34}S$, liver THg increases 324.81 and 125.49 ug/kg, respectively.

4. Discussion

Differences observed in THg concentrations among tissues were consistent with previous findings in both marine and terrestrial organisms. In this study, THg concentrations in liver and kidney were significantly higher than that in muscle tissue,

which has been widely reported in the literature (Sures et al., 2004; Agusa et al., 2011; Misztal-Szkudlińska, et al., 2010). Threshold levels of toxicological effects on terrestrial and marine mammals have been reported to be >25 and >60 ppm ww, respectively (Fisk et al., 2005; Law, 1996, Thompson, 1996), based on liver THg concentrations. While liver THg concentrations of the wolves did not exceed these levels, many of the values were relatively high, and may continue to increase with age, potentially resulting in high fetal and neonatal exposure.

Wolves greater than or equal to 12 months had significantly higher THg concentrations in liver and kidney, compared to individuals less than 12 months of age. Previously, Hg has been demonstrated to exhibit age-dependent bioaccumulation in many mammals (Teraoka et al., 2007; Endo et al., 2007; Gamberg & Braune, 1999; Pedersen & Lierhagen, 2006). However, despite the higher median concentrations in the older age class, one of the highest liver THg concentrations was actually observed in an individual female wolf from GMU 19 (interior) that was only 10 months of age (2226.8 ug/kg) (Figure 3). This individual had $\delta^{15}N$ and $\delta^{34}S$ values of 8.3 and 5.5 per mil, respectively, so it is not likely that marine prey sources were contributing substantially to its diet. The high mercury concentrations observed could, therefore, be due to individual exposure, individual physiological characteristics, or some combination of these factors. Maternal transfer of Hg to the fetus is known to occur (Endo et al., 2007), due to both transplacental and transmammary mechanisms, and some studies have shown that fetal liver Hg redistributes to other organs during postnatal development (Yoshida, 2002). However, maternal transfer of Hg is thought to contribute minimally to the

concentrations seen in young animals, and the greatest risk for exposure likely occurs once these animals switch from nursing to eating other food sources.

Other individuals from GMU 19 and 25, had kidney concentrations of 1,182.6 and 1,032.3 ug/kg, respectively. No isotope data were available from these animals, so it was not possible to speculate whether the high THg concentrations were due to dietary sources; however, both individuals were older than 3 years, so age-dependent THg accumulation may have contributed to the observed concentrations.

THg concentrations in the wolves from this study varied significantly based on GMU and presumed coastal proximity. Wolves from coastal GMUs had significantly higher THg concentrations in all tissues examined, compared to wolves from interior units, and many of the wolves from coastal units had liver and kidney THg concentrations exceeding 1 ug/g. It is likely that variation in THg concentrations is a reflection of the diverse dietary discretion of these animals. While wolves in North America are generally considered obligate predators of ungulates, they do occasionally prey on beaver (*Castor canadensis*), snowshoe hares (*Lepus americanus*), arctic ground squirrels (*Spermophilus parryii*), hoary marmots (*Marmota caligata*), and various birds (Mech et al. 1998). Alaskan wolves with coastal access likely utilize a wide variety of marine sources of prey, including harbor seals (*Phoca vitulina*), various marine mammal carcasses, anadromous eulachon smelt (*Thaleichthys pacificus*), and marine invertebrates (Szepanski, 1999). A number of wolves with the highest observed THg concentrations were from the coastal GMU 9, which is situated on the Alaska Peninsula. Pacific salmon have also been recognized as an important food source for wolves beyond coastal areas,

and may provide a substantial marine influence on inland wolf/prey systems (Adams et al., 2010).

Wolves from GMU 9 (Alaska Peninsula) also displayed significantly elevated $\delta^{34}S$ and $\delta^{15}N$ values, relative to wolves in other units. $\delta^{15}N$, $\delta^{13}C$, and $\delta^{34}S$ values have been used to show that marine sources of prey are being utilized by certain groups of wolves in Alaska (Adams et al., 2010). $\delta^{15}N$ values in marine organisms, including cephalopods and pollock, have ranged from 13.6 to 14.2 ‰ in certain studies (Dehn et al., 2006), and one terrestrial study determined that mean $\delta^{15}N$ values in arctic foxes ranged from 9.3 to 11.3 ‰ (Dehn et al., 2006). In the present study, mean $\delta^{15}N$ values in the liver of coastal and interior wolves ranged from 7.2 to 15.9 ‰ and 6.0-9.0 ‰, respectively. Given that $\delta^{15}N$ values increase predictably with successive trophic steps, these data alone show that marine resources are contributing to the diets of some of the coastal wolves. These $\delta^{15}N$ values compliment the THg concentrations observed, since a positive relationship exists between $\delta^{15}N$, Hg concentrations, and the trophic level of an organism (Capelli et al., 2008). These coastal wolves are likely preying on a wide variety of marine prey sources, including marine invertebrates, predatory fish species, and possibly scavenging on marine mammals. Coastal wolves have been reported scavenging on marine mammal carcasses including walrus, beluga, and sea otter (Watts et al., 2010); however, specific contributions of these foods to the diets of coastal wolves are difficult to quantify.

The results of the multiple linear regression analyses for both liver and muscle THg show that $\delta^{15}N$ is consistently a good predictor of host THg

concentrations. This lends further support to the fact that the high THg concentrations in coastal wolves are likely related to dietary exposure.

THg concentrations and $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ isotope values in various tissues provide four separate measures supporting the contention that Alaskan gray wolves, with access to marine resources, are relying on piscivory or exploitation of other organisms of marine origin. Additional studies quantifying the diet composition of individual wolves, including biomagnifying, non-essential elements such as Hg, will aid in our overall understanding of wolf-feeding ecology, likely via utilization of mixing model approaches. This work, and other reports, clearly demonstrate the need to better understand the variation in diet among Alaskan gray wolves, and how THg exposure varies spatially, based on the feeding habits of these animals.

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Table 1.1. Mean (±SD), median, and range of THg concentrations in Alaskan gray wolf tissues, expressed in ug/kg, wet weight (ww).

Organ/Tissue	n	Mean	Median	Range
Liver	145	455.7 (±1289.4)	34.7	5.54-7260.7
Kidney	145	627.6 (±1449.4)	101.9	8.3-11173.3
Skeletal Muscle	60	82.0 (±138.9)	12.5	4.39-545.9
Cardiac Muscle	16	216.0 (±153.8)	206.7	3.89-639.5

Table 1.2. Mean (±SD), median, and range of THg concentrations in Alaskan Gray wolves, expressed in ug/kg, wet weight (ww), based on coastal versus interior GMU.

Tissue		n	Mean	Median	Range	p-value
Liver						<0.001
	Interior	117	76.0 (225.2)	28.4	5.5-2221.2	
	Coastal	28	2042.2 (2326.9)	1159.4	33.8-7260.7	
Kidney						<0.001
	Interior	117	218.2 (523.2)	80.5	8.3-4567.3	
	Coastal	28	2338.3 (2503.4)	1939.2	78.3-11173.3	
Skeletal Muscle						<0.001
	Interior	47	19.2 (41.0)	10.9	4.4-258.4	
	Coastal	13	309.0 (130.8)	317.2	90.3-545.9	
Cardiac Muscle						<0.001
	Interior	3	62.4 (101.0)	4.22	3.9-179.0	
	Coastal	13	251.4 (143.5)	232.8	70.5-639.5	

Table 1.3. Mean (±SD), median, and range of THg concentrations in Alaskan Gray wolves, expressed in ug/kg, wet weight (ww), based on age class (<12 months or ≥ 12 months).

Tissue	n	Mean	Median	Range	p-value
Liver					<0.001
< 12 months	45	77.7 (±329.4)	15.3	5.5-6086.0	
≥ 12 months	97	608.7 (±1502.4)	48.4	13.3-7260.7	
Kidney					<0.001
< 12 months	46	67.2 (±76.1)	33.9	8.3-3373.4	
≥ 12 months	96	866.2 (±1672.1)	154.3	37.6-11173.3	
Skeletal Muscle					0.07
< 12 months	9	25.2 (±45.7)	8.1	4.4-545.9	
≥ 12 months	49	95.2 (±149.6)	13.0	5.0-465.6	
Cardiac Muscle					0.125
< 12 months	1	3.9 (n/a)	3.89	3.9-639.5	
≥ 12 months	15	230.1 (±148.0)	226.8	116.2-335.8	

Table 1.4. Correlation coefficients of variables including muscle THg, isotope values, and host characteristics.

	Age	Gender	Location	Muscle THg	$\delta^{13}C$	$\delta^{15}N$	$\delta^{34}S$
Gender	-0.04	--	--	--	--	--	--
Location	0.21	-0.03	--	--	--	--	--
Muscle THg	0.16	0.01	0.85	--	--	--	--
$\delta^{13}C$	0.28	-0.02	0.86	0.79	--	--	--
$\delta^{15}N$	0.21	0.02	0.91	0.92	0.83	--	--
$\delta^{34}S$	0.26	-0.08	0.86	0.78	0.76	0.79	--

Table 1.5. Regression model for THg in host Muscle, modeling differences based on age, coastal access, gender, and isotope values. P-value of the model was <0.0001 and r^2 = 0.86.

Variable	Coefficient	Std Error	p-value
Intercept	-89.07	206.44	0.67
Age	-19.63	21.27	0.36
Gender	2.00	14.65	0.89
Location	-30.42	54.69	0.58
$\delta^{13}C$	5.74	8.02	0.48
$\delta^{15}N$	37.96	6.34	<0.0001
$\delta^{34}S$	4.69	3.14	0.14

Table 1.6. Regression model for THg in Alaskan gray wolf muscle, modeling differences based on age, coastal access, gender, and isotope values, as well as their interaction terms. The p-value of the model was <0.0001 and $r^2 = 0.87$.

Variable	Coefficient	Std Error	p-value
Intercept	27.66	210.35	0.90
Age	-13.69	20.95	0.52
Gender	0.67	14.3	0.96
Location	-35.93	53.34	0.50
$\delta^{13}C$	7.11	7.85	0.37
$\delta^{15}N$	27.31	8.36	0.002
$\delta^{34}S$	-7.01	6.91	0.32
$\delta^{15}N*\delta^{34}S$	1.18	0.62	0.07

Table 1.7. Correlation coefficients of variables including liver THg, isotope values, and host characteristics.

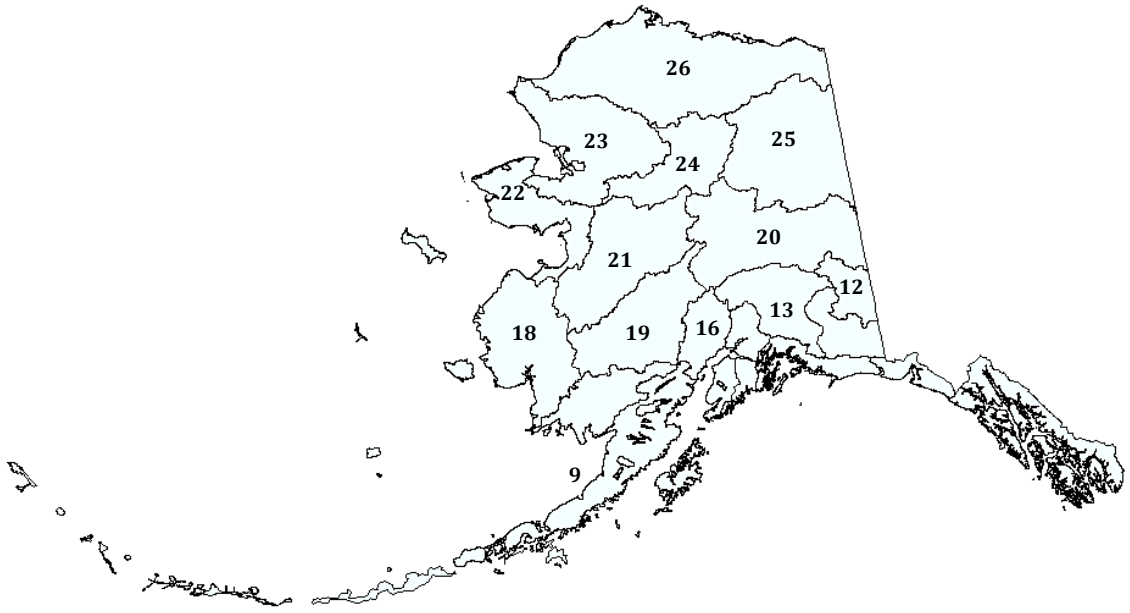
	Age	Gender	Location	Liver THg	$\delta^{13}C$	$\delta^{15}N$	$\delta^{34}S$
Gender	-0.02	--	--	--	--	--	--
Location	0.17	0.05	--	--	--	--	--
Liver THg	0.21	-0.08	0.59	--	--	--	--
$\delta^{13}C$	0.36	-0.01	0.70	0.53	--	--	--
$\delta^{15}N$	0.30	-0.05	0.73	0.71	0.79	--	--
$\delta^{34}S$	0.31	-0.01	0.70	0.68	0.78	0.79	--

Table 1.8. Regression model for THg in host liver, modeling differences based on age, coastal access, gender, and isotope values. The p-value of the model was <0.0001 and r^2 = 0.57.

Variable	Coefficient	Std Error	p-value
Intercept	-8922.91	3359.21	0.0094
Age	52.82	265.89	0.84
Gender	-190.68	221.90	0.39
Location	466.18	392.03	0.24
$\delta^{13}C$	-248.82	120.52	0.04
$\delta^{15}N$	324.81	87.41	0.0004
$\delta^{34}S$	125.49	42.25	0.004

Table 1.9. Regression model for THg in host Muscle, modeling differences based on age, coastal access, gender, and isotope values, as well as their interaction terms. The p-value of the model was <0.0001 and $r^2 = 0.67$.

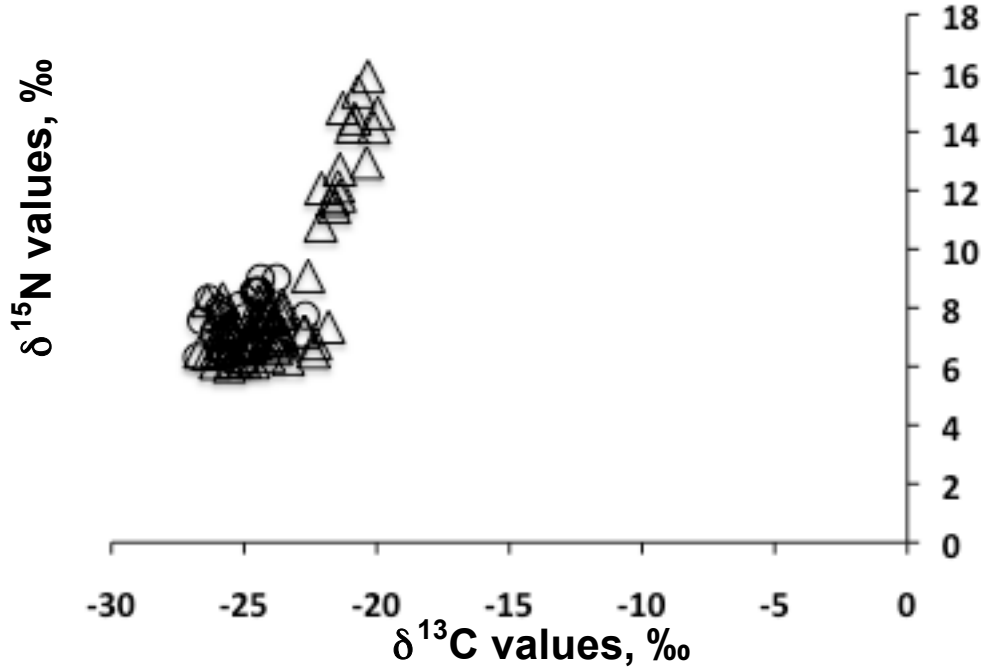
Variable	Coefficient	Std Error	p-value
Intercept	48381.96	13295.02	0.0005
Age	201.06	241.62	0.41
Gender	-216.90	197.58	0.28
Location	420.91	352.23	0.24
$\delta^{13}C$	1760.59	495.98	0.0006
$\delta^{15}N$	-5586.77	1644.24	0.0011
$\delta^{34}S$	-3560.88	895.11	0.0001
d13C*d15N	-198.37	62.09	0.002
d13C*d34S	-100.31	30.80	0.0016
d15N*d34S	157.53	30.94	<0.0001



Coastal Access	GMU	n
Coastal		
	9	20
	16	1
	18	1
	22	1
	23	5
	26	2
Interior		
	12	1
	13	3
	19	12
	20	106
	21	1
	24	2
	25	7

Figure 1.1. Game Management Units (GMUs) in the state of Alaska and number of wolves (n) sampled between 2006-2009.

A)



B)

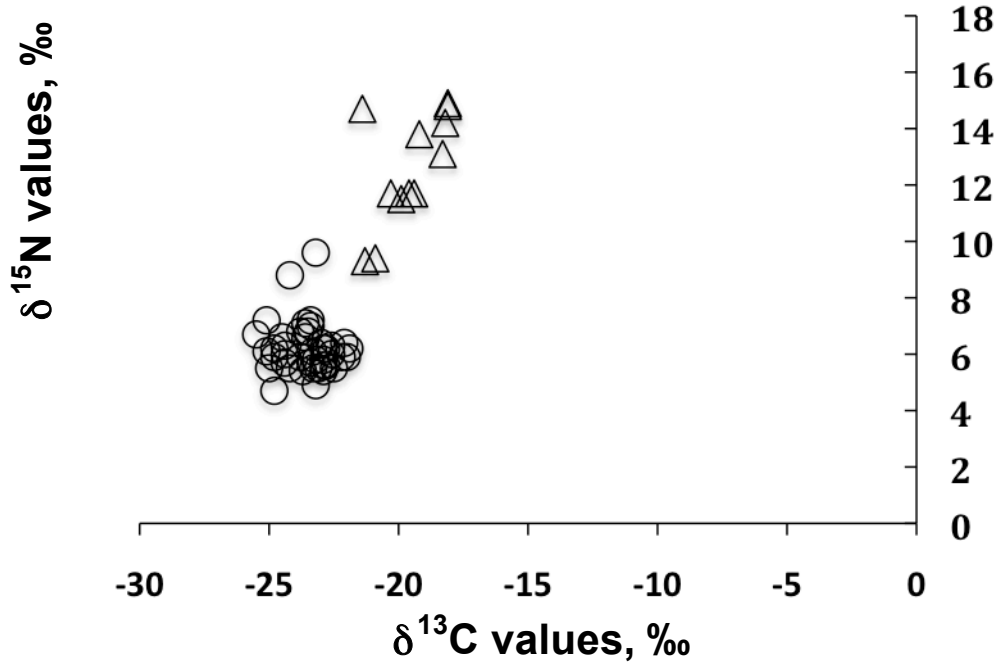
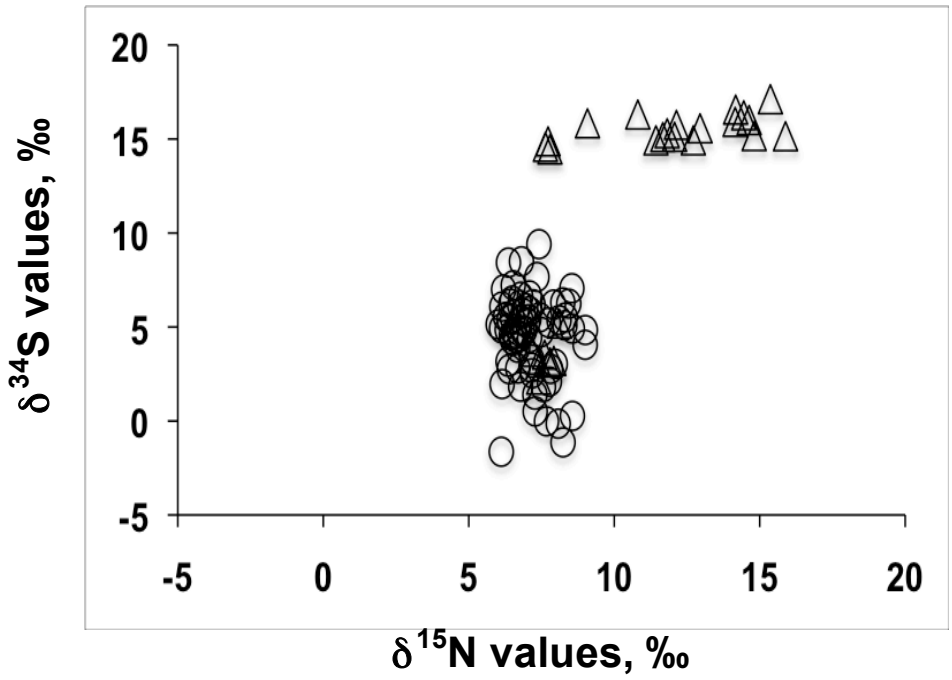


Figure 1.2. Isotope ratios (C and N) in A) liver, and B) skeletal muscle, based on coastal versus interior GMU. Data are expressed in per mil (‰); △ = wolves from coastal GMUs, ○ = wolves from interior GMUs.

A)



B)

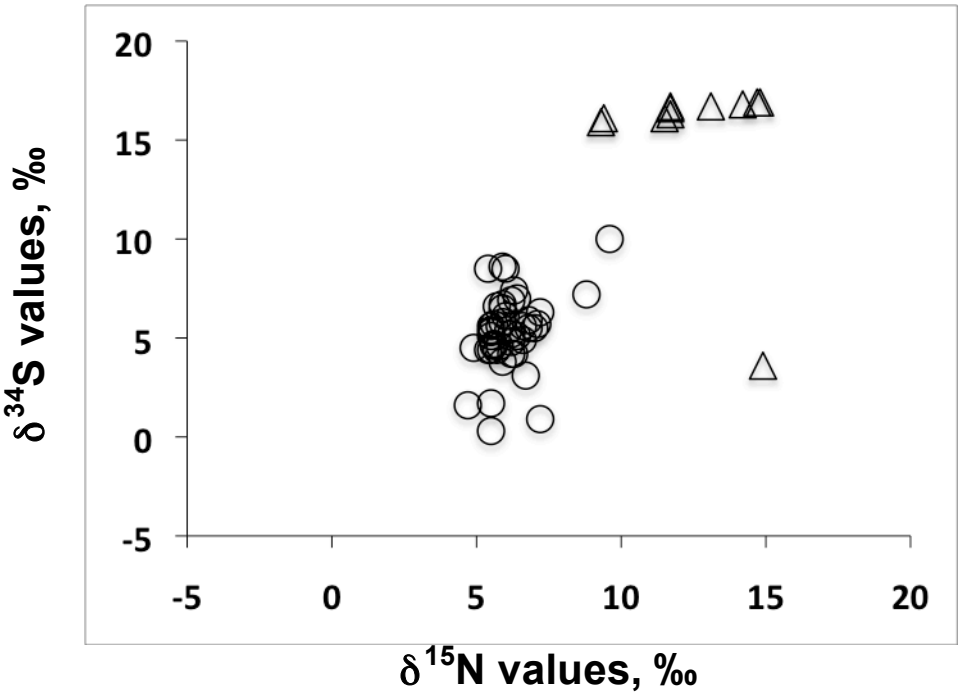
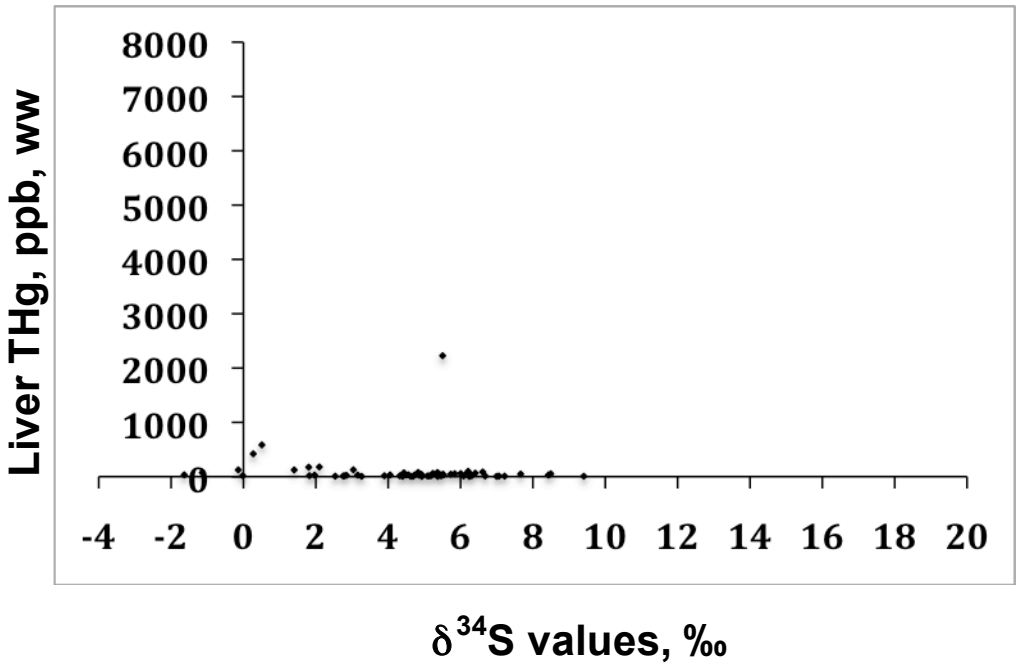


Figure 1.3. Isotope ratios (N and S) in A) liver, and B) skeletal muscle, based on coastal versus interior GMU. Data are expressed in per mil (‰); △ = wolves from coastal GMUs, ○ = wolves from interior GMUs.

A)



B)

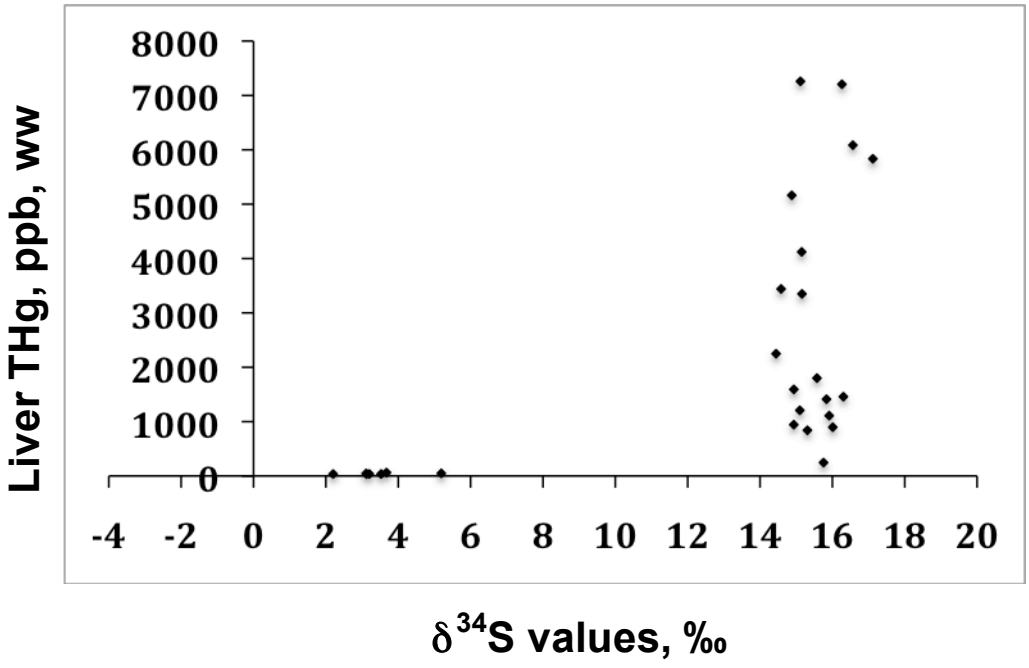


Figure 1.4. THg and $\delta^{34}S$ values (‰) of wolf liver, based on spatial context. A) Wolves from GMUs with no coastal access and B) Wolves from GMUs with coastal access.

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CHAPTER 2

Ecotoxicoparasitology of intestinal helminths in mercury dynamics of Alaskan gray wolves (*Canis lupus*)

Certain gastrointestinal (GI) helminths can bioaccumulate specific non-essential elements at concentrations that are orders of magnitude higher than those in host tissues. The objective of this study was to quantify the uptake of total mercury (THg) by GI macroparasites, and to elucidate their role in THg dynamics within the host. Intestinal tracts were processed from Alaskan Gray wolves (*Canis lupus*). All macroparasites were removed and weighed, and nematodes were enumerated; additionally, host lumen contents and various tissue samples were collected for total mercury (THg) analysis. Prevalence of cestodes and ascarids in the 88 intestinal tracts examined was 63.6% (56/88) and 20.5% (18/88), respectively. Nine wolves contained both nematodes and ascarids, out of 63 parasitized animals (14.3%). Ascarids from 15/18 individuals were identified, and prevalence of *Toxocara canis* and *Toxascaris leonina* was found to be 33.3% (5/15) and 80.0% (12/15), respectively. Two individuals were co-infected with both *T. canis* and *T. leonina.* All cestodes were of the genus *Taenia*. The highest wet weight (ww) THg concentration among cestodes was 107.34 ug/kg whereas the highest THg concentration observed in nematodes was 44.8 ug/kg, possibly suggesting a greater potential for uptake by cestodes. In this study, stable isotopes were used to better understand the trophic

interactions within the GI tract of the definitive host (DH), and to compare isotopic signatures between parasitic groups, relative to the host. $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ values in host tissues ranged from -29.2 to -21.8 ‰, 4.4 to 8.8‰, and -1.6 to 10.3‰, respectively. In parasites $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ values ranged from -25.6 to -22.8‰, 3.3 to 11.1‰, and -3.8 to 21.0‰, respectively. Initial results confirm that these parasites are capable of mercury (Hg) uptake, and suggest that macroparasites may play an important role in mercury distribution within the host.

KEY WORDS: stable isotopes, feeding ecology *Toxocara, Toxascaris, Taenia*

1. Introduction

Mercury (Hg) is a non-essential element that exists in numerous forms, occurring both naturally in the environment, and as a result of human activities. Major anthropogenic sources include the burning of fossil fuels (especially coal), municipal waste incineration (Virtenan et al., 2007), and other point sources such as amalgam waste from dental clinics (Shraim et al., 2011). Volcanoes, forest fires, and geological sources of Hg all contribute to natural emissions. Hg is not only globally ubiquitous, but also environmentally persistent (Balshaw et al., 2007), and can have detrimental effects on biological organisms.

While human activity can release inorganic Hg into the air, water, and soil (Bahn & Sarkar, 2005), microbially-mediated methylation results in a highly toxic for of Hg known as methylmercury (MeHg) (Virtenan et al., 2007; Kruzikova et al., 2008). MeHg has been shown to bioaccumulate in a variety of species, including fish, and often achieves high concentrations in the tissues of long-lived predatory animals such as pike

and sharks (Clarkson & Magos, 2006; Virtenan et al., 2007; Bridges & Zalups, 2010). Piscivorous marine mammals also exhibit some of the highest total mercury (THg) concentrations, facilitated by the biomagnifications of MeHg within food webs (Balshaw et al., 2007; Clarkson & Magos, 2006).

Some gastrointestinal (GI) helminths are known to bioaccumulate non-essential elements at concentrations that are orders of magnitude higher than those in host tissues (e.g., Sures et al., 1999). However, the process by which parasites acquire non-essential metals such as Hg is not understood. It is thought that exposure and uptake likely occur, at least in part, within the gastrointestinal tract in which they live. Some parasites feed directly on host intestinal contents, whereas others feed on host tissues, selectively absorbing specific biochemical compounds.

Ecological studies on food webs rarely include parasites, partly due to the complex nature of host-parasite interactions. (Gómez-Díaz and González-Solís, 2010). Parasites can utilize different feeding strategies, thereby making it difficult to interpret and elucidate trophic relationships using traditional methods. (Gómez-Díaz and González-Solís, 2010). Stable isotopes analyses have, therefore, been used in feeding ecology studies to elucidate food web length and structure (Gómez-Díaz and González-Solís, 2010), and may facilitate quick and accurate assessments of consumer feeding behavior (Daugherty and Briggs, 2007). One of the first isotopic studies of a host-parasite system showed gastrointestinal (GI) nematodes, *Graphidium strigosum* and *Passalurus ambiguus*, had increased nitrogen isotope values, relative to their host, the European rabbit, *Oryctolagus cuniculus*. In contrast, cestodes, *Cittataenia denticulata* and *Mosgovoyia pectinata*, had lower nitrogen isotope ratios than their definitive host,

suggesting different trophic relationships were displayed by these parasitic groups (Boag et al., 1998). Dubois et al. (2009) found no nitrogen fractionation occurring in the parasites, relative to their host, stating that results were in contrast to classical trophic enrichment reported in prey-predator systems, but were in agreement with the scarce literature regarding other parasite-host systems. For these reasons, stable isotope analyses were used in this study to better understand both trophic interactions within the host GI tract of Alaskan gray wolves, as well as how these interactions influence THg dynamics upon dietary exposure. Specifically, THg analysis and stable isotope values of carbon (C), nitrogen (N), and sulfur (S) were used to determine the effects of intestinal helminths on mercury dynamics of the Alaskan gray wolves (*Canis lupus*). It was hypothesized that parasites are affecting Hg distribution within the GI tract, thereby altering THg concentrations in host tissues, and potentially affecting its bioaccumulation potential and/or bioavailability.

2. Materials and Methods

2.1 Sample Collection

Eighty-eight grey wolves from across the state of Alaska, were collected by the Alaska Department of Fish and Game, between 2006 and 2009. At necropsy, gender and age class were determined, and tissues, including liver, kidney, and GI tract, were collected; 9/88 GI tracts were processed immediately. Wolves were divided into two age classes, pup (<12 months) and non-pup (>12 months), based on previous studies (Gese et al., 1997; Zarnke et al., 2004). Collected samples and remaining GI tracts were transferred to the University of Alaska Fairbanks and stored at -80 degrees until additional processing and further analyses could take place.

During processing, large and small intestines were opened longitudinally using stainless steel instruments, and all macroparasites were carefully removed, rinsed gently with ultrapure water, weighed, and enumerated. Individual cestode proglottids were frozen for subsequent identification using molecular techniques, and representative nematodes were fixed in 10% formalin for morphological identification. The remaining parasites were frozen for THg and C, N, and S stable isotope analyses. Lumen contents and full-thickness sections of GI wall were collected from proximal and distal small intestine, and the colon, and weighed prior to freezing at -80 C. All host tissues, lumen contents, and macroparasites were analyzed for THg and stable isotopes.

2.2 Total mercury (THg) analysis

All samples except parasites were thawed to room temperature and sub-sampled (70-150 mg) using stainless steel forceps and scissors. Instruments were washed with ultrapure water and dried between each sample. Samples were analyzed with a direct mercury analyzer (DMA) on a Milestone DMA-80 instrument (Butala et al., 2006; EPA 600-R-04-012). The method detection limit for THg determination was 0.005 ng/g, wet weight (ww). THg concentrations were determined from the standard reference material, DORM-3 (concentration of 0.382 +/- 0.060 mg/kg). To ensure quality control, 5 ng, 20 ng, and 100 ng standards were run, as well as a series of blanks (nickel boats). Analysis of the standard reference material was within 10% of the certified value for all metals. THg concentrations are reported on a ww basis in ug/kg.

Parasites were freeze-dried and homogenized using a mortar and pestle. THg was analyzed, initially, on a dry weight (dw) basis, and then converted to wet weight (ww) values based on percent moisture. In cases where percent moisture was unavailable

(<10% of cases) for a given sample, values representing the same type of parasite, from the same individual host were used. In cases where moisture data were unavailable from a given animal (2/109 parasite samples), average percent moisture was calculated based on parasite type (e. g. cestodes, nematodes). For nematodes, mean percent moisture was 77% (± 8%; n=19) and for cestodes, mean percent moisture was 80% (±8%; n=90). When cestodes or nematodes were found in both the proximal and distal small intestine, mean THg concentration was obtained for parasites of the same group, within a given individual host.

2.3 Stable isotope analyses

In preparation for stable isotope analyses, samples were freeze-dried and ground to a fine powder using a mortar and pestle, followed by further homogenization using a ball mill (mini-bead beater; BioSpec). Approximately 1.5-2.0 mg of liver and muscle tissue were loaded into 5 x 9 mm tin capsules for C and N analyses; ~5.5 mg of sample was amended with 2.0 mg of V_2O_5 for S stable isotope analysis. All samples were then analyzed for stable C, N, and S isotopes by continuous-flow isotope ratio mass spectrometry using an elemental analyzer (Carlo Erba NC1500 or Thermo Flash 2000) interfaced to a mass spectrometer (Micromass Optima or Thermo-Finnigan Delta Plus XP), as described by Fry et al. (1992). Isotope values are reported in delta (δ) notation:

$$\delta X = (R_{sample} / R_{standard}) - 1$$

X represents ^{13}C, ^{15}N, or ^{34}S in parts per thousand (‰) deviation relative to a standard (monitoring) gas and R represents $^{13}C/^{12}C$, $^{15}N/^{14}N$, or $^{34}S/^{32}S$ for samples and standards respectively. Isotopic data were normalized to V-PDB, Air, and V-CDT using the primary standards USGS 40 (-26.24‰ and -4.52‰ for $\delta^{13}C$ and $\delta^{15}N$ respectively),

USGS 41 (37.76‰ and 47.57‰ for $\delta^{13}C$ and $\delta^{15}N$ respectively), NBS127 (21.1‰ for $\delta^{34}S$), and IAEA-SO6 (-34.05‰ for $d^{34}S$). Analytical error was assessed by replicate measures of primary standards (<0.2‰ for all three isotopes across all analytical sequences) and quality control assessed using several secondary standards analyzed several times within individual analytical sequences (<0.3 ‰); accuracy was assessed using primary standards as unknowns and was within 0.2‰ for all three isotopes. Sample reproducibility was generally better than 0.2‰ for all three isotopes. Sulfur isotope data were available for a small subset of parasites (n = 16) and GI lumen contents (n = 33).

2.4 Helminth Identification

Nematodes were identified, based on morphological criteria (Averbeck et al., 1995; Bowman, 1987; Bowman, 2009). For identification of cestodes, genomic DNA was isolated from individual proglottids according to manufacturer's instructions (Qiagen, DNA Blood and Tissue Kit). Primers targeting a fragment of the NADH Dehydrogenase subunit I (ND1) gene, based on the protocol of Bowles & McManus (1993), was used to amplify an approximately 471 bp product. Amplification products were purified, according to manufacturer's instructions (Qiagen, QIAquick Gel Extraction Kit), prior to sequencing (Protemonics and Metabolomics Facility, Colorado State University, Fort Collins, CO).

2.5 Statistical Analyses

Prevalence and 95% confidence intervals (Bush et al., 1997; Rickard et al., 1999) were determined for all helminths. For comparisons between hosts, data distributions were first assessed for normality using graphical techniques including histograms and

box-and-whisker plots, supplemented by quantile-quantile plots (Henderson, 2006). Data were determined to be non-normal. A Chi square test was used to determine whether gender or age class was associated with the presence/absence of nematodes alone, cestodes alone, macroparasites (cestodes and/or nematodes), or both cestodes and nematodes. The Mann-Whitney U test was used to determine differences in liver and kidney THg concentrations in animals with and without parasites, and to compare THg concentrations between cestodes and nematodes. Bioaccumulation factors were calculated by dividing the THg concentrations in either cestodes or nematodes by the concentration of THg in the lumen contents from where the parasites were derived. Statistical analyses were performed using StatCrunch5.0 statistical software (Integrated Analytics LLC, Pearson Education, 2007-2009). For all tests, $p < 0.05$ was used to evaluate statistical significance.

3. Results

Prevalence of GI macroparasites was 63/88 (71.5%; 95% CI =59.8%, 79.7%). Cestodes were present in 56/88 (63.6%; 95% CI=52.6%, 73.4%) animals, and ascarids were present in 18/88 (20.5%; 95% CI=12.9%, 30.7%). Nine of 63 parasitized wolves contained both ascarids and cestodes (14.3%; 95% CI=7.1%, 25.9%). Mean intensity of ascarids was 27.1 (1-91).

In individual animals that only had a single ascarid, species identification could not be performed without adversely affecting THg analysis. Therefore, ascarids from 15/18 individuals were identified. *Toxocara canis* was found in 5/15 (33.3%; 95% CI=13.0%, 61.3%) and *Toxascaris leonina* in 12/15 (80.0%; 95% CI=51.4%, 94.7%) animals. Two wolves contained both *Toxocara canis* and *Toxascaris leonina*.

Proglottids were collected from 54/56 wolves containing cestodes. Sequences obtained from 5/54 proglottids were uninterpretable. Of the remaining 49, 20 were *Taenia krabbei* (40.8%; 95% CI=27.3%, 55.8%), and 29 were *Taenia hydatigena* (59.2%; 95% CI=44.3%, 72.7%).

Female wolves were more likely to harbor macroparasites (e.g. nematodes and/or cestodes) than male wolves (X^2= 4.5, p=0.03, df=1); however, no significant association was observed on the basis of age class. On the basis of gender or age class, there was no significant association between the presence/absence of cestodes or nematodes alone, and there was no significant difference in nematode intensity. The presence/absence of cestodes had no significant effect on THg concentrations in the host liver or kidney (data not shown); however, animals with nematodes had significantly lower kidney THg concentrations (p=0.02; Tables 1 & 2). THg concentrations in liver or kidney did not vary significantly based on the presence/absence of macroparasites, or based on the presence of both cestodes and nematodes occurring together in one host. There was no difference in median cestode THg concentrations (7.44 ug/kg; n=50) vs. median nematode THg concentrations (7.67 ug/kg; n=12); however, the maximum THg concentration in cestodes was 107.34 ug/kg, whereas the maximum THg concentration in nematodes was 44.8 ug/kg (Fig 1). Cestodes found more proximally in the small intestine, had a wider range of THg concentrations, although median THg concentrations in the proximal vs. distal small intestine lumen contents were similar (data not shown). The same trend was observed for nematode THg concentrations (data not shown).

Median THg concentrations were significantly higher in cestodes and nematodes, relative to the host proximal SI wall or lumen contents, distal SI wall, and colon contents.

In contrast, THg concentrations were significantly higher in the feces than in either parasite group (Figure 1, Table 3), and no siginificant difference existed between THg in distal SI lumen contents and either cestodes or nematodes. Median bioaccumulation factors (BAFs) in cestodes and nematodes were 1.26 (0.03-13.62) and 1.09 (0.74-4.35), respectively. Because median BAFs in both paraitic groups were ≥ 1, these results indicate bioaccumulation of THg is occuring in these organisms.

There was more variation in $\delta^{13}C$ values in the distal small intestine wall (dSI) and large intestine wall (LI), compared to other tissues or lumen contents, and median $\delta^{13}C$ values were increased in intestinal wall samples, relative to lumen contents (Figure 2A). A greater range of nitrogen isotope values existed in the small intestine wall, compared to lumen contents in the proximal and distal SI (Figure 2B). Median parasite (cestode or nematode) $\delta^{15}N$ values were lower than host liver and muscle, as well as proximal small intestine (SI) wall and proximal SI lumen contents (Figure 3). Of the two parasitic groups, the widest range of $\delta^{15}N$ values was observed for cestodes.

Mean $\delta^{34}S$ values in parasites and lumen contents were 4.6‰ (-3.8 to 21.0), and 5.9‰ (2.7 tp 10.3), respectively. Because only 2/33 GI samples were from coastal wolves, and 1/16 parasites was a nematode, $\delta^{34}S$ values were not compared based on location (Coastal vs. Interior habitat) or taxonomic group. While high $\delta^{34}S$ values were observed in two parasite samples, these elevated values did not appear to relate to high $\delta^{15}N$ or $\delta^{13}C$ values, or THg concentrations.

4. Discussion

The taeniids and ascarids identified in this study are common parasites of wolves (Craig and Craig, 2005; Rausch & Williamson, 1959). No differences in cestode or nematode prevalence, or nematode counts were observed based on host gender, age class, or location. Similarly, Bagrade et al. (2009) found no differences in the intensity or prevalence of any helminth species among wolves, based on age and gender, although *Taenia multiceps* has been reported to be more prevalent in adults than in juveniles (Bagrade et al., 2009).

Different helminth species have been investigated with respect to essential and non-essential metal accumulation, and selective uptake of metals by certain parasitic groups has been demonstrated; nonetheless, the influence of heavy metals on the host-parasite system is poorly understood. The findings of this study provide unique insight into the uptake of THg by parasites representing different taxonomic groups.

These results showed that wolves with nematode infections had significantly lower median THg concentrations in the host kidney, but THg concentrations in host organs were not affected by the presence of cestodes. Previous studies have shown the intensity of nematode parasites to be positively correlated with both THg and MeHg concentrations in birds (Wayland et al., 2001), and cestode and acanthocephalan intensities have been demonstrated to be positively correlated with selenium and Hg concentrations, respectively (Sagerup et al., 2009). Nematode intensity in this study was not associated with THg concentrations, although their presence was related. This may suggest that individual nematodes were contributing more substantially to the decreased

THg concentrations observed in host kidney, rather than factors such as nematode intensity.

The maximum BAF among cestodes was 13.62 indicating that THg was over 13 times higher in the cestodes of at least one host, relative to the lumen contents from which it was derived. In contrast, the maximum BAF observed in nematodes was only 4.35. While bioaccumulation appears to be occuring in both cestodes and nematodes, this would suggest that nematodes may not take up THg as effectively as cestodes and/or may not retain THg in their system to the same extent as cestodes. It is also plausible that nematodes are capable of biotransforming THg, but do not sequester it in high concentrations. Vertebrates possess multiple mechanisms for processing and/or eliminating THg from their system; for example, Hg can be depurated in the hair, fur, feces, and eggs. While the ways by which parasites may process non-essential elements are largely unknown, their ability to take up heavy metals suggests that mechanisms for eliminating metals could potentially exist.

Demethylation of Hg by intestinal microflora is thought to be a major factor determining the excretion rate of Hg from consumer organisms (Rowland et al., 1984). In ruminants, demethylation has been shown to occur in the presence of both aerobic and anaerobic rumen bacteria. On the basis of Hg^{2+} sensitivity, demethylation is likely mediated by two enzymes, a $CH3\text{-}Hg^{+}$ hydrolase and a Hg^{2+} reductase (Kozak & Forsberg, 1979). Despite this evidence, few studies have examined the possibility that parasites in the intestinal tract contribute to the demethylation of Hg to a less toxic form. Robinson et al. (2010) demonstrated that nematodes accumulated (and thus sequestered) some of their cormorant hosts' body burden of MeHg; however, they did not dramatically

reduce their hosts' accumulation of MeHg (Robinson et al., 2010). In this study, maximum THg concentrations were higher in taeniids than ascarids. Though the ascarids are not bioaccumulating THg to the same extent as taeniids, ascarids may have the capacity to demethylate the Hg into a form more readily eliminated by the host. If the ascarids are playing a role in biotransformation, then Hg could be more readily eliminated from the host, which may account for the lower median THg concentrations seen in the kidney of those hosts with ascarids. Conversely, the diet of the individual host may also contribute to the THg concentrations seen in parasites.

Given the potential for certain forms of Hg to bioaccumulate and/or biomagnify in food webs, determining the trophic interactions within the GI tract and the feeding ecology of intestinal helminths is essential to understanding Hg dynamics within the host. Indirect parasite life cycles follow predator–prey linkages, as definitive hosts consume infected intermediate hosts (Lafferty et al., 2008). When evaluating the ecologic-like interactions within the GI tract, it is therefore critical to consider how parasitism differs from predation as a trophic strategy, and how parasites differ from each other.

Stable isotope values in parasites have been explored on a limited basis. $\delta^{15}N$ values of the cestodes and nematodes in this study were decreased relative to host tissues and lumen contents, and median values were comparable to each other. In other reports, parasites have been shown to display higher or lower nitrogen signatures, relative to definitive host tissues based on the feeding strategy employed and the assimilation of nutrients. For example, slightly greater $\delta^{15}N$ values have been reported in the trematode, *Drepanocephalus spathans*, compared to their hosts or the co-occurring nematodes *Contracaecum* spp. (Robinson et al., 2010), and lower $\delta^{13}C$ values have been shown in *D.*

spathans compared to *Contracaecum* spp. (Robinson et al., 2010). Isotope enrichment may differ across parasite species within the same host, or and may also differ within a single species infecting multiple hosts.

Other studies have reported that isotopic signatures of parasites did not closely correspond to that of the tissue with which the parasite was found or closely associated, or on which the parasite was thought to be feeding (Deudero et al., 2002). Explanations that have been proposed include selective feeding by parasites on specific amino acids or lipids (Lafferty et al., 2008), migration of the parasite among different fish tissues, and migration between different host animals (Deudero et al., 2002). Differential ^{15}N enrichment patterns in parasites may also be attributed to differences in parasite and host metabolism, particularly the anaerobic and aerobic natures of their respective metabolisms, (Power and Klein, 2004).

$\delta^{34}S$ values have not previously been reported in parasites. While no clear trends were observed relating to C and N isotopic data, or THg concentrations, these values will provide a basis for future comparisons. The wide range of $\delta^{34}S$ values in parasites was unexpected, and determining what factors contribute to a parasite's sulfur signature should be pursued. Due to limited sample numbers, it was unclear whether $\delta^{34}S$ values in parasites reflect marine contributions in the diet of its host.

5. Conclusions

The influence of parasites on the distribution of THg within the host has been demonstrated, and their potential role in biotransforming THg to a less toxic form has been discussed. This work shows that THg uptake occurs in the intestinal helminths of Alaskan gray wolves, and these toxicant-parasite interactions may ultimately affect the

host-toxicant interface. The toxicant-parasite interactions described depend not only on type of parasite and toxicant, but also on the complex ecological-like interactions within the host's body. Elucidating whether parasites are demethylating mercury needs to be determined in order to more fully understand the THg dynamics in this system.

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Table 2.1. Median THg concentrations, expressed in ug/kg wet weight (ww), in host liver and kidney based on the presence or absence of nematodes. Sample number is denoted by n.

Host Organ	Wolves With Nematodes	Wolves Without Nematodes	p-value
Liver	28.3 (5.7-125.9) n=15	45.8 (5.8-2226.8) n=59	0.088
Kidney	45.1 (13.2-261.3) n=15	130.8 (13.5-11173.3) n=54	0.016

Table 2.2. Median THg concentrations, expressed in ug/kg wet weight (ww), in host liver and kidney based on the presence or absence of cestodes. Sample number is denoted by n.

Host Organ	Wolves With Cestodes	Wolves Without Cestodes	p-value
Liver	41.59 (5.7-2,226.83) n=45	34.93 (5.8-585.36) n=29	0.930
Kidney	129.76 (13.17-11,173.3) n=45	93.43 (20.96-585.36) n=29	0.582

Table 2.3. THg in tissues and lumen contents of the host intestine. Mean, median, and range of THg concentrations in Alaskan wolf tissues, expressed in ng/g, wet weight (ww); standard deviation of the mean is indicated in parentheses.

Tissue	n	Mean	Median	Range
Stomach	3	2.2 (±1.2)	2.5	0.8-3.2
pSI Wall	84	8.6 (±21.0)	4.1	0.0-177.8
pSI Lumen	64	5.0 (±8.1)	5.0	0.4-64.0
dSI Wall	86	7.3 (±11.9)	3.5	0.0-79.6
dSI Lumen	60	12.6 (±11.0)	9.3	1.2-48.3
Colon Wall	87	6.3 (±9.2)	3.4	0.0-49.3
Colon Contents	79	38.5 (±65.0)	23.2	2.8-462.9

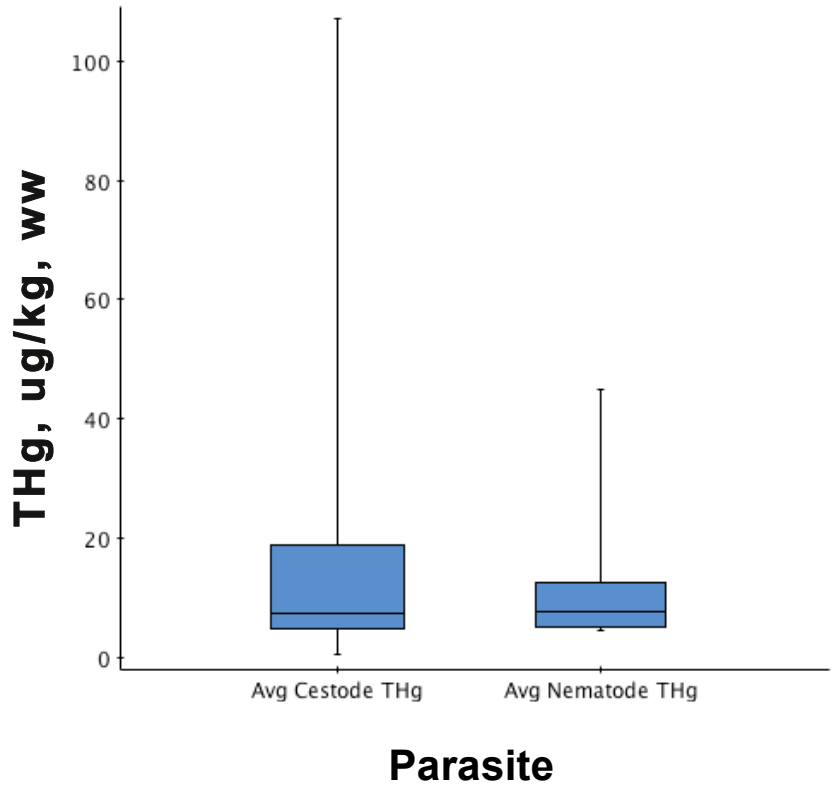
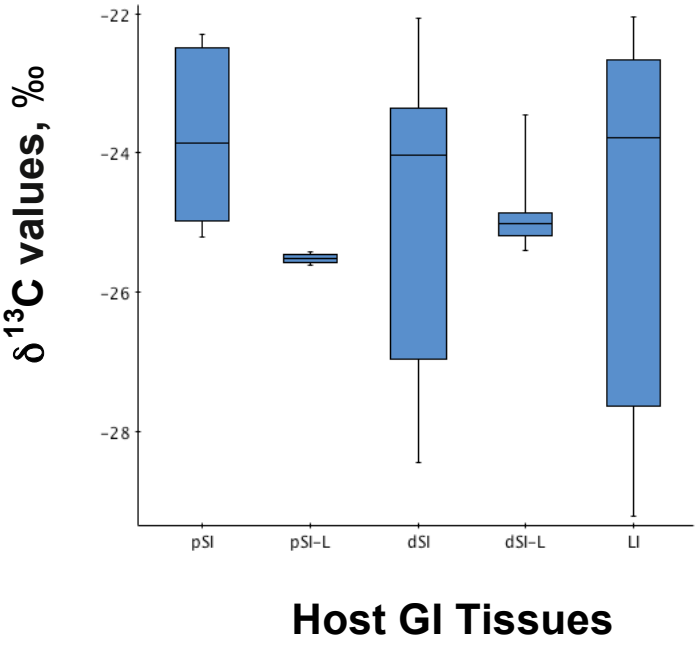


Fig 2.1. THg concentrations (ug/kg, ww) in cestodes and nematodes.

A)



B)

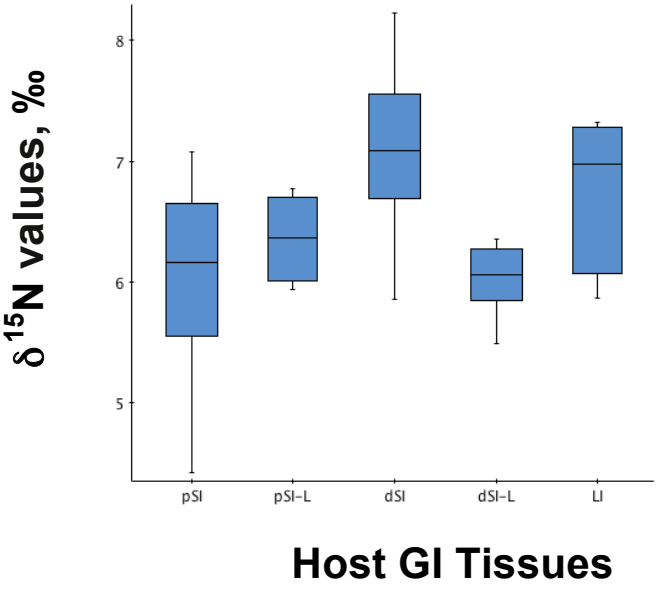


Figure 2.2. $\delta^{15}N$, $\delta^{13}C$ values in host gastrointestinal (GI) wall and GI-lumen contents. A) $\delta^{13}C$ values in proximal small intestine wall (pSI), lumen contents from the proximal small intestine (pSI-L), distal small intestine wall (dSI), lumen contents from the distal small intestine (dSI-L), and large intestine wall (LI). B) $\delta^{15}N$ values in pSI, pSI-L, dSI, dSI-L, LI.

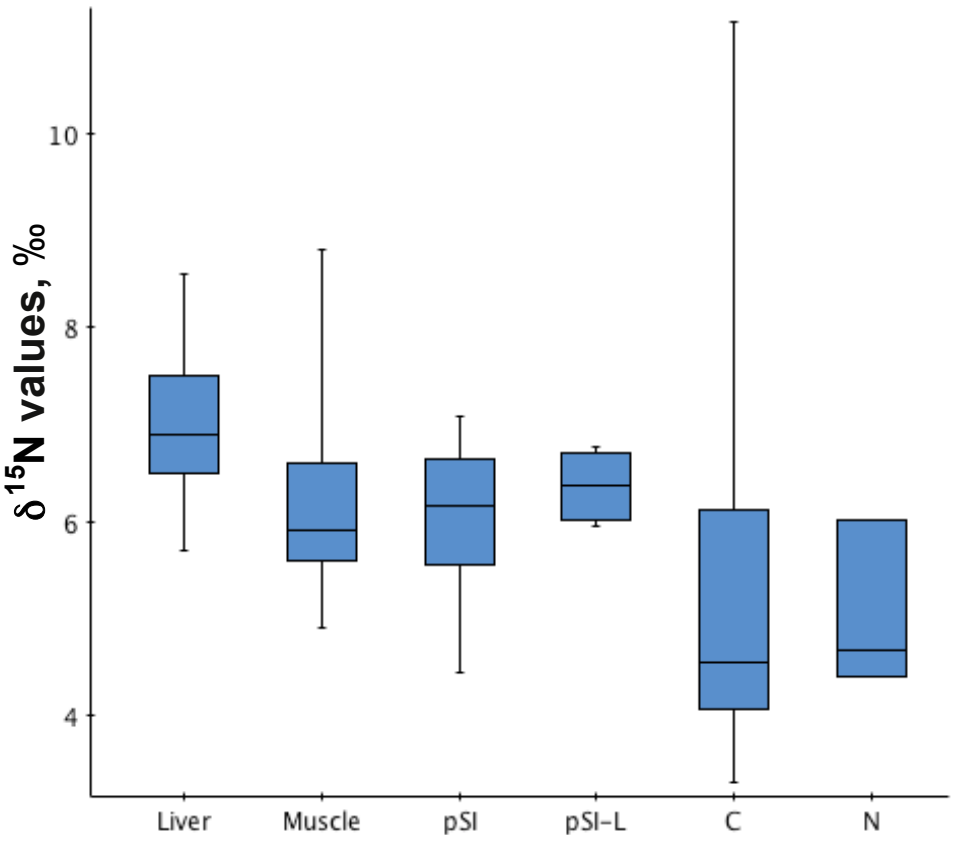


Figure 2.3. $\delta^{15}N$ values (‰) in Alaskan gray wolves. Values are displayed for host liver, muscle, proximal small intestine (pSI), proximal small intestine lumen (pSI-L), cestodes (C), and nematodes (N). Data are expressed in per mil.

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CHAPTER 3

The ecotoxicoparasitology within the gastrointestinal tracts of pinnipeds from Alaska and California

Mature acanthocephalans, cestodes, and some species of nematodes acquire nutrients from the lumen contents in the gastrointestinal (GI) tract of their definitive host. These parasites are exposed to toxicants, such as mercury (Hg) through passive or active feeding mechanisms; therefore, understanding the effect of parasites on Hg bioavailability within a pinniped host was the focus of this study. Total mercury (THg) distribution in tissues and lumen contents from 22 California sea lions (*Zalophus californianus*), 15 ringed seals (*Phoca hispida*), and 4 spotted seals (*Phoca largha*) was determined. Additionally, stable isotope values were obtained to determine the trophic status of host and parasites, and define the ecologic-like interactions within the GI tract. THg concentrations in sea lions and their parasites were related as follows: large intestine (LI) acanthocephalans >> gastric nematodes = small intestine (SI) cestodes = SI acanthocephalans. Concentrations in large intestine acanthocephalans (LI-A) were significantly higher than any other samples from the host GI tract (lumen contents or intestinal wall), and all other parasites except SI nematodes. $\delta^{15}N$ values in all parasites, except those inhabiting the large intestine, were significantly lower relative to host liver, whereas $\delta^{13}C$ values did not differ significantly from host tissues. In all pinnipeds, median THg concentrations were elevated in LI-A, relative to other parasitic groups.

Parasites are likely altering the distribution of Hg absorption within GI tract, and toxicant-parasite interactions appear to depend on both parasitic group as well as their location within the host.

KEYWORDS: stable isotope**,** *Corynosoma*, *Diphyllobothrium*, anisakid, mercury

1. Introduction

Mercury (Hg) is a non-essential metal that exists naturally in the environment, most commonly as the mineral cinnabar (mercury sulfide). Volcanic activities, forest fires, and the natural weathering of rocks all contribute to mercury emissions. Among anthropogenic causes, coal burning is by far the largest source; however, Hg is also present in the ores used in metal production and smelting industries. Despite limited anthropogenic activity in northern regions, mercury can be transported in the environment by winds, ocean currents, and rivers. The occurrence of Hg in some marine food webs, and concentrations observed in biota across the Arctic, are therefore, thought to be attributed to anthropogenic sources in southern regions (Muir et al., 1999).

Exposure to Hg in the arctic, as well as other regions, can have important implications on health of human and wildlife populations. Hg has a wide spectrum of effects, depending upon the chemical form and modes of exposure. Hg may be a contributing factor in ischemic heart disease (Hansen & Gilman, 2005), and both mercury vapor (Hg^0) and methylmercury (MeHg) are well recognized as neurotoxic agents (Satoh, 2003). MeHg can be produced by microbial methylation of inorganic Hg in water sediment, and becomes integrated into the food web at lower trophic levels. Upon entering the food web, it may bioaccumulate and/or biomagnify in fish (Kruzikova et al.,

2008) and upper trophic-level organisms such as marine mammals (Kemper et al., 1994). Fish, therefore, play an integral role in the transport of Hg in a marine food web, serving as a primary source of MeHg in human food (Kruzikova et al., 2008), and a dietary source of Hg exposure in pinnipeds (Kemper et al., 1994). Hg is of particular concern to indigenous peoples of the Arctic, who depend on marine mammals and other wildlife species for not only nutritional purposes, but also for economic and cultural reasons (Moses et al., 2009).

As top predators in a marine food web, marine mammals generally possess elevated total mercury (THg) concentrations in their tissues (Braune et al., 2005; Moses, 2009), and have adapted mechanisms for tolerating these higher concentrations (Ikemoto et al., 2004). Metallothioneins (MTs**)** are low molecular weight proteins that are found in a vast range of taxonomic groups, including pinnipeds, and it has been suggested that these proteins modulate the bioavaibility of physiological cations and the toxicity of non-essential elements, such as Hg. Marine mammals are also known to detoxify Hg by forming mercury selenide (HgSe, tiemannite), mainly in the liver (Nakazawa et al., 2011).

Determining the trophic interactions that exist in a system can aid in determining the effects of dietary exposure to Hg, since Hg can bioaccumulate and/or biomagnify in organisms. Stable isotopes have increasingly been used in feeding ecology studies to evaluate food web structure and energy pathways (Campbell et al., 2005). Stable isotope analysis has had applicability in understanding the interactions in not only terrestrial environments, but in marine ecosystems as well. $\delta^{13}C$ values can reveal the contributions of various carbon sources in the diet of a population or individual. $\delta^{15}N$ values tend to

increase in predictable amounts with each trophic level, and may be indicative of an organism's position in a food web (Peterson and Fry, 1987). THg concentration and burden have also been shown to be dependent on $\delta^{15}N$ values (Brookens et al., 2008). For these reasons, THg concentrations, in conjunction with stable isotope values, can be used to determine the ecotoxicologic relationships in a system.

Although parasites are known to alter the physiological responses of their hosts to toxicants, there are relatively few investigations examining the interface of ecotoxicology and parasitology in marine ecosystems. The purpose of this study was, therefore, to determine the distribution of Hg, and the toxicant-parasite interactions, within the GI tracts of California sea lions (*Zalophus californianus*) and two species of ice seals (*Phoca hispida* and *Phoca largha*). The former provides insight into a population of individuals that originally stranded, and either died in rehabilitation, or were euthanized due to their poor health status; the latter represents a healthy, wild population from the Arctic.

2. Materials and Methods

2.1 Sample Collection

Twenty-two California sea lions (*Zalophus californianus*) that initially stranded in one of seven counties along the central coast of California, were transported to The Marine Mammal Center (TMMC) (Sausalito, CA) in June, 2010. Individuals that stranded dead or died in transport underwent a post-mortem exam upon arrival, whereas animals that were alive at stranding underwent rehabilitative efforts at TMMC for 1-15 days prior to death or euthanasia. Necropsies and gastrointestinal (GI) tract processing were performed at TMMC and samples were collected within 24 hours of death.

Seals were collected in the fall of 2009 (13 ringed seals and 2 spotted seals) and 2010 (5 ringed seals and 2 spotted seals) in Kotzebue Sound, AK. Seals were sampled from the indigenous subsistence hunts (logistics support provided by the Native Village of Kotzebue), collected under permit No. 932-1905-00IMA-009526 issued by the National Marine Fisheries Service (NMFS) and the U.S. Fish and Wildlife Service (USFWS) under the authority of the Marine Mammal Protection Act (MMPA) and Endangered Species Act (ESA). Seal samples collected in 2009 were frozen immediately at -20 degrees Celsius, transferred to UAF, and stored at -80 degrees Celsius until analysis could take place, whereas seal samples from 2010 were processed within 24 hours of death, in the native village of Kotzebue, AK.

At necropsy, sex was determined for each animal, and samples were collected, including liver, kidney, and GI tissue samples. During GI processing, stomach, large intestine (LI) and small intestine (SI) were opened longitudinally using stainless steel instruments, and all macroparasites were carefully removed, sorted by helminth type, and weighed. Parasites were also gently rinsed with ultrapure water. Nematodes and acanthocephalans were enumerated, and all parasites were frozen for THg measurement and C and N stable isotope analysis. Lumen contents and GI tissue sections (2 x 2 cm) were collected from stomach, proximal and distal small intestine, and colon. Representative acanthocephalans and nematodes were fixed in formalin, and later identified based on distinguishing criteria (Van Cleave, 1953; Anderson et al., 1974). Prevalence and mean intensity were also determined (Bush et al., 1997).

2.2 Total mercury (THg) analysis

Samples were thawed at room temperature and liver, kidney, skeletal muscle, and

cardiac muscle was sub-sampled (70-150 mg) using stainless steel forceps and scissors. Instruments were washed with ultrapure water and dried between each sample. THg concentrations are reported on a wet weight (ww) basis in ug/g. Results were also compared on a dry weight basis to ensure that significant differences observed were not affected by moisture content. Samples were weighed before and after being dried so that percent moisture data could be obtained, thereby allowing for conversion between ww and dry weight (dw) concentrations. Samples were analyzed with a direct mercury analyzer (DMA) on a Milestone DMA-80 instrument (Butala et al., 2006; EPA 600-R-04-012). The method detection limit for THg determination was 0.005ng/g. Parasites were freeze-dried, and then homogenized using a mortar and pestle. THg in parasites was analyzed on a dry weight basis and then converted to ww values, based on percent moisture, whereas all other samples were run on a wet weight basis.

Quality assurance and quality control (QA/QC) samples included instrument and method blanks, standard reference materials (SRMs), check standards, and sample duplicates. All samples were run in duplicate and re-analyzed if the percent difference between sample was >10%. The SRM utilized was DORM-3 fish protein homogenate (National Resource Council Canada; 0.382 +/- 0.060 ng Hg/g). Percent recovery for check standards (5, 20, and 100 ng aqueous Hg) was >90%. Analysis of the standard reference material was within 10% of the certified value for Hg.

2.3 Stable isotope analyses

In preparation for stable isotope analysis, samples were freeze-dried and ground to a fine powder using a mortar and pestle. Samples were further homogenized using a

mini-bead beater (BioSpec). Approximately 1.5-2.0 mg of liver and muscle tissue were loaded into 5 x 9 mm tin capsules for carbon (C) and nitrogen (N) isotope analyses.

All samples were analyzed for stable C and N isotopes by continuous-flow isotope-ratio mass spectrometry using an elemental analyzer (Carlo Erba NC1500 or Thermo Flash 2000) interfaced to a mass spectrometer (Micromass Optima or Thermo-Finnigan Delta Plus XP), as described by Fry et al. (1992). Isotope values are reported in delta (δ) notation:

$$\delta X = (R_{sample} / R_{standard}) - 1$$

where X represents ^{13}C, or ^{15}N in parts per thousand (‰) deviation relative to a standard (monitoring) gas and R_{sample} and $R_{standard}$ represent the ratio of $^{13}C/^{12}C$ or $^{15}N/^{14}N$, for sample and standard, respectively. Isotopic data were normalized to V-PDB or Air, using the primary standards USGS 40 (-26.24‰ and -4.52‰ for $\delta^{13}C$ and $\delta^{15}N$ respectively), USGS 41 (37.76‰ and 47.57‰ for $\delta^{13}C$ and $\delta^{15}N$ respectively). Analytical error was assessed by replicate measures of primary standards (<0.2‰ for all three isotopes across all analytical sequences) and quality control assessed using several secondary standards analyzed several times within individual analytical sequences (<0.3 ‰). Accuracy was assessed using primary standards as unknowns, and was within 0.2‰ for all three isotopes. Sample reproducibility was generally better than 0.2‰ for all three isotopes.

2.4 Statistical analyses and calculations

THg concentrations and stable isotope values were analyzed statistically. Data were non-normal; therefore, non-parametric analyses were employed. Prevalence and 95% confidence intervals (Bush et al., 1997; Rickard et al., 1999) were determined for all helminths. A Kruskal Wallis test was used, and then Mann Whitney-U tests, with Bon-

ferroni-corrected alpha values, were used to determine differences in THg concentrations, based on various binomial variables. Multiple linear regression analyses were carried out to determine the significant associations between THg concentrations or isotope values in various host tissue types or parasitic groups. The dependent variable was liver THg, and independent variables included THg in LI acanthocephalans, colon contents, and the interaction of the two variables. For all tests, $p < 0.05$ was considered significant. Due to the small sample number of spotted seals (n=4), spotted seal data are presented, but were not analyzed statistically. Statistical analyses were performed using StatCrunch 5.0 statistical software (Integrated Analytics LLC, Pearson Education, 2007-2009).

Bioaccumulation factors (BAF) are calculated as the ratio of the toxicant concentration in the consumer, relative to the toxicant concentration in the food-source. Therefore, BAFs for this study were determined by dividing the THg (ww) concentration in a group of parasites, within a given region of the host GI tract, by the THg concentration (ww) in the lumen contents of that same region. THg concentrations in the SI were obtained by taking the mean concentration of THg in the proximal and distal small intestine. Mean ratios of THg concentrations in intestinal wall compared to lumen contents were also determined for each section of the GI tract, for reference purposes.

3. Results

3.1 Parasite prevalence

Cestodes, nematodes, and acanthocephalans were all commonly found in the California sea lions, whereas in seals, acanthocephalan prevalence was higher, relative to the other two parasite groups (Tables 1, 2). One sea lion had *Corynosoma obtuscens* in both the large and small intestine, and 5/22 sea lions had both *C. obtuscens* and *C.*

strumosum. Of the 20 sea lions with acanthocephalans, 19 had acanthocephalans in the SI (3/19, *C. obtuscens*; 16/19, *C. strumosum*) and 7 had acanthocephalans in the large intestine (LI) (7/7, *C. obtuscens*; 0/7, *C. strumosum*). Mean intensity of *C. obtuscens* in the LI was 101 (1-591), and 109 (1-318) in the SI. Mean intensity of *C. strumosum* in the small intestine (SI) was 104 (9-386). Cestodes recovered from all pinnipeds were *Diphyllobothrium* spp., with *Diphyllobothrium pacificum* identified in one animal. Nematodes were identified as anisakid-type.

3.2 THg analyses in sea lions and their parasites

THg concentrations were higher in host liver relative to other tissues, and were higher in LI acanthocephalans, relative to the other parasitic groups or host GI samples (Table 3). For comparative purposes, THg concentrations were also compared on a dry weight basis. Both relative trends and variability within a given tissue or parasitic group were similar to results obtained with wet weight (ww) values. THg in the stomach wall and in gastric nematodes were significantly higher than in the stomach contents; however, there was no significant difference between concentrations in the stomach wall and in gastric nematodes. THg concentrations in acanthocephalans of the large intestine were significantly higher than in either the large intestine (LI) wall or the colon contents, and THg in the feces were significantly higher than concentrations in the LI wall.

There was a significant positive correlation between THg concentrations (ug/kg) in the lumen contents of the proximal small intestine (pSI) and those in both acanthocephalans ($r = 0.49$) and cestodes ($r = 0.53$) in the same area. Within parasitic groups, LI acanthocephalans had significantly higher THg concentrations than did gastric nematodes, SI acanthocephalans, and cestodes. THg in gastric nematodes was

significantly higher than in cestodes ($p = 0.0261$), but not significantly higher than THg in acanthocephalans or nematodes of the SI. Within the SI, THg in parasites was related as follows: nematodes = acanthocephalans > cestodes.

Bioaccumulation factors demonstrated effective THg uptake by parasites (Figure 1). Mean ratios of THg concentrations in intestinal wall to lumen contents were also determined for each section of the GI tract for reference, to determine the degree of influence of lumen contents on BAFs. These ratios were determined to be 3.1, 1.2, and 0.8 for stomach, small intestine, and large intestine, respectively. THg concentrations were also evaluated based on number of days in rehabilitation at the marine mammal center, and no associations were identified for any host tissue or parasite. Bivariate analyses between lumen contents and host liver or kidney showed that there is a stronger relationship between liver and kidney THg than between THg in these host organs and THg in GI lumen contents (Table 4).

3.3 THg analyses in ice seals and their parasites

As was seen in California sea lions, THg concentrations were higher in the liver of both species of seals relative to other tissues, and were higher in LI acanthocephalans, relative to the other parasitic groups or host GI samples (Table 5). Data were also compared in dry weight (dw) basis to ensure that moisture content was not affecting the relative concentrations in tissues and parasites. Similar trends were observed in seals when data were compared on a ww and dw basis (data not shown). There was no significant difference between THg concentrations in stomach contents, and those in gastric nematodes. There was also no significant difference in THg concentrations in

lumen contents of the proximal- or distal-small intestine, SI acanthocephalans, or SI nematodes.

In the large intestine, THg concentrations in the acanthocephalans were significantly higher than concentrations in the intestinal wall; and, as was seen in the California sea lions, THg concentrations in colon contents were elevated relative to the intestinal wall of the LI. Median THg concentrations in the LI acanthocephalans were also significantly higher than those concentrations observed in SI acanthocephalans.

Bioaccumulation factors were highest in LI acanthocephalans and gastric nematodes (Figure 2). Mean ratio of THg concentrations in intestinal wall:lumen contents were determined to be 1.6, 1.3, and 0.7 for stomach, small intestine, and large intestine, respectively.

3.4 Stable isotope analyses

Nitrogen isotope ratios revealed that while most parasites occupy the same trophic level as the host, SI acanthocephalans appear to be feeding approximately one trophic level lower than the host (Figure 3). Median nitrogen isotope ratios from host tissues represented one trophic level.

4. Discussion

The acanthocephalans, cestodes, and nematodes found in both the seals and sea lions in this study were expected based on host species and geographic location. *Corynosoma semerme, C.strumosum,* and *C. wegeneri* (Heinze, 1934) have all been reported previously in phocids from arctic regions, and *C. strumosum* and *C. obtuscens* are both characteristic species in California sea lions (Van Cleave, 1953; Margolis & Dailey, 1972).

THg in the stomach wall and in gastric nematodes of the California sea lions were significantly higher than in the stomach contents; however, there was no significant difference between THg concentrations in the stomach wall compared to THg in gastric nematodes. Greatest variability was seen in the nematodes, relative to host tissues, in all pinnipeds; however, maximum THg concentrations were higher in sea lions, compared to seals. In contrast to the findings in the California sea lions, in the ringed seals, there was no significant difference between THg in stomach contents and THg in gastric nematodes. THg concentrations in the stomach contents and stomach wall were relatively low in both seals and sea lions.

THg concentrations in acanthocephalans of the large intestine of the California sea lions were significantly higher than in either the wall of the LI, or the colon contents, and THg concentrations in the colon contents were significantly higher than concentrations in the LI wall. It was clear from these data that the LI acanthocephalans in both seal and sea lions have the greatest capacity for THg uptake, and these parasites demonstrated higher maximum THg concentrations than any other GI samples (host lumen contents or intestinal wall from any region of the GI tract).

The LI acanthocephalans may be preferentially taking up more mercury than other parasites, or may simply be exposed to higher concentrations of THg as it is eliminated from the host. Both MeHg and inorganic Hg are retained by the liver and kidney of the host, and can be recirculated throughout the body, or excreted in the feces and urine as part of the distribution and excretion process (Morton et al., 2004). There was at least one example in which THg concentrations in the large intestine acanthocephalans (LI-A) did not seem to relate to exposure. In this instance, *C.*

obtuscens was found in both the small and large intestine in one California sea lion, and parasite THg concentrations were 175 ug/kg and 176 ug/kg, respectively. In this same animal, THg in lumen contents ranged from 40 ug/kg in the proximal small intestine, to over 15,000 ug/kg in the colon contents. In this instance, the unchanged THg concentrations in *C. obtuscens* in the SI and LI could be due to preferential nutrient uptake/depuration in this species, the parasite's attainment of a steady-state of THg, or an inability of the parasites to bioacumulate THg due to maturity status of the acanthocephalans in that individual host.

Moving proximally to distally in the intestinal tract, no discernible trends in THg concentrations were noted in sea lions; however, THg was slightly elevated in colon contents, and significantly higher in LI acanthocephalans, compared to regions more proximal in location. In both ringed and spotted seals, median THg concentrations remain fairly constant throughout the entire GI tract. As was seen in the sea lions, acanthocephalans in the distal GI tract had the highest THg concentrations.

In seals, nitrogen isotope values in all parasites were decreased relative to host lumen contents. Among parasites, nitrogen isotope values were highest in gastric nematodes and LI acanthocephalans. BAF values greater than 1.0 demonstrate that parasites in both seals and sea lions are bioaccumulating and/or biomagnifying THg. In sea lions, BAFs suggest that THg concentrations in LI acanthocephalans and gastric nematodes are nearly 15 times higher than concentrations in their food source (lumen contents). Furthermore, the ratio of THg in intestinal wall compared to lumen contents decreases moving proximally to distally, which is not associated with the patterns seen in BAFs moving proximally to distally. Therefore, it may be that the BAFs observed are

more likely based on parasite uptake, rather than strictly on concentrations in the lumen contents. Gastric nematodes and LI acanthocephalans had the highest BAFs. In sea lions, the greatest variability was observed in LI acanthocephalans; and in seals, BAFs varied most in acanthocephalans of the large and small intestine. Within parasitic groups, significant differences in THg concentrations were related as follows: LI acanthocephalans > SI acanthocephalans = gastric nematodes > SI cestodes. Within the SI, nematodes and acanthocephalans both had significantly higher THg concentrations than cestodes, but THg in nematodes and acanthocephalans did not differ significantly. Parasite uptake of THg may be multi-factorial, depending on not only parasite type and specific location in the GI tract, but also on host species, geographical location, and other factors. The relevance of metal concentrations in marine mammals is not well understood, and relating concentrations between stranded and free-ranging populations has been difficult (Stavros et al., 2011). While the pinnipeds in this study represent two distinct populations in two different geographical locations, it is worthwhile to note the similarities and differences that exist between these two different groups of animals.

Marine mammals are known to both sequester and biotransform Hg. The percentage of hepatic and renal Hg bound to metallothionein (MT) is very low (generally less than 10%), and this metal is mainly associated with selenium (HgSe), as a detoxified form in the insoluble fraction of the tissues (Das et al., 2000). Selenium is thought to form seleno–protein complexes with Hg, and Se-related pathways such as the glutathione peroxidase enzymes may be used to mitigate the toxic effects of Hg in fish, birds, and mammals (Campbell et al., 2005). Inorganic Hg binds to selenium (Se), forming inert complexes (HgSe) that can be retained indefinitely in liver of the animal (Martoja and

Berry, 1980). Demethylation is another mechanism by which marine mammals process toxicants, and it appears to be influenced by both abiotic (photochemical) and biotic processes. Some species of marine mammals may be better adapted than others at converting MeHg into the less toxic inorganic form (Daoust et al., 1998). For example, fish do not have the same ability to eliminate mercuric species as mammals do (Bridges & Zalups, 2010). Actual rates of demethylation have not been measured in marine mammals.

Most information about Hg methylation and bioaccumulation is from studies of freshwater bodies. Little is known about where and how mercury is methylated in the open oceans, and there is currently a debate as to whether MeHg concentrations in marine fish have increased along with global anthropogenic mercury emissions (Kraepiel et al., 2003). Some, but not all, sulfate-reducing bacteria are thought to be the primary methylators of Hg in sediment (Eckstrom et al., 2003). However, recent evidence suggests that other bacteria, including iron reducers, methylate Hg as well (Slowey and Brown 2007). A lower percentage of MeHg, with increasing organ concentrations of THg in adults, implies that dietary uptake of MeHg in some species of seals remains in equilibrium with physiological detoxification processes for MeHg (Dehn et al., 2005). Furthermore, hair is known to function as an excretory tissue for harbor seals, because considerable amounts of toxic substances can be removed from the blood and retained in hair (Wenzel et al., 1993). Mammals, including pinnipeds, can demethylate MeHg into inorganic mercury via intestinal flora (Norseth and Clarkson, 1971), tissue macrophages, and liver (National Research Council, 2000).

These data confirm that parasites effectively bioaccumulate and/or biomagnify mercury within the host GI tract. There is great variability in within-parasite THg concentrations, and uptake is not necessarily associated with concentrations in host lumen contents, liver, or kidney. THg distribution within the host is altered by the presence of parasites; and consequently, bioavailability of THg to the host may also be altered. The mechanisms by which composition and structure of parasite infrapopulations affect the balance of ecotoxicoparasitological interactions within the definitive host should be further explored.

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Table 3.1. Parasite prevalence and mean intensity (MI) in California Sea Lions. Acanthocephalans (A), Cestodes (C), and Nematodes (N) are displayed.

Parasite	Prevalence (%; 95%CI)	MI
Acanthocephalans	20/22 (90.9%; 69.4%, 98.4%)	135.4 (1-909)
Corynosoma strumosum	16/20 (80.0%)	104.2 (9-386)
Corynosoma obtuscens	9/20 (45.0%)	79.8 (1-591)
Cestodes	16/22 (72.7%; 49.6%, 88.4%)	N/A
Nematodes	20/22 (90.9%; 69.4%, 98.4%)	60.8 (2-422)
Acanthocephalans + Cestodes	14/22 (63.6%)	--
Acanthocephalans + Nematodes	18/22 (81.8%)	--
Nematodes + Cestodes	15/22 (68.2%)	--
Acanthocephalans + Cestodes + Nematodes	3/22 (13.6%)	--

Table 3.2. Parasite prevalence (95% confidence interval) and mean intensity (MI) in ringed seals.

Parasite	Prevalence (95%CI)	MI
Ringed Seals		
Acanthocephalans	15/15 (100%; 74.7%, 100%)	89.7 (2-174)
Corynosoma strumosum	9/14 (64.3%)	
C. semerme	9/14 (64.3%)	
C. wegeneri	1/14 (7.1%)	
Cestodes	1/15 (6.7%; 1.2%, 29.8%)	N/A
Nematodes	7/15 (46.7%; 22.3%, 72.6%)	7 (2-19)
Acanthocephalans + Cestodes	0/15	
Acanthocephalans + Nematodes	7/15 (46.7%)	
Nematodes + Cestodes	0/15	
Acanthocephalans + Cestodes + Nematodes	1/15 (6.7%)	
Spotted Seal		
Acanthocephalans	4/4 (100%; 39.6%, 100%)	874.3 (362-1818)
Corynosoma strumosum	1/3 (33.3%)	
Corynosoma semerme	2/3 (66.7%)	
Cestodes	0/4 (0%; 0%, 60.4%)	N/A
Nematodes	3/4 (75%; 21.9%, 98.7%)	10.7 (3-17)
Acanthocephalans + Cestodes	0/4	
Acanthocephalans + Nematodes	3/4 (75%)	
Nematodes + Cestodes	0/4	
Acanthocephalans + Cestodes + Nematodes	0/4	

Table 3.3. THg concentrations (ug/kg, ww) in tissues and parasites from California sea lions.

Tissue or Parasite	n	Mean	Median	Range
Liver	20	9259.7	5052.2	(1623.4-34945.5)
Kidney	20	1173.1	1070.8	(431.9-2436.0)
Stomach Wall	22	113.0	100.3	(55.7-232.3)
Stomach Contents	18	76.4	43.0	(6.5-199.4)
Proximal SI Wall	21	98.5	96.6	(48.6-190.7)
Proximal SI Lumen	21	89.7	72.8	(21.5-211.4)
Distal SI Wall	22	173.5	114.4	(30.3-784.4)
Distal SI Lumen	19	226.4	244.9	(62.2-550.6)
LI Wall	22	103.3	97.4	(30.1-192.8)
Colon Contents	22	226.6	195.6	(31.2-765.8)
Gastric Nematodes	17	180.8	116.1	(40.0-1135.6)
SI Acanthocephalans	18	138.7	114.3	(33.1-298.5)
Cestodes	14	87.5	72.1	(46.3-201.5)
SI Nematodes	4	222.7	229.5	(164.1-268.0)
LI Acanthocephalans	5	1227.4	1554.5	(175.8-2174.3)

Table 3.4. Bivariate analysis of host tissues and lumen contents in California sea lions. Bivariate relationships between host liver, kidney, and lumen contents of the small intestine.

	Proximal SI Lumen	Distal SI Lumen	Kidney
Distal SI Lumen	0.54	- -	- -
Kidney	0.60	0.1	- -
Liver	0.62	0.32	0.72

Table 3.5. THg concentrations in tissues and parasites from ringed seals and spotted seals.

Tissue or Parasite	n	Mean	Median	Range
Spotted Seals				
Liver	4	2054.7	2266.8	901.5-2783.5
Kidney	4	477.0	410.4	264.8-822.6
Stomach Wall	2	85.2	85.2	71.0-99.3
Stomach Contents	2	77.0	77.0	59.6-94.3
Proximal SI Wall	4	96.1	67.8	44.0-204.9
Proximal SI Lumen	4	50.9	51.0	42.2-59.2
Distal SI Wall	4	87.9	89.7	45.0-127.2
Distal SI Lumen	4	90.6	76.5	71.5-137.8
LI Wall	4	73.3	79.5	39.8-94.3
Colon Contents	3	174.3	191.2	96.7-235.1
Gastric Nematodes	2	40.5	40.5	21.7-59.2
SI Acanthocephalans	4	45.0	28.2	15.7-107.9
Cestodes	0	N/A	N/A	N/A
LI Acanthocephalans	4	88.6	88.2	35.4-142.6
Ringed Seals				
Liver	15	775.1	354.6	122.4-3291.7
Kidney	15	161.8	159.4	10.2-347.0
Stomach Wall	5	29.7	36.6	11.7-42.3
Stomach Contents	2	21.8	21.8	12.8-30.7
Proximal SI Wall	15	30.0	29.0	13.0-47.8
Proximal SI Lumen	15	22.6	17.2	9.1-83.4
Distal SI Wall	14	30.7	29.6	13.0-67.6
Distal SI Lumen	14	29.6	25.4	8.1-57.1
LI Wall	14	26.6	28.1	9.9-50.1
Colon Contents	15	50.5	50.4	12.1-124.3
Gastric Nematodes	4	70.4	46.7	28.0-160.3
SI Acanthocephalans	14	38.3	24.5	2.6-168.9
Cestodes	0	N/A	N/A	N/A
SI Nematodes	5	30.4	19.2	12.1-8-.6
LI Acanthocephalans	9	105.8	87.1	28.7-218.5

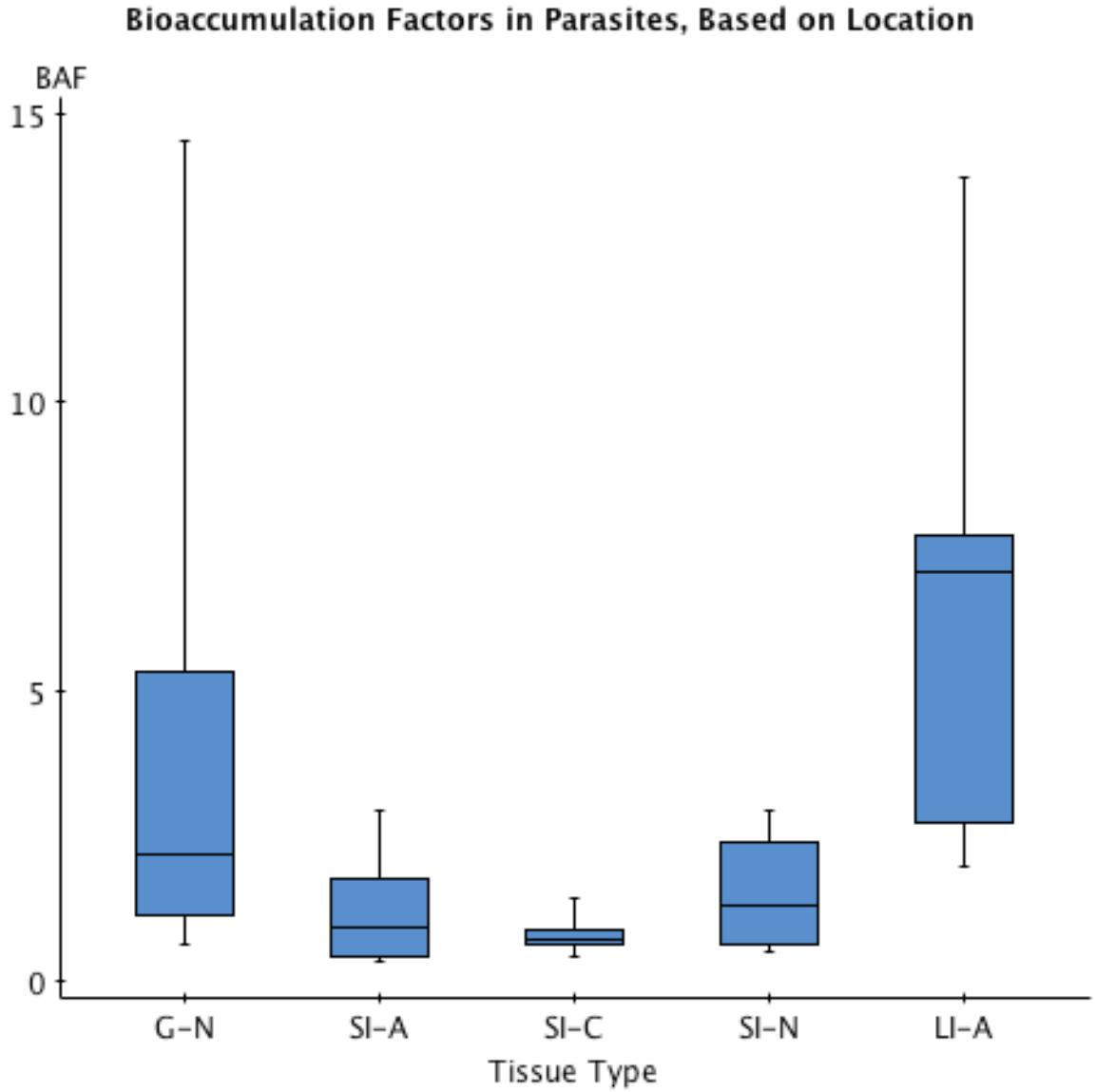

Figure 3.1. BAF in the parasites of California sea lions. BAF is expressed as the ratio of THg in the parasite, compared to the THg concentration in the lumen contents on which the parasite feeds, within a given region of the host GI tract. Tissue types are defined as follows: gastric nematodes (G-N), SI acanthocephalans (SI-A), cestodes (SI-C), SI nematodes (SI-N), and LI acanthocephalans (LI-A).

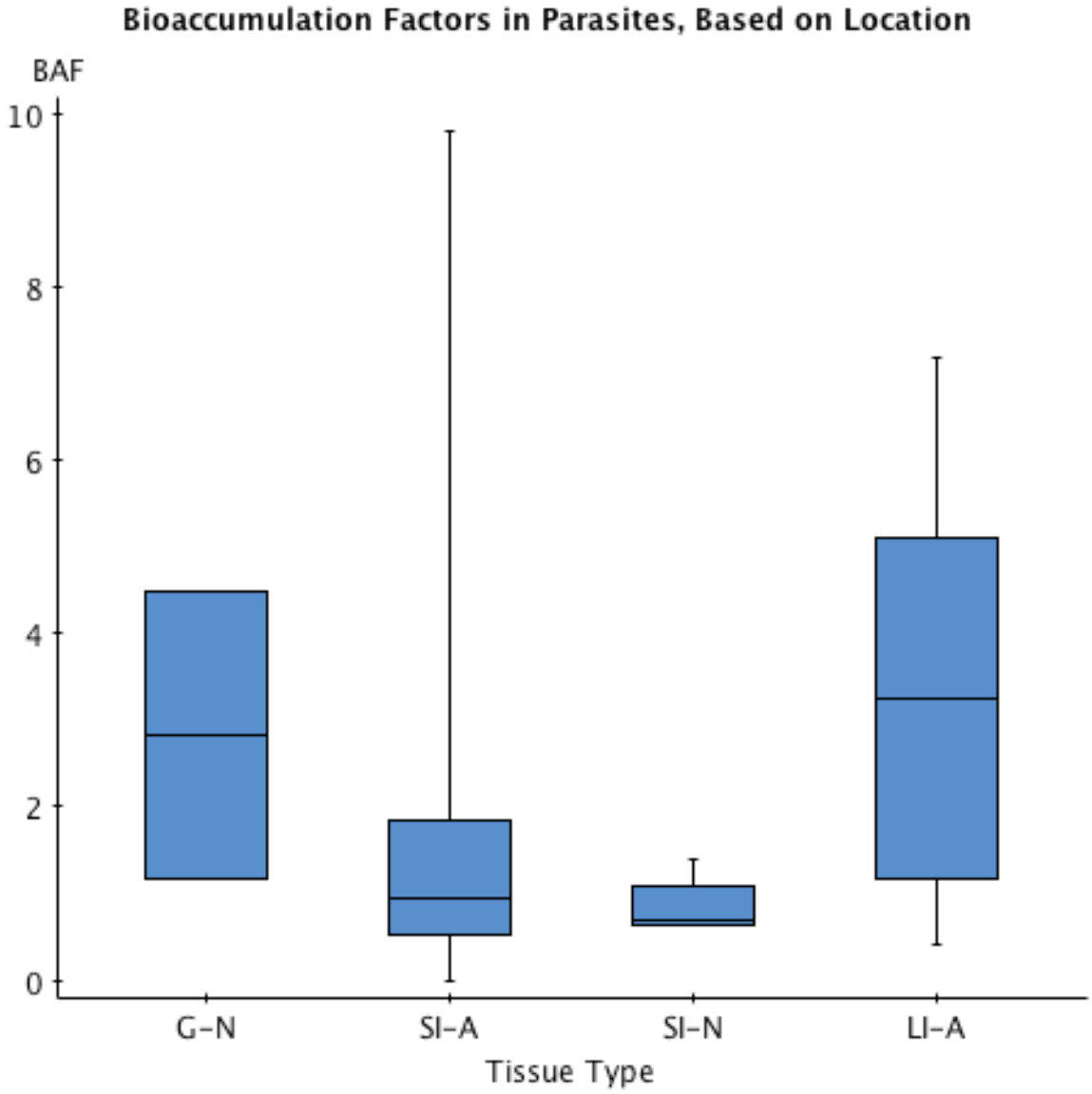

Figure 3.2. BAF in the parasites of ringed seals. BAF is expressed as the ratio of THg in the parasite, compared to the THg concentration in the lumen contents on which the parasite feeds, within a given region of the host GI tract. Tissue types are defined as follows: gastric nematodes (G-N), SI acanthocephalans (SI-A), SI nematodes (SI-N), and LI acanthocephalans (LI-A).

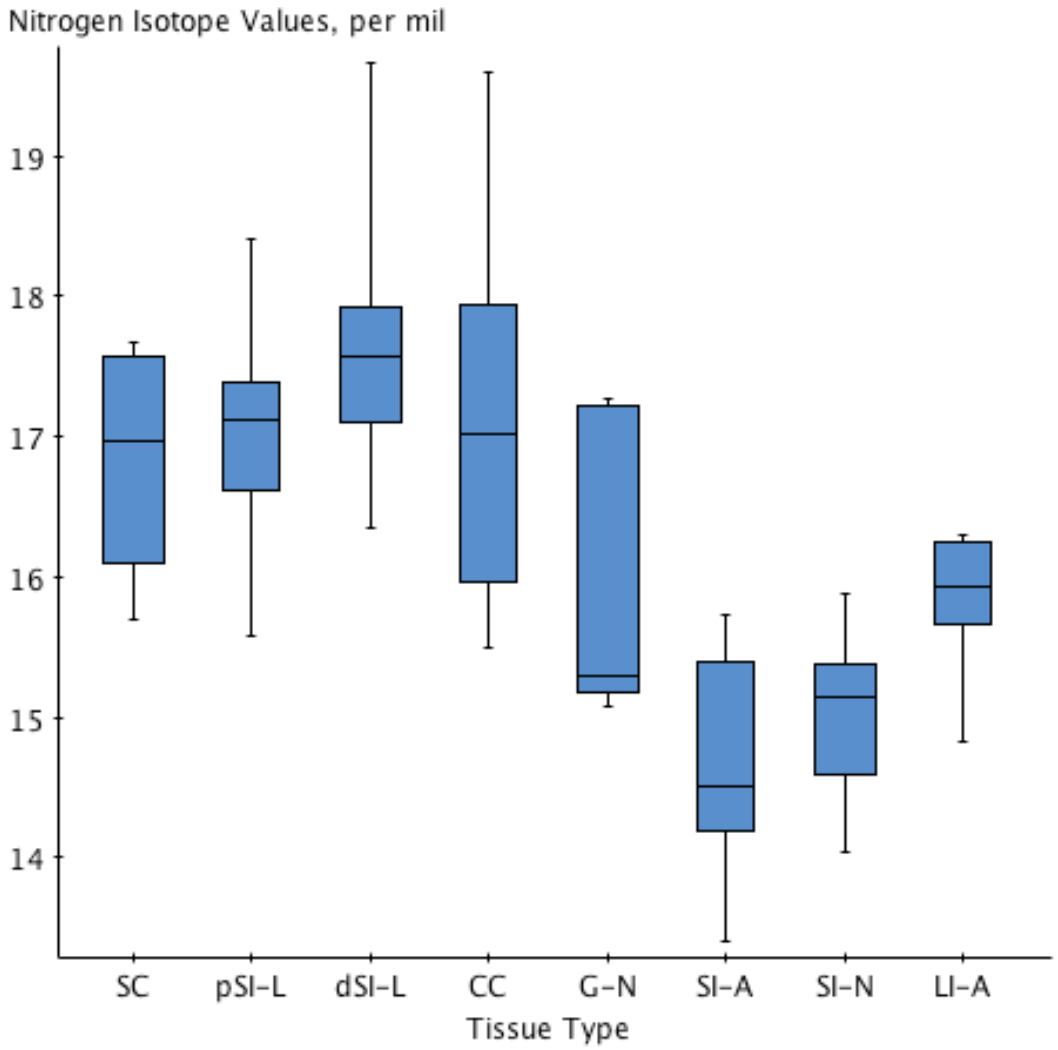

Figure 3.3. Nitrogen isotope values in seals. $\delta^{15}N$ values (‰) in host stomach contents (SC), proximal small intestine lumen contents (pSI-L), distal small intestine lumen contents (dSI-L), feces, gastric nematodes (G-N), SI nematodes (SI-N), SI acanthocephalans (SI-A), and LI acanthocephalans (LI-A).

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CHAPTER 4

Modeling as a tool for understanding the effect of parasites on THg distribution within piscivorous mammalian hosts: concepts and demonstrations

Certain intestinal macroparasites bioaccumulate and/or biomagnify some essential and non-essential elements at significantly higher concentrations than those of their fish host tissues. Therefore, we hypothesized that toxicant-parasite interactions within the gastrointestinal (GI) tract of a piscivorous mammal may affect the distribution and bioavailability of the total mercury (THg) within the host. The objective of this study was to explore the use of epidemiologic modeling in complex systems as a tool for addressing ecological questions. Specifically, a conceptual model is presented, and the application of agent-based modeling in evaluating the effect of parasites on THg distribution within piscivorous mammalian hosts is discussed.

1. Introduction

Hg is a non-essential element, known to be environmentally ubiquitous. Both wildlife and humans are exposed to the many forms of Hg, primarily elemental mercury (Hg^0), divalent mercury (Hg^{2+}), and methylmercury ($MeHg^+$) (Dietz, 2009). Among the various chemical forms of this element, Hg vapor and MeHg are well known and established as neurotoxic agents (Satoh, 2003). While this has been long recognized, in the last 10 years, Hg has also been cited to cause potentially harmful effects on the

cardiovascular system (Virtenan et al., 2007; Roney et al., 2011). Of the different routes of exposure, most humans are exposed to Hg following ingestion of food contaminated with MeHg. In its methylated form, Hg is readily absorbed by the gastrointestinal (GI) tract, and can enter systemic circulation and be delivered to target organs (ATSDR, 2007; Bridges & Zalups, 2010). While certain forms of Hg can also be inhaled or absorbed through the skin and mucous membranes (Kruzokova et al., 2008), dietary sources of MeHg are among the most important routes of exposure. In fact, nearly all of the Hg present in fish is in the form of MeHg. The distribution of MeHg in the body is related to the complexing of Hg with the amino acid cysteine. (Clarkson & Magos, 2006). The structure of this complex resembles that of a large neutral amino acid, methionine, and thereby gains entry into cells on the large neutral amino acid carrier. (Clarkson & Magos, 2006). Following exposure, Hg may accumulate in numerous organs, including brain, intestine, kidneys, liver, and placenta.

Agents of disease, including parasites, make an important difference in the way organisms respond to toxicant exposure (Morley, 2010), and interactions between parasitic agents and toxicants are recognized for their complex nature. Parasites can induce changes in the host that may benefit either the host or the parasite (Campbell et al., 2010), or both. Their elimination may also be disruptive, considering the important homeostatic relationships that exist between host and their normal flora or fauna of the gut and interactions with components of the diet. They affect the physiological homeostasis of their hosts, and therefore, may represent a confounding factor in ecotoxicological studies (Minguez et al., 2009).

Certain intestinal helminths are known to sequester non-essential elements, at concentrations exceeding those in host tissues or the environment, and may invoke adaptive changes in the host. For example, parasitized individuals of the freshwater bivalve *Pisidium amnicum* were shown to have increased tolerance towards contaminants such as polychlorobiphenyls (Minguez et al., 2009). In a changing environment, the link between ecology, toxicology, and parasitology will therefore be important to explore and understand. Climate change is expected to affect both bioavailability and toxic effects of contaminants (Schiedek et al., 2007 in Morley, 2010), as well as disease transmission, and host susceptibility (Harvell et al., 2002 in Morley, 2010). For these reasons, understanding the effect of toxicant-parasite interactions on the distribution of Hg in the host is of importance.

Development of a model should begin with defined objectives, a detailed description of desired outputs, and a clear understanding of the data needed to develop model parameters. The presented framework for the model design, therefore, serves as a foundation for model development. THg data may be evaluated based on the presence and/or effect of various combinations of different parasite groups in order to assess how population dynamics within the GI tract affect THg distribution in the host. The objective of this chapter was to review the application of modeling as a tool in understanding complex system interactions, and to propose a general approach for modeling the affect of gastrointestinal (GI) macroparasites on mercury (Hg) distribution within the GI tract of piscivorous mammals.

2. The application of agent-based models (ABM) in ecological studies

The goal of agent-based models (ABMs) is to better understand a system by studying the relationships of the components within the system (Gilbert & Troitzsch, 1999). Some have described this form of modeling as a mindset, rather than a technique, as it describes the system from the perspective of its units (Bonabeau, 2000). It captures emergent properties, resulting from the constituent components and interactions; the whole is more than the sum of its parts because of these interactions (Bonabeau, 2000). ABMs are comprised of a set of agents that encapsulate properties of various individuals that make up a system (Parunak et al., 1998). The repetitive, competitive interactions between agents are a feature of ABM that relies on the power of computers to explore the dynamics that are inaccessible through mathematical methods alone (Bonabeau, 2002). The system is essentially modeled as a collection of autonomous decision-making entities (agents), which assess their situation and make decisions based on a set of rules (Bonabeau, 2000). ABMs implement a "bottom-up" approach, in which a landscape can be created and populated with this heterogeneous group of agents (Carpenter & Sattenspiel, 2008). The ABM approach can be used to explore the movement of toxicants in a system, by modeling the interaction between the toxicant of interest and the organism that is exposed. In this study, the toxicant of interest is mercury (Hg), and the organisms are parasites, living within the gastrointestinal (GI) tract of a piscivorous definitive host.

2.1 Model design

The following discussion describes ways in which an agent-based model could be used to determine how parasitic infrapopulations affect the distribution of THg in the host's body. Modeling may be used to evaluate the influence of specific characteristics of parasites, and the communities they form, on THg dynamics within the host, as well as the influence of ecological-like interactions existing within the GI tract on THg concentrations/uptake in the parasites themselves.

NetLogo software[1] is proposed as a platform (NetLogo 4.1.2, 2010) for exploring such interactions. The landscape to be modeled is the GI tract of a piscivorous host. Grid size will be three units representing the three parts of the GI tract where the parasites live: stomach (S), small intestine (SI), and large intestine (LI). The two agents represented in the system, include THg and parasites. THg concentrations should minimally be available for host liver, kidney, and various compartments within the host GI tract (i.e. stomach, small intestine, large intestine), but THg concentration data from additional tissue types would be beneficial in providing more information about the system. Parasite population characteristics include presence/absence of 3 primary parasite groups (cestodes, acanthocephalans, and nematodes), mean intensity and biomass of each group, and their distribution throughout the 3 defined GI tract compartments and host organs.

Model parameters can vary over a range, or be set at a specific value in an ABM. Contact between the two agents in this system and uptake of THg by parasites as well as the movement of THg and parasites will be defined by probability functions. These functions can be either fixed, if a deterministic ABM is desired, or by using distributions,

if a stochastic ABM is applied. Known characteristics of the host population including host age, gender, and morphometric measurements are essential elements in the construction of the model. The parameter "movement" is defined by the distribution of agents (e.g. THg and parasites), the probability of their contact, and the probability of a parasite leaving the population.

The basic components necessary for building the framework of an agent-based model include the use of information to design a landscape, creation of a [1]conceptual map or flow-diagram, determination of demographic attributes for all agents of the system, and outlining structure and assumptions of the model. Basic structure should be defined by grid-size, time each simulation runs, number of iterations to run, definition of outputs, and basic assumptions of the model.

Parameters (input variables) are selected based on background information and research questions, and can vary over a range, or be set at a specific value. Examples include contact, movement, and THg uptake. Population is defined as the number of agents in various locations within the GI tract, maturity status, sex, etc. Contact is defined as the number and location of agents. Movement describes and quantifies the distribution of THg and probability of contact, as well as probability of parasites leaving the population.

2.2 Assumptions of the Model

Inter-specific competition is only assumed to take place in portions of the small intestine in which multiple types of parasites co-exist. Parasites must be mature in order to acquire THg, and males and females are equally capable of THg uptake and

[1] Wilensky, U. 1999. NetLogo. http://ccl.northwestern.edu/netlogo/. Center for Connected Learning and Computer-Based Modeling, Northwestern University. Evanston, IL.

depuration. In the state-transition conceptual model (Figure 2), the rate by which a parasite is eliminated by the host (v) is assumed to be constant, and can occur at any point in time, during any state. The probability of uptake is the probability that if contact occurs, it will be adequate. Constant dietary exposure to THg and its allocation to various compartments in the body should be assumed to be constant. THg concentrations, measured in ug/kg represent parts per billion, which can be expressed as percentage of contacts with THg; for example, if the THg concentration is 150 ug/kg, then 150/1,000,000,000 interactions can be assumed to result in THg uptake by the parasite. Therefore, THg concentration will be directly proportional to interaction of the probability of contact, given a constant set of parasitic characteristics, when THg is present in a given region.

2.3 Conceptual Model

The structure of any agent-based model depends on the underlying characteristics of the population being modeled, their daily activities, and the environment in which those activities occur (Carpenter & Sattenspiel, 2008). The proposed modeling approach will include considerations for addressing the affect of gastrointestinal macroparasites on THg distribution within the gastrointestinal tract, and determine whether parasites alter bioavailability of this non-essential metal to the host. The flow of THg in the host is described (Figure 1). The GI tract is divided into three primary compartments: Stomach (S) comprised of the stomach wall (S_W) and stomach contents (S_L); small intestine (SI) is comprised of the small intestine wall (SI_W) and SI lumen contents (SI_L); and large intestine (LI) consists of the large intestine wall (LI_W) and colon contents (LI_L). Parasitic groups include nematodes (N), cestodes (C), and acanthocephalans (A). Solid arrows

indicate direction of movement for total mercury (THg) within the system, whereas dashed arrows indicate general direction of Hg flow within the GI tract, irrespective of the Hg cycling that occurs with other organs. Rectangles represent host liver and kidney, or other organs or tissues (e.g. muscle). The blood compartment is depicted with a hexagon to illustrate that Hg moving from the GI tract to other host organs must first enter circulation. From the kidney, Hg can be excreted through the urine, or can re-enter the bloodstream and be distributed elsewhere. Hg in the liver can re-enter the GI tract through the bile, via post-hepatic cycling. Within the GI tract, THg can exist in the lumen contents or in the intestinal wall. THg can be taken up by parasites directly from the lumen contents, and parasites may be able to depurate THg back into the lumen, through any elimination processes these organisms may possess.

The proposed framework for the development of epidemiologic simulation models uses a state transition diagram to provide the basis for building a detailed ABM. Probabilities of transitioning from one state to another can be fixed or stochastic in nature; the latter of which are often important when the number of individuals involved is small (Daley & Gani, 2000). Determination of state will be carried out using probability density functions. The state of each parasite, and parasite THg concentrations, consequently determine THg that is available to the host. Building stochasticity into a model allows randomness to be taken into consideration (Carpenter & Sattenspiel, 2008). To illustrate this point, random number generators can be used in conjunction with probability functions within the program. In this model, the probability of contact between two agents (THg and parasite) and the probability of THg uptake upon contact are two stochastic measures that will be considered.

2.4 Data Sources

Previously collected data are used in designing the conceptual flow diagram, and also serve as a foundation for understanding how to derive probabilities and relationships during the model development phase. The following results are based on data collected from California sea lions, and provide example calculations that can be used to obtain probabilities that will be needed for building the model on a greater scale or with different populations of piscivorous hosts. These probabilities and proportions relating to THg distribution are specifically important during the programming phase of model development.

During GI processing, parasites were collected from the S, SI, or LI, as described. Lumen contents and intestinal wall samples, however, were collected from not only the proximal small intestine (pSI), but also the distal small intestine (dSI). The gastrointestinal (GI) tract was broken into 3 subsections: S, SI, and LI. The overall mean of the THg concentrations were calculated per compartment, where compartment was defined to include intestinal wall (S_W, SI_W, LI_W) and either stomach contents (S_L) or lumen contents ($SI_{L)}$, LI_L) for each subsection. Proximal SI wall was designated as SI_{pW}, and proximal small intestine lumen was designated as SI_{pL}; distal small intestine was designated as SI_{dW}, and distal small intestine lumen was designated as SI_{dL}. Average THg concentrations for the SI were obtained by taking the mean THg concentrations obtained in proximal and distal regions of the small intestine. This was done to ensure that data from lumen contents could be easily compared to data from parasites, whose small intestine origin was not sub-divided into proximal and distal designations.

3. Results

A state transition flow diagram is depicted (Figure 2) to illustrate the 4 states in which a parasite can exist, based on its exposure/contact with THg. The cycle is entered when the parasite enters the host and moves into the GI tract. The cycle will continue as long as THg and parasites are present together in the system. The schematic represents the temporal transition of THg to the parasites. Parasites exist in only one state at a given time, but the THg with which they enter a state may be based upon the state from which they have come. For example, a parasite may progress to state (D), and eliminate part of the THg it has acquired; subsequently, it may enter state (B) with some percent of the THg that was acquired in state (C), but not effectively eliminated in state D. State (A) represents a state in which the parasite within the host has not yet had contact with THg. This could occur under one or both of the following conditions: 1) the parasitic infection may be recently established, and exposure to THg has not yet taken place, or 2) THg is not present in the system due to a lack of dietary exposure, assuming that parasitic contact with THg is solely from contact with THg in host lumen contents. In state (B), contact with THg occurs, but no uptake occurs. This could have a temporal basis, in which a parasite must spend a given amount of time in state (B) before uptake and progression to state (C) can take place; or conversely, there are circumstances in which the parasite is incapable of THg uptake (e.g. based on maturity status) and will remain in state (B) indefinitely until it is eliminated from the host at rate v. In state (C), both contact and uptake occur, and uptake is directly from the host lumen contents. The transition between state (C) and (D) is unidirectional because after eliminating some percentage of THg that has been acquired, the parasite cannot take up more THg without

exposure again taking place. Therefore, a parasite that has eliminated some of the THg it has acquired, must transition again to state (B), and then (C) and/or (D), or it can alternatively remain in state (D) before being eliminated from the host. A parasite in state (D) cannot move to state (A) unless it moves into a new definitive host, but this possibility is not explored within the context of this conceptual model. The arrow between state (D) and (B) is bidirectional because while the transition from (D) to (B) is somewhat intuitive, a parasite can also move from (B) to (D). In the latter case, a parasite that has eliminated some percentage of THg may come into contact with more THg, but be unable to acquire any more. It is unknown whether a steady state of uptake is eventually achieved in these organisms; but if it does, it would be represented at this point in the state transition diagram. The parasite would then transition back to state (D) and continue to eliminate the THg that was initially sequestered. THg in an individual parasite in state (D) will always be less than its concentration was in state (C). The parasites may leave the host at constant rate v at any point, and leaving the host breaks the cycle. The host does not become repopulated with the same individual parasite during a simulation.

Stochastic probabilities, under certain circumstances, may depend on the number of parasites in a previous state. For example, if competition exists between parasites (thereby limiting the amount of THg available for uptake), then the number of parasites in state (A) might prevent parasites in state (B) from being exposed to THg more distally in the GI tract. Finally, because the simulation model is stochastic in nature, each run or iteration is different, with parameters held constant.

The number of samples that contributed to the overall compartmental averages was based on the number of sea lions that had data available for that compartment, out of the total 22 California sea lions sampled. For example, in the calculation for THg in compartment S (below), 18/22 sea lions had stomach contents (S_L), whereas all 22 individuals had stomach wall samples (S_W).

i. Calculation of average THg in the stomach compartment
    a. $[((\sum S_L)/(18))+((\sum S_W)/(22))]/(2) = 94.7$ ug/kg, wet weight (**w**w)
ii. Calculation of average THg in compartment SI
    a. SI Lumen Contents
        i. $[((\sum SI_{pL})/(21))+((\sum SI_{dL})/(19))]/(2) = 158.1$ ug/kg, ww
        ii. $[(\sum SI_{pL} + SI_{dL})/(40)] = 154.6$ ug/kg, ww
            1. The average THg concentration of compartment SI_L was therefore determined to be: $[(158.1 + 154.6)/(2)] = 156.3$ ug/kg, ww
    b. SI Intestinal Wall
        i. $[((\sum SI_{pW})/(21))+((\sum SI_{dW})/(19))]/(2) = 136.0$ ug/kg, ww
        ii. $[(\sum SI_{pW} + SI_{dW})/(40)] = 136.9$ ug/kg, ww
            1. The average THg concentration of compartment SI_W was therefore determined to be: [(136.0.3 + 136.9)/(2)] = 136.4 ug/kg, ww
    c. Therefore the overall average THg in compartment SI was:
        i. $[(SI_L)+(SI_W)/(2)] = 146.4$ ug/kg, ww

iii. Calculation of average THg in compartment LI

   a. $[((\sum LI_L)/(22))+((\sum LI_W)/(22))]/(2) = 164.9$ ug/kg, ww

iv. Calculation of average THg ratios between compartments S, SI, and LI

   a. Stomach:Small Intestine = 0.65

   b. Small Intestine:Large Intestine = 0.89

   c. Stomach: Large Intestine = 0.57

3. Discussion

A real system is variable, and contains uncertainty, so sensitivity analyses are important to carry out in order to assess influence of certain parameters on model outcomes. The concept of verification has been suggested to be encapsulated in the question: "was the model right?", whereas the concept of model validation has more to do with whether the "right model was built." It is always important to recognize the capabilities as well as limitations of modeling, and to select the most appropriate approach, given the data available. While there is never an entirely objective or accepted approach to epidemiologic modeling, it is important to retain the perspective that the intent of the model is to generate relevant questions and addressable hypotheses, so that it can be used to make predictions about a real system. The applications of the model relate to the quality and quantity of the data available, and outcomes should be considered based on best-case and worse-case scenarios, and primary as well as more indirect outcomes should be anticipated.

An ABM can aid in elucidating the role of macroparasites in toxicant distribution within piscivorous hosts. ABMs are tools that will be useful in future studies that explore the interface of toxicants and parasites in the ecological context of the host GI tract. A better understanding of the ecological-like interactions in this system will allow for predictions to be made about the effects of parasites on host health, especially in a host that is exposed to toxicants via dietary sources. Effective model design will have wide applicability to other systems as well, since toxicant-parasite interactions are likely to contribute to the ecology and health of other populations, including both humans and wildlife.

Table 4.1. Data and probabilities needed to build the ABM proposed.

THg Concentrations	Parasite Characteristics	Host Characteristics	Probabilities
Liver	Presence/absence	Morphometrics	THg-parasite contact
Kidney	Age	Sex	Parasite exiting host
Skeletal Muscle	Mean Intensity		Inter-state transitions
SI Wall	Biomass		
LI Wall	Location		
Stomach Wall			
Stomach Contents			
SI-Lumen Contents			
Colon Contents			

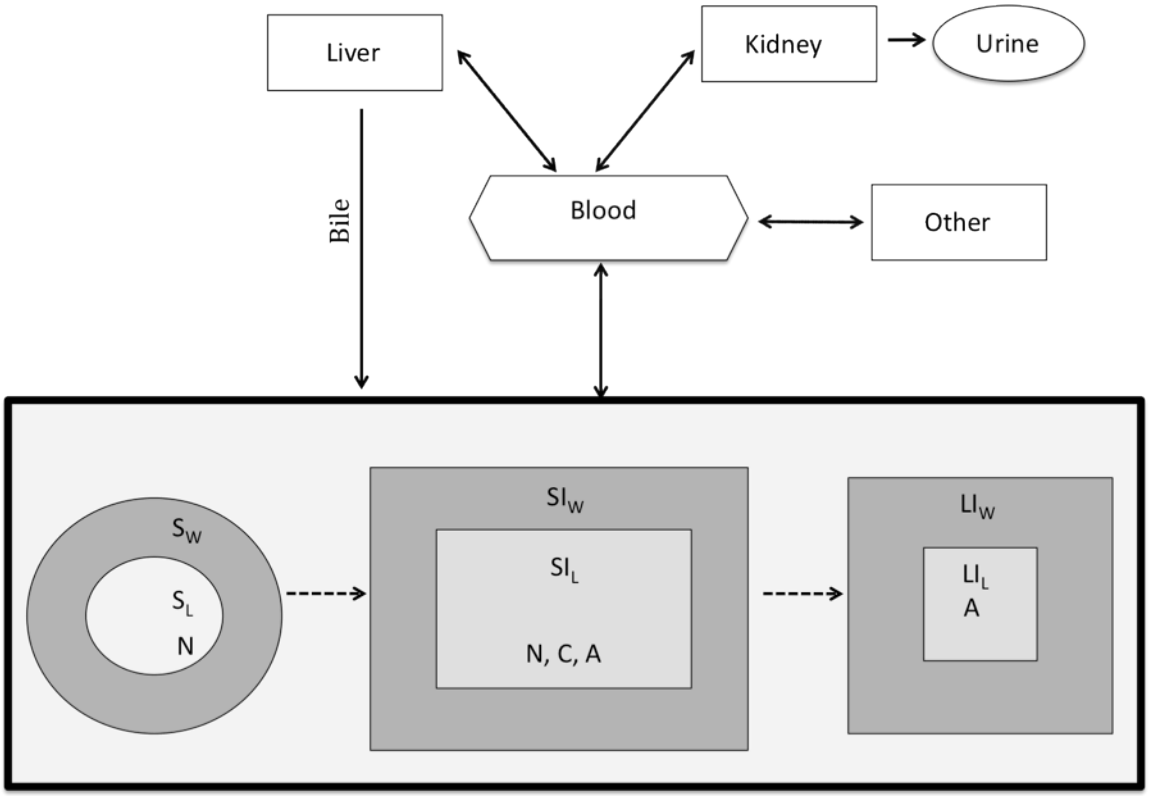

Figure 4.1. Depiction of Hg distribution in a host system. Compartment (S) is comprised of the stomach wall (S_W) and stomach contents (S_L), compartment (SI) is comprised of the small intestine wall (SI_W) and SI lumen contents (SIL), and compartment (LI) consists of the large intestine wall (LI_W) and LI lumen contents (LI_L). Parasitic groups include nematodes (N), cestodes (C), and acanthocephalans (A). Solid arrows indicate direction of movement for total mercury (THg) within the system, whereas dashed arrows indicate general direction of Hg flow within the GI tract, irrespective of the Hg cycling that occurs with other parts of the body. Rectangles represent host organs or other organs aside from liver and kidney. The blood is depicted with a hexagon to illustrate that Hg moving from the GI tract to other host organs must first enter circulation. From the kidney, Hg can be excreted through the urine, or can re-enter the bloodstream and be distributed elsewhere. Hg in the liver can re-enter the GI tract through the bile via post-hepatic cycling.

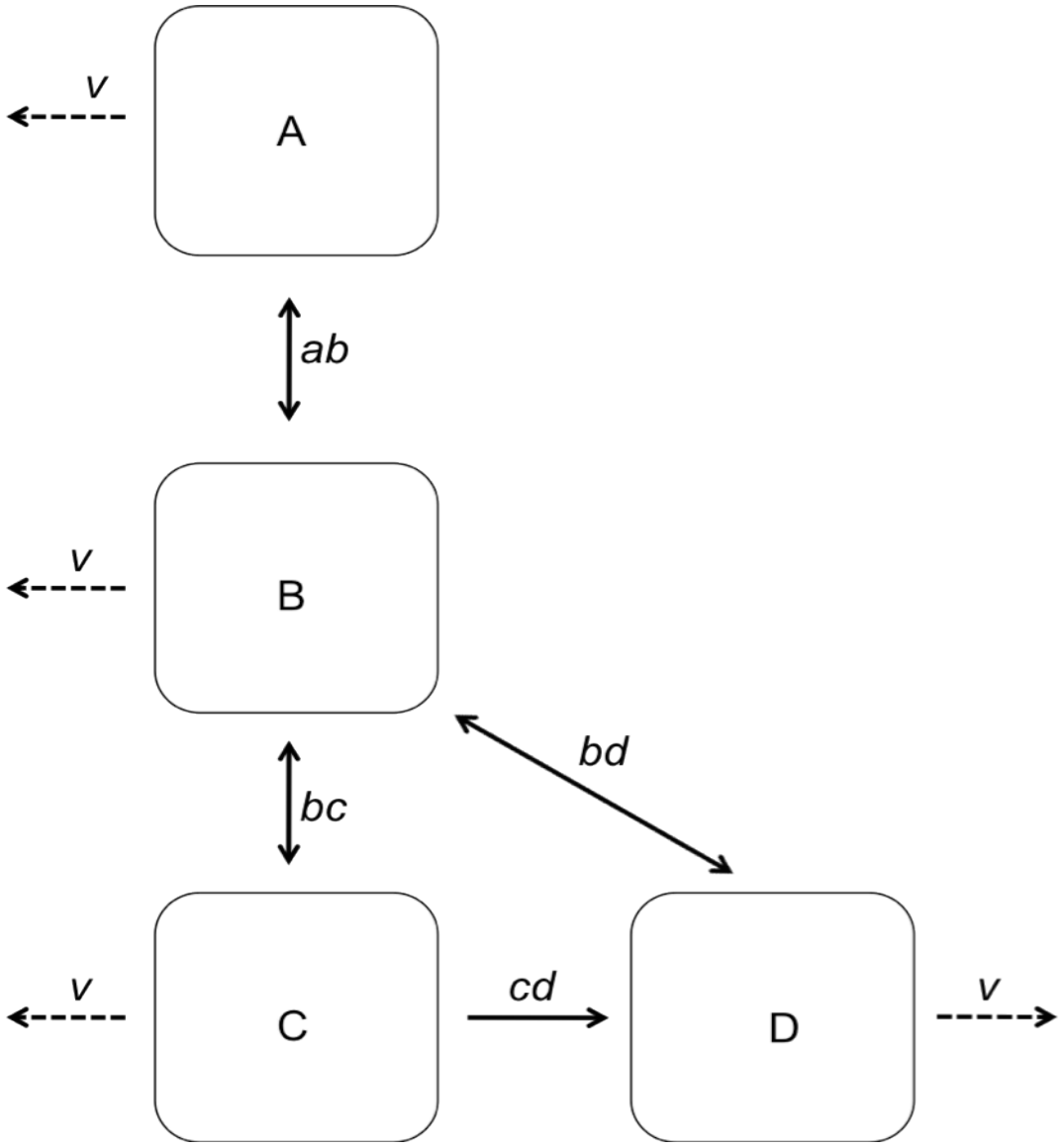

Figure 4.2. Depiction of a state-transition diagram for individual parasites based on their interactions with THg within the host. State are defined as follows: (A) No within-host contact with THg has occurred, (B) Contact with THg has occurred but parasite has not acquired any THg, (C) Contact between THg and parasite has occurred, and THg has been acquired by the parasite, (D) Parasite has eliminated at least some THg that was acquired in state (C). The rate at which a parasite leaves the host (during any state) is represented by *v*. The probability of transitioning between any two states (in either direction) are denoted by *ab*, *bc*, *bd*, *cd*.

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CONCLUSIONS

In modern medicine, the long-standing common practice has been to remove parasites from the host organism—both human and domestic animals. Parasites have largely retained their historical reputation for being unwanted "guests" of their host. The interactions and ecological relationships described in these studies, however, suggest that the symbiosis existing between host and parasite may be far more dynamic than originally supposed, and these interactions may serve an integral role in complex systems, affecting all levels of biological organization.

Many lab models evaluating host-parasite interactions are inappropriate for learning about free-ranging populations, especially since many of the relationships in wildlife populations are thought to be well-established and long-standing in nature. For this reason, interdisciplinary approaches, and the utilization of tools such as epidemiologic modeling can greatly aid in answering complex ecological questions. In these studies, THg and stable isotope analyses revealed the influence of geographic location on THg distribution in Alaskan gray wolves. These two techniques were used in both wolves as well as pinnipeds to demonstrate how trophic relationships affect THg distribution within an individual host. Data provided reinforcing evidence that wolves can display highly opportunistic feeding strategies, and some of the Alaskan gray wolves may be at risk for higher Hg exposure, as a result of these prey choices. The feeding ecology of parasites was also determined, and insight gained has led to more questions relating to the ways in which parasites process the toxicants/nutrients to which they are exposed. Finally, results showed that, even between two vastly different pinniped

populations (varying based on species and geographic location), markedly similar patterns exist relating to THg distribution and trophic relationships.

THg concentrations, and $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values in various wolf tissues, represented four separate measures used to confirm that certain coastal wolves in Alaska are relying on piscivory, or exploitation of organisms within marine-based food webs. THg ranges in some of the coastal wolves may have important health implications at individual, community, and population levels. However, this assessment of potential adverse impacts is beyond the scope of this work. THg concentrations in coastal wolves, reflected by their spatial distribution and dietary exposure, suggest that THg could potentially pose a risk for piscivorous hosts that occupy top trophic positions.

The concurrent evaluation of isotope ratios provided unique insight into not only wolves, but also of feeding ecology of parasites. This work, in conjunction with other studies that have looked at toxicant-parasite interactions, demonstrates that toxicant-parasite interactions depend not only on the specific toxicant and parasitic group, but also on the complex ecological-like interactions within the host's body. These studies suggest that parasites may play a role in biotransformation of Hg within the host, and rather than sequestering THg, certain parasites may demethylate Hg to a less-toxic and more easily eliminated form. Whether parasites can shed THg in eggs, and/or whether THg is present in the parasites when they exit the body of the definitive host remains to be determined.

Cestodes and nematodes were both shown to bioaccumulate THg, although greater variability in cestode THg was noted, with maximum THg in cestodes exceeding maximum THg concentrations in the host small intestine (SI wall or SI lumen). All parasitic groups bioaccumulated THg to varying degrees. The differences observed may

reflect the varying nutritional need of these organisms, and could potentially occur as a result of inter-specific competition within the GI tract. While competition was not directly evaluated in these studies, stable isotope analyses provided insight relating to the trophic relationships of parasites within the GI tract of the host. No proximal-to-distal trends were noted in nitrogen isotope values, and nitrogen values in parasites were decreased relative to host liver, muscle, and GI samples. This finding was particularly surprising, since certain hosts have been reported to excrete lighter isotopes. While the fractionation process in parasites may differ from mammalian hosts, stable isotope analysis (C and N) is nonetheless relevant in providing information about the relative trophic positions among parasites. The acanthocephalans were consistently shown to have the greatest potential for THg bioaccumulation in pinnipeds. Acanthocephalans have been recognized to display dominance and exhibit highly competitive interactions in the presence of other parasitic groups; these studies, therefore, support what little is known about the ecology of this phylum. The results of this project suggest that the acanthocephalans, especially those inhabiting the large intestine, may occupy a higher trophic position relative to other helminths within a given host. Acanthocephalans are known to seek out specific niches within the host GI tract, and may inhabit the large intestine based on nutrient availability and interactions with other co-inhabitating helminths.

Cestodes and nematode prevalence and intensity, and THg concentrations were higher in California sea lions (CSL), relative to ice seals. These differences likely relate to geographic location and /or biological or physiological host characteristics. Few ice seals contained more then one type of helminth, but when two or more taxonomic groups

were represented in one individual, acanthocephalans and nematodes were most commonly found to co-exist. Despite these differences, the trends in bioaccumulation, THg distribution, and trophic interactions strongly suggest that the ecologic influence of parasites on a system exist across populations, and parasites may play a more defined role in ecologic relationships and toxicant exposure in the host than previously assumed.

The degree to which parasites may be altering toxicant dynamics within their host is yet to be determined; however, their influence on host-toxicant interactions should be acknowledged, and recognizing the role they can play in better understanding toxicant distribution in biological systems will be essential in the future.

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