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Title: Mercury Bioaccumulation in Remote Subalpine Lakes: Assessing the Influence of Invasive Trout on Salamanders

Abstract approved:

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Collin A. Eagles-Smith

Mercury (Hg) contamination is a global conservation threat to freshwater ecosystems, detected in even the protected lakes of Olympic National Park (Washington, USA). Although considered some of the most remote and pristine US ecosystems, these lakes are currently subject to multiple environmental stressors, including climate change and fish introductions; adding contaminants, such as Hg, could exacerbate the effects of these stressors on native species. Amphibians are undergoing large worldwide declines, which include declines in protected areas, such as our National Parks. Our goal is to better understand the effect of historically stocked fish in Olympic National Park on native amphibian Hg bioaccumulation. To minimize the need for lethal sampling of this already threatened group, we first examine how Hg concentrations in non-lethally sampled amphibian tissues correlated with whole-body Hg concentrations, depending on species and the different type of non-lethal tissue sample used. Second, we aimed to understand how a food web altered by fish impacted Hg bioaccumulation in native species. We measured Hg and stable isotopes of carbon and nitrogen in organisms across trophic levels from 18 subalpine lakes. We detected small shifts in salamander food web indices and minor changes to salamander Hg concentration associated with the presence of fish. Importantly, we found increased mercury trophic magnification slopes in lakes with fish compared to fishless lakes. This research provides evidence that historic fish introductions can elevate mercury exposure in pristine ecosystems compared to natural fishless lakes.

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Mercury Bioaccumulation in Remote Subalpine Lakes: Assessing the Influence of Invasive Trout on Salamanders

by

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Adam Pfleeger, Author

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Mercury Bioaccumulation in Remote Subalpine Lakes: Assessing the Influence of Invasive Trout on Salamanders

CHAPTER 1 – Introduction

This study examines the influence of historic fish introduction on mercury (Hg) accumulation in aquatic amphibian species from remote subalpine lakes in the Pacific Northwest. In chapter 2, we examined how Hg concentrations in non-lethally sampled amphibian tissues correlated with whole-body Hg concentrations. This included sampling four species of salamanders and one species of frog. In salamanders we collected tail clips in three size classes and in frogs we clipped toes. We then determined the best tail clip size and toe location in terms of predicting the whole body total mercury (THg) concentration. Using these non-lethal sampling techniques for salamanders, we studied the influence of fish presence on salamander foraging ecology and THg concentration.

Few studies have integrated the effects of non-native introduced fish and their corresponding influences on both food-web structure and contaminant concentrations in aquatic top predators (Swanson et al. 2003, Eagles-Smith et al. 2008). We measured food web components (fish, salamanders, zooplankton, and benthic invertebrates) from remote subalpine lakes in Olympic National Park for Hg concentration and stable carbon and nitrogen isotope ratios. We used these measures to examine how different compositions of aquatic apex predators (fish-only lakes, fish and salamanders, and salamander-only lakes) affect the food web structure and Hg concentrations of those organisms.

To understand this food web, it is important to understand the sources, pathways, and bioavailability of Hg and how they interact with non-native fish introductions. In this chapter, I describe the pathway of Hg emissions to the accumulation of Hg in aquatic food webs. I review historic fish stocking in Olympic National Park and the impacts fish stocking can have on food web structure, and how stable isotopes can link changes in the structure of the food web to Hg concentration in aquatic predators.

Mercury contamination is a global conservation threat to freshwater ecosystems worldwide and is commonly detected in even the most pristine remote aquatic ecosystems (Fitzgerald et al. 1998). Although commonly detected, the pathway from Hg deposition to bioaccumulation through the food web is complex. Mercury enters the atmosphere from natural and anthropogenic sources, which has resulted in a threefold increase in worldwide atmospheric Hg levels in the last 150 years (Mason et al. 1994). The leading contributor to atmospheric Hg is the combustion of coal. Currently, coal power plants contribute 50% of anthropogenic Hg pollution to the global Hg pool (Pacyna et al. 2003). Once Hg pollution has entered into the atmosphere, elemental Hg can have a residence time of 0.5-2 years before precipitating out into the ecosystem (Swartzendruber and Jaffer, 2012), explaining its presence at ecologically relevant levels in remote areas.

Once Hg has been deposited into terrestrial and aquatic ecosystems from the atmosphere, two main processes are known to regulate its availability and uptake into food-webs. The initial process is the production of methylmercury (MeHg) in the environment, followed by the bioaccumulation of MeHg through the food web. In the first process, MeHg must be converted from its ionic state (inorganic Hg) to a bioavailable and bio-accumulative form, MeHg (Mason et al. 1994). This process is associated with the activity of microbial groups, specifically sulfate reducing bacteria. In anoxic environments, sulfate reducing bacteria binds ionic mercury to waste methyl groups as a byproduct from energy production, discarding the methylmercury complex into the environment (Ullrich et al. 2001). The second process begins once Hg is methylated; MeHg can then be absorbed into lower food web where it is retained in organisms by binding with protein complexes (Jakubowski et al. 1970). Biomagnification of Hg occurs when the MeHg in prey items is consumed by predators, transferring Hg from prey to predator (Kidd et al. 2012).

Predator-prey relations can be strongly influenced by the presence of non-native species. Fish have been commonly introduced throughout the world for sport

and subsistence purposes; over 60% of all lakes in the western US have been stocked (Bahls, 1992). While there is overwhelming support that introduced fish can affect the food web structure (Zanden et al. 1999; Knapp et al., 2001; Baxter et al. 2004) far less research has been conducted on how those food web shifts relate to mercury concentration in top aquatic predators (Swanson et al. 2003, Eagles-Smith et al. 2008)

Decades of stocking fish in alpine lakes has caused widespread impacts to native aquatic taxa (Lizz et al. 1995). Amphibian species are particularly vulnerable to introduced non-native trout, as trout impact the feeding ecology and behavior of the amphibians (Hoffman et al. 2004). As in many aquatic environments in the Pacific Northwest, historic fish stocking was widespread in Olympic National Park lakes prior to its establishment. Legal fish stocking prior to the 1970's was conducted aggressively by backpack, horse, mule train, fixed-wing aircraft, and helicopter (Meyer and Olsen 1994). Although official fish stocking practices ceased in the 1970's, following National Park Service policy change, illegal stocking likely continued at a small scale, as fish have continued to be found in isolated lakes that were previously known to be fishless. Fish species that have been historically stocked and currently documented in the parks include eastern brook trout (*Salvelinus fontinalis*), cutthroat trout (*Oncorhynchus clarkii*), and rainbow trout (*Oncorhynchus mykiss*) (Hagen 1961).

Lakes with high densities of nonnative fish, in comparison with fishless lakes, have been shown to have altered amphibian, zooplankton, and benthic macroinvertebrate community composition, which can have cascading impacts to the trophic ecology of the ecosystem (Knapp 2005, Lisse et al. 1995). In many fishless lakes, the salamander (*Ambystoma gracile* or *Ambystoma marcodactylum*) are considered the primary vertebrate predator (Taylor 1983). With the presence of introduced trout species, amphibians likely change foraging time and locations to avoid predation and competition for food resources (Tyler 1988, Kenision 2014). Accordingly, any induced shift in amphibian foraging ecology in response to fish

predation and resource limitation may also shift Hg bioaccumulation, as Hg is linked with energy flow in food webs.

The use of stable isotopes, in particular nitrogen ($^{15}N/^{14}N$) and carbon ($^{13}C/^{12}C$), has greatly increased the scientific community's ability to determine feeding relationships and energy flow through food webs (Finlay 2001, Jardine et al. 2006). By assessing the ratio of $^{13}C/^{12}C$ in aquatic ecosystems, one can determine if predators are consuming prey resources from pelagic or benthic origin. The ratio of carbon isotope in consumers is similar to those of their food sources, with trophic enrichment factors ranging from 0-1‰ (Vander Zanden and Rasmussen 2001). Phytoplankton and benthic algae in freshwater lakes have distinct δ^{13} C values because benthic algae has generally lower ^{13}C fractionation during carbon fixation than do phytoplankton, and organisms living in the profundal zone in lakes often are highly negative δ^{13} C due to the fixation of respired CO_2 (France 1995). Thus, the δ^{13} C of aquatic consumers can be used to determine whether prey sources were consumed from pelagic or benthic origin. Nitrogen isotopes on the other hand, have been widely documented to become enriched, generally 3-4‰, as nitrogen moves from the prey to the consumer (Post 2002). This stepwise enrichment of ^{15}N can then be used to determine trophic positions of organisms within a site. When comparing between sites δ^{15} N in consumers can range from 1-13‰ (Jardine et al. 2006). As a result of the wide range of observed δ^{15} N, consumer's δ^{15} N values are used to baseline correct for site differences. Primary consumers, rather than primary producers, are often chosen as a baseline δ^{15} N value due to their larger mass and greater longevity, reducing effects of seasonality in δ^{15} N value (Post 2002). Once properly baseline corrected, isotopes can provide evidence of where an aquatic predator is obtaining energy resources from, what trophic level it resides at, and compare those values across sites.

Utilizing stable isotopes to determine food web pathways, this study aims to examine the integrated effects of historically stocked fish on amphibian foraging

ecology and Hg biomagnification in subalpine lakes. It is well known that amphibian species are in worldwide decline (Pounder and Master 2009). These declines cannot be contributed to just one cause but a combination of multiple stressors, such as contaminants, habitat degradation, and climate change (Sih, and Kerby 2004). By researching the links between contaminants bioaccumulation and effects of non-native trout on amphibians, we hope to lend land managers and the greater scientific community a stronger understanding of how Hg is distributed in altered food-webs and the impacts to amphibians under these combined stressors.

CHAPTER 2 – From tails to toes: developing nonlethal tissue indicators of mercury exposure in amphibian species

Abstract:

Amphibian decline is a serious global conservation threat that is a result of a suite of stressors. Exposure to environmental contaminants is one stressor known to impact amphibian reproduction, growth, and survival. In aquatic ecosystems, mercury (Hg) is a particularly widespread contaminant that biomagnifies in amphibians. This is especially concerning in the Pacific Northwest because the region has some of the highest atmospheric Hg deposition rates of anywhere in the country. Due to the sensitive status of amphibian species, there is a need to develop non-lethal tissue sampling techniques to quantify Hg exposure in amphibians. For many taxa, nonlethal tissue sampling, for example toe clipping frogs, is a common technique used in population-genetic research. However, it is unclear if these methods can adequately characterize Hg exposure. We examined the relationships between Hg concentrations in non-lethally sampled tissues and whole-body Hg concentrations from the same individuals in five amphibian species. Specifically, we examined 1cm, 2 cm, and 4 cm tail clips from four species of salamander and toes from one species of frog. We found that the relationship between tail clip total mercury (THg) concentration and whole-body THg differed among species, suggesting that species specific equations should be applied when possible. We also found that the strength of the relationship was dependent upon the specific tail clip section sampled, with the 0-2 cm segment generally performed the best across all species. Lastly, THg concentrations in frog toe clips were correlated with whole-body THg concentration for some toes but not others, with the three outer toes of the hind foot having the most consistent relationships. These findings suggest non-lethal sampling of tails and toes has potential applications for monitoring contaminant exposure and risk in amphibians, but care must be taken to ensure consistent collection and interpretation of samples.

Introduction:

Amphibian decline is a global conservation crisis, attributed to numerous and diverse stressors (Krest et al. 2003, Stuart et al. 2004, Sparling et al. 2010). Among those stressors is exposure to environmental contaminants. Recent work has shown that mercury (Hg) in particular is both geographically widespread and can elicit deleterious effects on amphibian behavior (Burke et al. 2010), fitness, and survival (Unrine et al. 2004, Bergeron et al. 2011). Mercury is frequently detected in some of the most pristine remote lakes and wetlands due to atmospheric Hg deposition (Fitzgerald et al. 1998, Landers et al. 2008, Eagles-Smith et al. 2014). In the United States, some of the highest annual atmospheric Hg deposition rates occur in Pacific Northwest (National Atmospheric Deposition Program), where numerous sensitive amphibian species occur (Ryan et al. 2014) and there are abundant wetland habitats that can facilitate Hg methylation (Driscoll et al. 2007). Once mercury has entered into an aquatic ecosystem from atmospheric deposition, the methylated form biomagnifies through the food-web (Watras et al 1998, Lavoie et al. 2013). Organisms occupying top trophic levels of aquatic food-webs, such as many amphibian species, will assimilate this form of mercury, increasing their risk and exposure.

Due to the potential risk posed by Hg to amphibians, it is important to better understand the spatial extent and magnitude of amphibian Hg exposure. However, the determination of Hg exposure through tissue sampling often employs lethal methods, which may not be preferred or even feasible because of the sensitive conservation status of many species. Thus, there is great utility in developing nonlethal sampling techniques for determining Hg exposure in a range of amphibian taxa. Additionally, nonlethal sampling methods facilitate field-based studies on the toxicological effects of Hg to amphibians, thus improving our understanding of the risks posed to amphibian populations. Nonlethal sampling methods are well established for fish (Lake et al. 2006, Ackerson et al. 2014), mammals (Malvandi et al. 2010), birds (Eagles-Smith et al. 2008, Jackson et al. 2011), and reptiles (Hopkins

et al. 2001, Hopkins et al. 2011). In amphibians, recent work has shown that nonlethal sampling can also be used (Bergeron et al. 2010, Todd et al. 2012) but relatively less is known about the utility of these methods across different species or how subtle methodological differences can influence their utility. Additionally, some of the non-lethal methods that have been developed, such as cardiocentesis in anurans (Bergeron et al. 2010), are complicated and often lethal if performed incorrectly.

Mercury concentrations in tail clips and toes from salamanders and frogs, respectively, have been shown to correlate with whole-body or blood concentrations (Townsend and Driscoll 2013, Bergeron et al. 2010, Todd et al. 2012), but little information exists on the optimal section of tail or the smallest amount of tissue needed to adequately predict whole-body Hg exposure in salamanders, nor is it clear whether all toes adequately predict whole-body Hg concentrations in frogs. Additionally, little is known about whether there are species-specific differences in these relationships. In this study, we evaluated the relationships between total Hg (THg) concentrations of different tail sections or different toes and individual's respective whole-body THg concentrations in five species of amphibian species to determine, (a) if the correlation between tail THg and body THg concentrations differs among species, (b) how whole-body prediction estimates vary with tail clip section, (c) the smallest tail clip section needed to adequately predict whole-body THg concentrations, and (d) if there are particular toes that are better predictors of whole-body THg concentrations in frogs.

Methods:

Sampling Sites

We collected four salamander species [northwestern salamander (*Ambystoma gracile*, n= 133), long-toed salamander (*Ambystoma marcodactylum*, n= 11), rough-skinned newt (*Taricha granulosa*, n= 39), coastal giant salamander (*Dicamptodon*

tenebrosus, n= 42)] and one frog species [Cascades frog (*Rana cascadae*, n= 30)] from sites in Mount Rainier National Park Washington, Olympic National Park Washington, the headwaters of the Trask River watershed in northwest Oregon, and Tongass National Forest, Prince of Wales Island, Alaska.

Specimens were collected from lakes and wetlands by hand and using dipnets with the exception of pacific giant salamanders, which were collected from streams. After capture, each species was identified and immediately euthanized using an overdose (>250 mg/L) of buffered MS-222 tricaine methane sulfonate. Each specimen was then placed into individually labeled polyethylene bags and stored on snow or ice packs until transport to the laboratory, after which they were frozen at -20 ˚C until processing.

Laboratory Processing

Salamanders

Specimens were thawed, then rinsed in deionized water and patted dry using lint free wipes. Using a small fish board, we measured snout to vent length and total length to the nearest mm, and weighed each sample to the nearest 0.001 g. Initial dissections were made at the 0-2 cm and 2-4 cm marks, but to test for the smallest possible tail clip section that can be used to effectively predict whole-body Hg concentrations, later dissections were made at the 0-1cm, 1-2 cm, and 2-4 cm marks (Figure 2.1). Tail segments were dissected and sectioned using stainless steel scalpels cleaned with dilute nitric acid and deionized water. If the salamander tail was less than 4 cm long, only 0-2 cm or 0-1 cm and 1-2 cm sections were dissected. Each tail segment and the remaining whole-body were then weighed to the nearest 0.001 g and dried at 50°C until a constant mass was achieved (typically 48 hours). Samples were then cooled to room temperature in a desiccator, after which a dry mass was recorded to the nearest 0.0001 g. Once dried, the remaining whole-body amphibians were

homogenized using acid-cleaned stainless steel scissors and ceramic mortar and pestle. Dried tail segments were analyzed whole and thus not homogenized.

Frogs

Cascade frogs were processed in a similar fashion as salamanders. Briefly, frogs were thawed, rinsed and patted dry. Then snout-to-vent length (mm) and total length were measured, and wet weight was recorded to the nearest 0.001 g. All toes, excluding thumbs, were dissected in front of the webbing using stainless steel scalpels cleaned with dilute nitric acid and deionized water (Figure 2.2). We chose not to dissect thumb toes from frogs as they are used for courtship and mating practices and thus are not commonly sampled in non-lethal surveys. Dissections were made on each toe at the point past the webbing of the foot. Each clip was labeled with a unique code to track the toe and associated frog from which it was excised, and then the remaining carcass and toes was weighed to the nearest 0.001 g. Whole-body homogenization was conducted as described above for salamanders and toes were analyzed whole.

Total Mercury Analysis

We use THg as a proxy for methylmercury (MeHg) concentrations because THg concentrations have been shown to be positively correlated with MeHg concentrations in amphibians, and MeHg represents most (approximately 70%) of THg in amphibian tissues (Townsend and Driscoll, 2013). We determined THg concentrations on a dry weight basis using combustion and gold amalgamation coupled with cold vapor atomic absorption spectrometry, using a tri-cell DMA-80 Direct Mercury Analyzer (Milestone Inc., Monroe, CT USA), following U.S. Environmental Protection Agency method 7473 (U.S. Environmental Protection Agency, 1998). The detection limit for THg analysis was 0.01 ng Hg in a sample. Certified reference material (dogfish muscle tissue [DORM-4; National Research Council of Canada, Ottawa Canada] and lobster hepatopancreas tissue [TORT-2; National Research Council of Canada, Ottawa Canada]), calibration verifications (liquid standards), duplicates and blanks were included with each run of 40 samples.

Recoveries (mean ±1 standard error) were 100.7±0.7 % (n=168) and 100.4±0.7 % (n=158) for certified reference material and calibration verification, respectively. Absolute relative percent difference for all duplicates averaged 8.4±1.0 % (n=44).

Statistical Analysis

We conducted statistical analyses using R 2.15.1 (R Core Team, 2013). We applied a log-10 transformation to all THg concentrations in order to meet the assumptions of analysis of variance (ANOVA). After Hg analysis, we reconstructed whole-body THg concentrations in salamanders using the equation adapted from Ackerman and Eagles-Smith (2009), which factors the weighted proportion of THg in each tail segment into the remaining whole-body THg. We herein refer to reconstructed salamander whole-body THg as whole-body THg. For cascade frogs, we did not reconstruct whole-body THg concentrations because dry tissue mass from toes accounted for only a small proportion of dry mass in whole-body (mean 3.9%, range 2.3-5.0%).

Salamanders

We employed a hierarchical stepwise approach to the statistical analyses because we analyzed several different tail segments for four different salamander species. First, we constructed a series of analysis of covariance (ANCOVA) models to examine the relationship between THg concentrations in each tail segment and the THg concentrations in the associated whole-body homogenate. Each model contained species as a categorical factor, tail clip THg as a covariate, and the interaction between species and tail segment THg concentration to test for slope differences between tail clip THg and whole-body THg among species. Because we found significant interactions between species and tail segment THg concentrations (see results) we subsequently developed species-specific linear regression models of tail clip THg vs. whole-body THg concentrations for each species and each tail clip size. We then examined the relationships between different tail segment THg and whole-body THg concentration for each species individually.

Frogs

For Cascade frogs, we first examined the variation in THg concentrations among all of the different toes using a repeated measures ANOVA framework. To do so, we constructed a model that included toe THg concentration as the dependent variable nested within foot (front left, front right, back right, back left) as a categorical factor, and individual frog identification as a random effect to account for lack of independence among toes within individual frogs. Because there were significant differences between individual toe positions (see results), we then examined individual linear models correlating each toe positions's THg concentration with its associated whole-body THg concentration. When examining the linear regression equations for individual toe position THg concentration to whole-body THg concentration there were two frogs that consistently had disproportional high toe THg concentration to whole-body THg concentration compared to all other samples. Although we could not rule out those two organisms as laboratory or analytical errors, we developed linear regression equations of toe THg concentration to whole-body THg concentration that included and excluded those two organisms to show the influence those two frogs had on the relationship.

Results:

Salamanders

Total Hg concentrations (ng/g dry weight) ranged 34-fold between 19-649 ng/g in whole-body homogenates of salamanders. Tail clip THg concentrations ranged 107-fold (14-1503 ng/g), 40-fold (15-602 ng/g), and 107-fold (3-321 ng/g) for 0-4 cm, 0-2 cm, and 0-1 cm segments, respectively.

We found that THg concentrations in all three tail clip segments were positively correlated with their paired whole-body THg concentrations (0-1 cm tail clips: $F_{1,78}=354.2$, $P<0.0001$; 0-2 cm tail clips: $F_{1,206}=1613.4$, $P<0.0001$; 0-4 cm

tail clips: F1,127 =482.2, P<0.0001). However, the significant species by tail clip segment interactions indicate that the relationships between tail clip THg and whole-body THg concentrations differed among species. Specifically, THg in all tail clips had significant interactions with species (0-1 cm tail clips: $F_{3,878}$=4.7, P=0.004; 0-2 cm tail clips: $F_{3,206}$=7.7, P<0.0001; 0-4 cm tail clips $F_{2,127}$=3.2, P=0.05). These interactions between species and tail clip THg indicate that it may be more appropriate to use species-specific regression equations to predict whole-body THg concentrations from tail clips than a global equation that can be applied across taxa. Therefore, we subsequently analyzed the relationship between whole-body THg concentrations and tail clip THg concentrations separately for each species and each tail clip section (Figure 2.3; Table 2.1). For northwestern salamanders, R^2 values from the regression equations were 0.92, 0.82 and 0.68 for 0-1 cm, 0-2 cm and 0-4 cm tail clip segments, respectively. Long-toed salamander and rough-skinned newts both had higher R^2 values when using the 0-2 cm tail clip segment (0.87 and 0.86 respectively) in the regression model compared to the 0-1 cm tail clips R^2 values (0.82 and 0.62) or the 0-4 cm tail clip R^2 value of the rough-skinned newt (0.67). Conversely, the R^2 values for coastal giant salamanders were lower for the 0-1 cm tail clip (0.68), than either the 0-2 cm (R^2 = 0.92) or 0-4 cm tail clip R^2=0.97). Importantly, the slopes of these regressions varied both among and within species, ranging from 0.50 to 1.29 ng/g increases in whole-body THg for every 1 ng/g unit increase in tail clip THg. Only three regression models had slopes >1, Northwestern salamander 0-1 cm segment, and the rough-skinned newt 0-1 cm and 0-2 cm (1.22, 1.02, 1.29 respectively), these slopes indicate that Hg concentrations become increasingly higher in whole-body as tail Hg increases. The remaining eight regression models had slopes <1, indicating the ratio between whole-body THg and tail THg decreases with increasing tail Hg.

Frogs

Total Hg concentrations (ng/g dry weight) in Cascade frogs ranged 4-fold (80-345 ng/g) in whole-body homogenates and 25-fold (38-968 ng/g) in frog toes.

While controlling for toe-location and individual identification code, there were no differences in least-squares mean THg concentrations among feet (front left, front right, back right, back left; $F_{3,12.07}$=0.7955, P= 0.79). However, THg concentrations did differ among individual toes within each foot ($F_{15,422.68}$=4.9125, P<0.0001). Additionally, for each foot the toe THg concentrations exhibited bilateral symmetry between left and right sides in both the anterior and posterior (Figure 2.5). Specifically, the inner-most toe on each foot (A_L1, A_R1, P_L1, P_R1) had the highest least square mean THg concentrations compared to the other toes within the respective feet. Additionally, these inner toes that had the highest THg concentrations also had some of the lowest R^2 values for the relationships between THg concentrations in toes and whole-bodies (Figure 2.6, Table 2.2). Among specific toes, there was a wide range of correlation coefficients between THg concentration in toes and THg concentration in whole-bodies. Generally, toes towards the outside of a foot (regardless of the side of body clipped) had higher R^2 values then inside toes, and posterior toes had higher R^2 values than anterior toes. In fact, regardless of left or right, posterior toes in locations 3, 4, and 5 had consistently higher correlation coefficients in comparison to all other toes. These same three posterior toe locations also have slopes that are closer to 1, ranging from (0.65 to 1.05) compared to all other toes (Table 2), which indicates that the ratio between whole-body THg and tail THg concentrations increase at similar rates.

Discussion:

We found strong correlations between tail clip THg and whole-body THg in salamanders, and between toe clip THg and whole-body THg in frogs. However, we also found substantial variation in the tissue versus whole-body relationships among species of salamander and newt, suggesting that species-specific regressions may be needed to predict whole-body Hg concentrations from tail clips for individual taxa. Additionally, the size of the tail clip and the specific toe used as a nonlethal indicator can have a large influence on estimates of whole-body Hg. These results indicate that

nonlethal indicator tissues can be a robust approach to evaluating amphibian Hg exposure. However, an *a priori* understanding of the relationship between nonlethal tissues and whole-body Hg may be necessary for amphibian ecotoxicological studies, and care and consistency must be applied in selecting indicator tissues.

Our results suggest that when possible, tissue to whole-body relationships should be made on an individual species basis. In the 0-2 cm tail clip segments, slopes varied among species from above 1 for rough-skinned newts (1.29), and below 1 for northwestern salamanders (0.91), long-toed salamanders (0.50), and coastal, giant salamanders (0.94). This indicates that the ratio between whole-body THg and tail THg increased in rough-skinned newts, are very similar in northwestern and coast giant salamanders, and the ratio decreases in long-toed salamanders. If species specific regression equations where not developed, the mean 0-2cm THg (111.6 ng/g) of all species would predict whole THg concentrations to be (139.0), using equations in Table 1. When species specific regression equations of northwestern salamander, pacific giant salamander and rough skinned newt using the same 0-2cm THg (111.6 ng/g) predicted whole THg concentrations are (133.2 ng/g, 157.4 ng/g, 129.2 ng/g, respectively. If species specific regression equation were not used to predicted whole-body THg concentrations, concentrations would be off by 4.1-13.3%, further indicating the importance of species specific regression equations. Although we only analyzed the toe to whole-body relationship for a single frog species, comparison of data from the American toad (*Bufo americanus*) suggests that species-specific determinations may also be important for frogs when using toe clips to estimate whole-body Hg concentrations. Specifically, the slope of the relationship between toe-clip THg and whole-body THg in American toad (1.245; Todd et al. 2002) was 17.8% higher than the slope we measured for toe P_R4 THg and whole-body THg, our highest R2 values in cascade frogs. However, Todd et al. randomly selected and then combined one anterior and one posterior toe clip for THg analysis, potentially confounding our comparison.

The mechanisms for species differences in the relationship between tail clip THg and whole-body THg concentrations are unclear, but may be due to the variation in physiological routing of Hg within a species. Bergeron et al. (2010), also found different slopes for the correlation between body and tail THg concentration in two salamander species in the South River (VA, USA). Combined, this indicates that some species may have different physiological or developmental processes that change where THg is apportioned in the body, particularly in tails. Species have different growth rates and functions for tails, which then could alter the relative amount of skin vs protein and boney structure (Lannoo, 2005). Because bone, muscle, and whole-bodies often have different THg concentrations (Goldstein et al, 1996), these structural differences may be the reason why certain tissues segments performed better than others to predict whole-body THg concentration.

We found substantial within-species differences in the predictive strength of different tail clip sizes and toes. In regards to salamanders, differences among species are likely attributed to the change of bone structure as you move toward the terminus of the tail or other physiological processes, as stated above. We found no difference when comparing THg concentrations between the left and right feet of frogs, which is not surprising as there is no biological reason that mercury would partition differently on the right of left side of the body. More unexpectedly, we found large among-toe differences in the relationship between frog toe THg whole-body THg concentrations. Least-squared mean frog toe THg ranged from 98.9-138.2 ng/g with a standard deviation of ±11.3, despite the fact that there was substantial bilateral symmetry in the toes. The bilateral symmetry of the least squared means from the inner to outer toes points out it is less important from what side of the organism you collect a tissue sample from but more important which toe is clipped. We found a consistent gradient from inner to outer toe in terms of THg concentrations and the strength of the relationship to whole-body THg concentrations (Figure 2.5). Specifically, our study shows that 3 toes in particular, posterior locations 2, 3, and 4, had consistently higher correlations to whole-body THg, in comparison to all other toes. Why certain toes

demonstrated to be better predictors of whole-body concentration is unclear; however, we hypothesize that the difference in toes THg concentration could be due to the developmental timing of the toe, as the developmental timing of toes has been well described and matches the differences of THg concentration we found between toes (Alberch and Gale, 1985). Although the literature is scant, current literature has lacked reporting which exact toe was used, by not consistently comparing between toe, there may be an unintentional increased error associated with the toe clip THg to whole-body THg relationship.

In salamanders we found that THg in the 0-2 cm clip was the most consistently correlated with THg in the whole-body. Therefore, at least in the species we examined, the 0-2 cm segment of the tail is likely the best option for non-lethally monitoring Hg concentrations. Although we found some evidence that a 0-1 cm tail clip could be used, it should be used with caution as the quality of the relationship was inconsistent across species. However, with robust data sets even smaller tissue samples might be able to be utilized, thus further reducing stress to the sampled individuals. For cascade frogs, we suggest using posterior toe clips located in positions 3, 4, or 5 since these provided the most robust relationship to whole-body THg concentrations. Importantly, we only examined lake and stream-dwelling frogs and salamanders and some caution should be used when relating these regression equations to terrestrial adults or tadpoles, as the understanding of how mercury partitions in tissue during these life stages has not been well described. Future research should continue to examine these relationships with additional species, particularly in frogs. This research increases our ability to understanding Hg risk to amphibians, allowing for field based studies on potential toxicological effects due to Hg exposure.

Acknowledgments:

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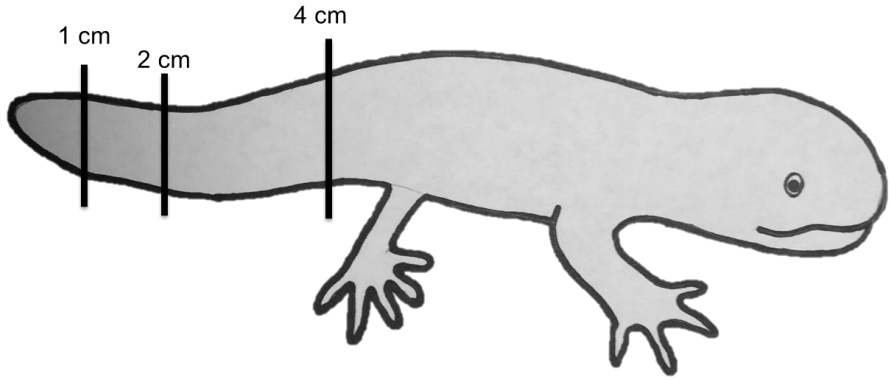

Figure 2.1. Diagram of salamander tail clip locations. Image is not to scale.

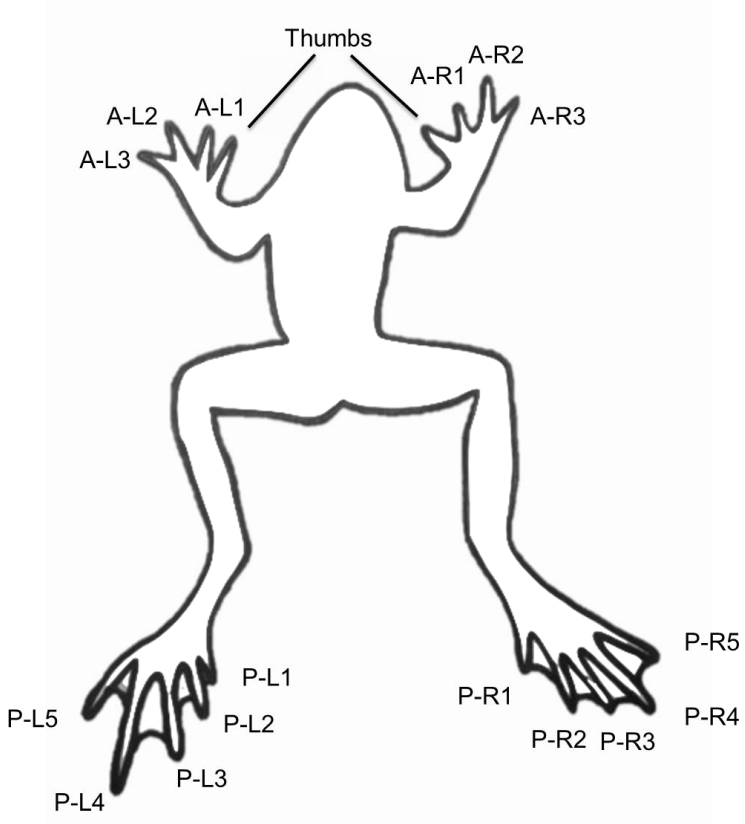

Figure 2.2. Diagram of frog toe locations A = anterior, P = posterior, L = left, R = right. Thumbs were not dissected. Image is not to scale.

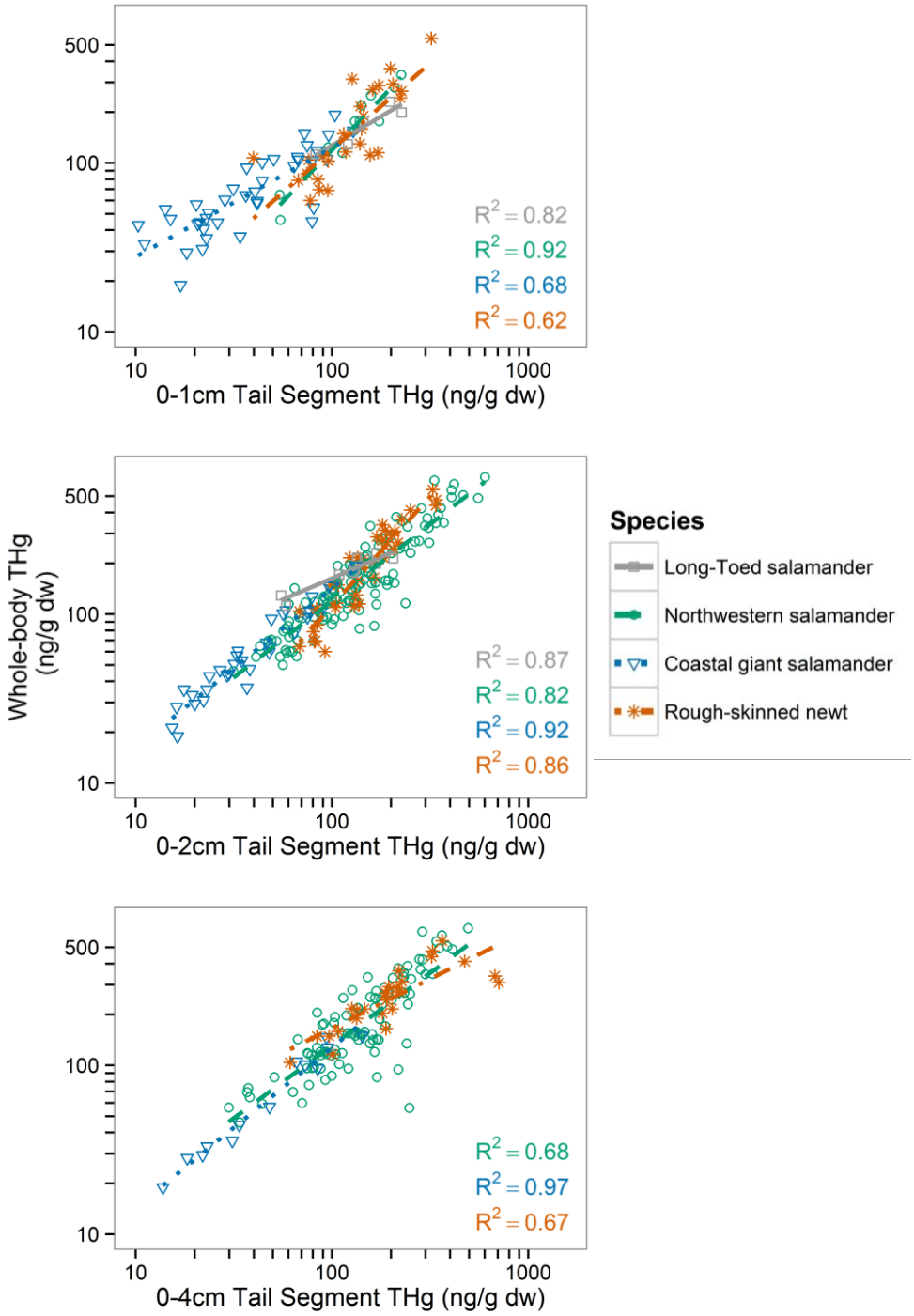

Figure 2.3. Linear regression models for total mercury (THg) concentrations (dry weight ng/g) in 0-1, 0-2, and 0-4 cm salamander tail clips versus whole-body THg concentration. Each color and symbol combination represents a different species.

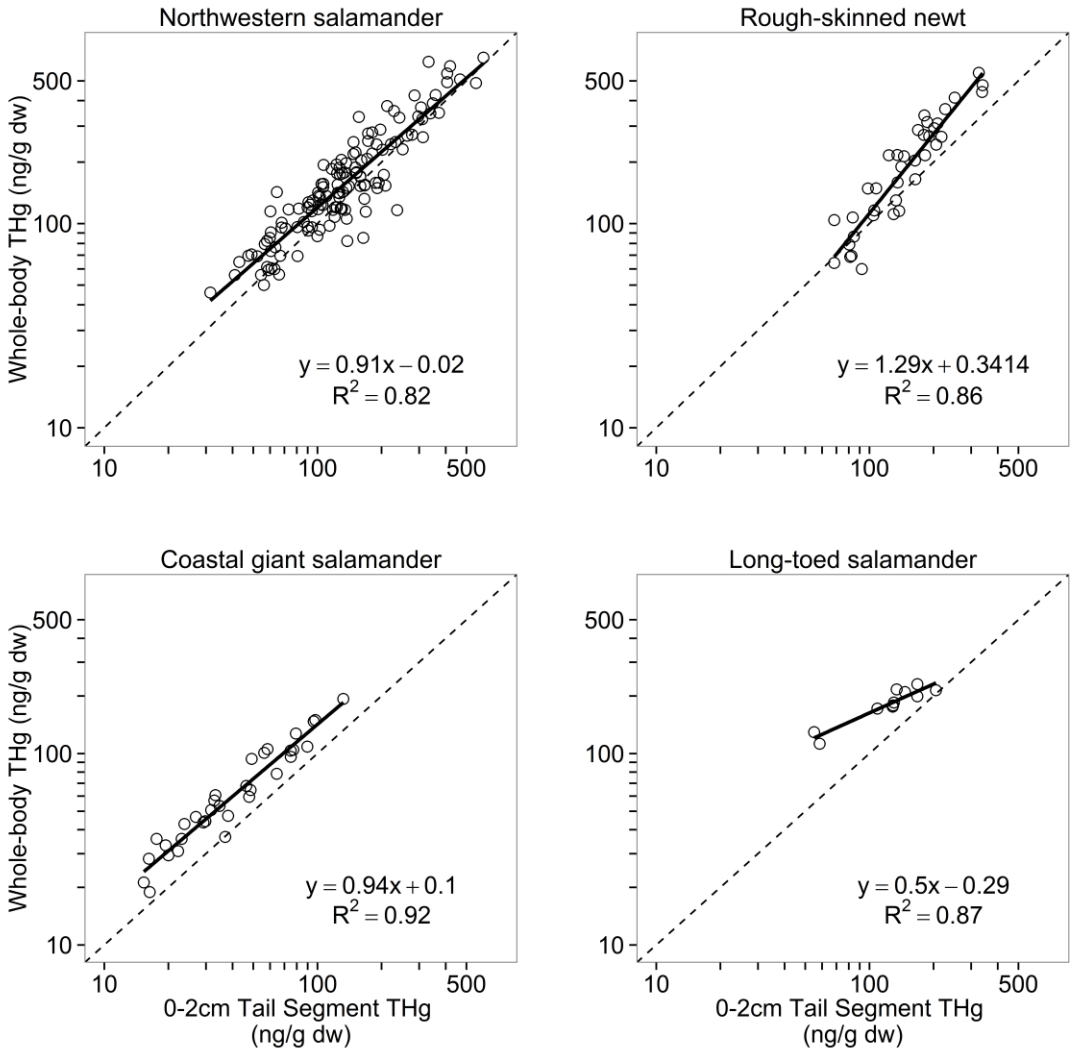

Figure 2.4. Linear regression models 0-2 cm tail segments total mercury (THg) concentration (dry weight ng/g) and whole-body THg concentrations in four species of salamanders.

Table 2.1. Regression parameters and associated correlation coefficients defining the relationship between salamander whole-body THg concentrations and tail segment THg concentrations. Equations for the relationship are: log(salamander whole-body THg concentration) = log(tail clip segment THg concentration)*slope + intercept.

Species	Segment	Slope	Intercept	R^2	P-value
All species combined	0-1 cm	0.70	-0.18	0.79	<0.0001
	0-2 cm	0.90	-0.01	0.87	<0.0001
	0-4 cm	0.86	-0.02	0.78	<0.0001
Long-toed salamander	0-1 cm	0.69	-0.21	0.82	0.0225
	0-2 cm	0.50	-0.29	0.87	<0.0001
Northwestern salamander	0-1 cm	1.22	0.30	0.92	<0.0001
	0-2 cm	0.91	-0.02	0.82	<0.0001
	0-4 cm	0.86	-0.02	0.68	<0.0001
Coastal giant salamander	0-1 cm	0.61	-0.32	0.68	<0.0001
	0-2 cm	0.94	0.10	0.92	<0.0001
	0-4 cm	0.95	0.05	0.97	<0.0001
Rough-skinned newt	0-1 cm	1.02	0.01	0.62	<0.0001
	0-2 cm	1.29	0.34	0.86	<0.0001
	0-4 cm	0.58	-0.20	0.67	<0.0001

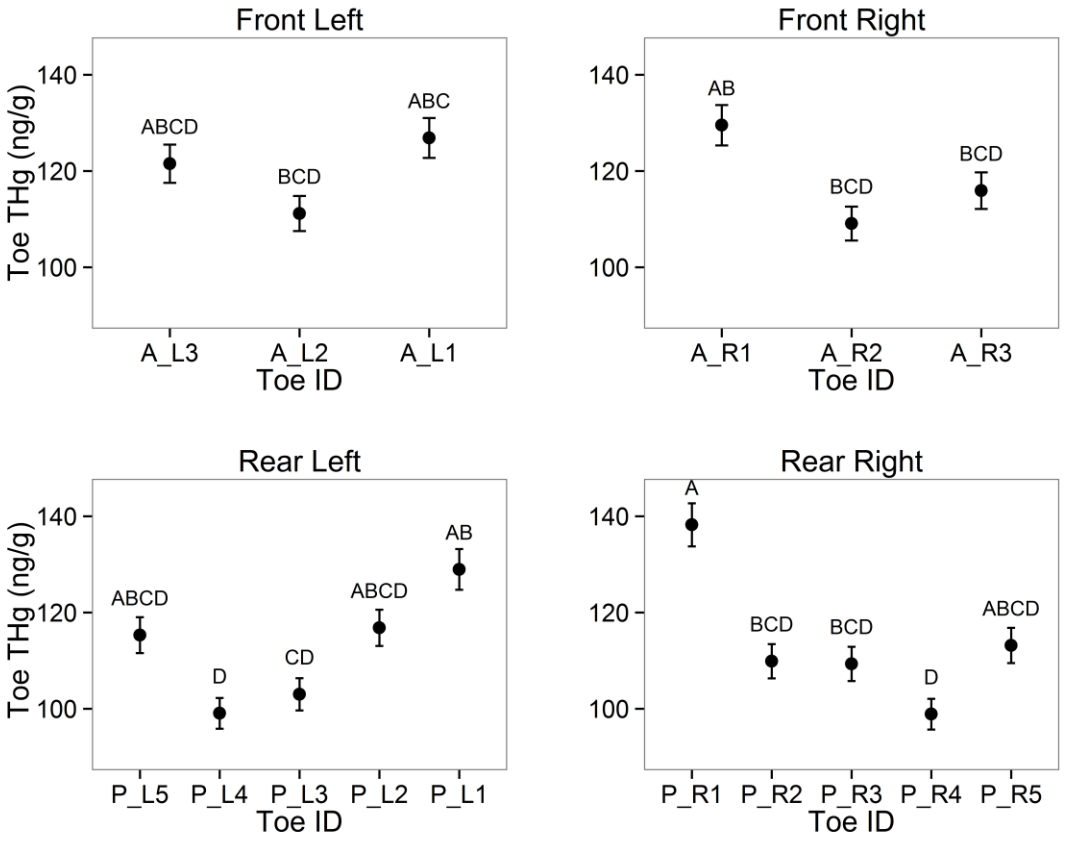

Figure 2.5. Back transformed least squared means of dry weight total mercury (THg) concentration (dry weight ng/g) of cascade frog toes, n=16. Error bars calculated by the Delta method, letters above the error bars indicate pairwise differences.

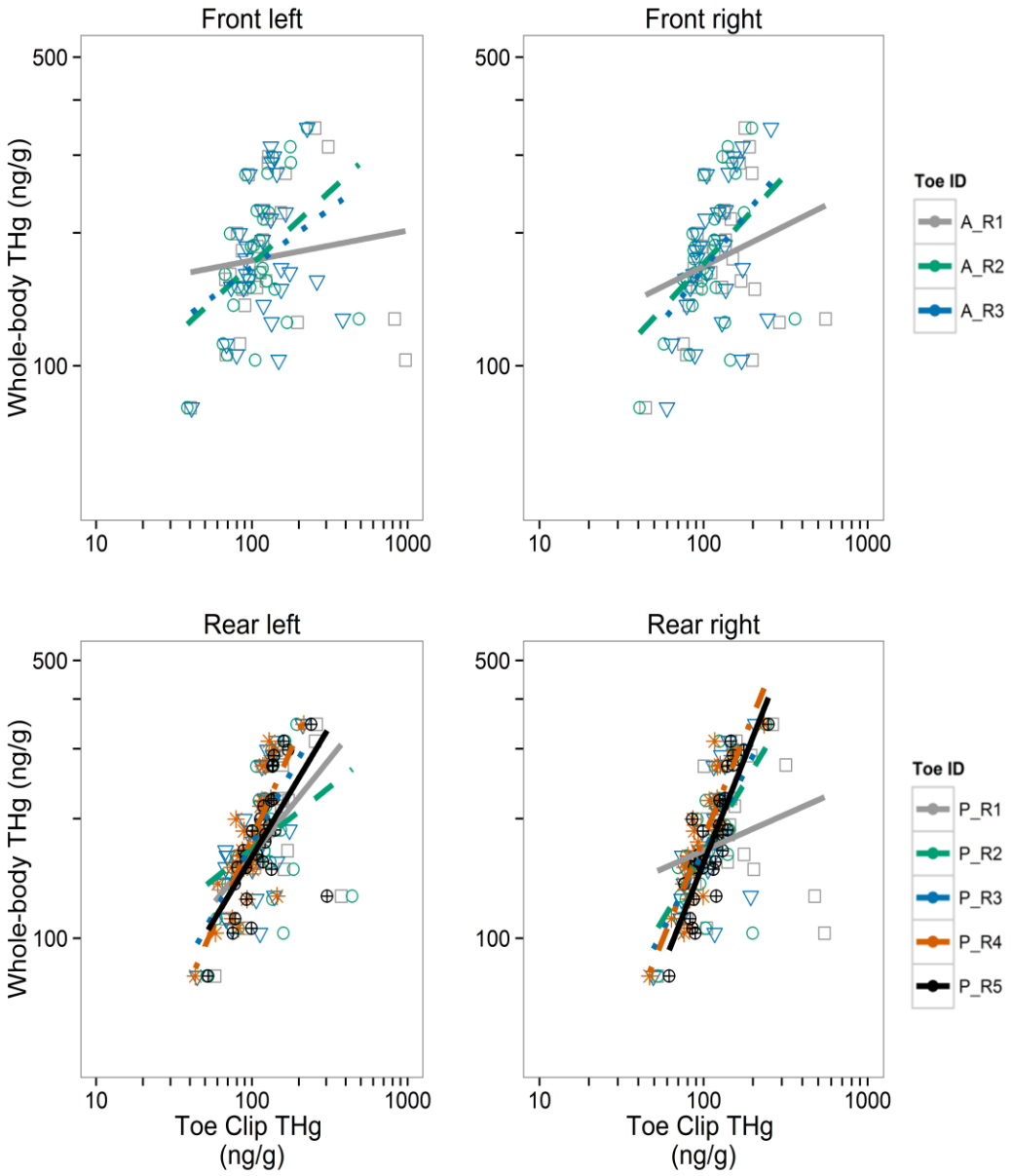

Figure 2.6. Linear regression models of toe total mercury (THg) concentrations (ng/g dry weight) and whole-bodies THg concentrations for cascade frogs. Colors and symbols represent different toes.

Table 2.2. Regression equations for each toe of cascade frog, defined by the relationship between whole-body THg concentrations and toe THg concentrations, and associated correlation coefficients. Equations for the relationship are: log(frog whole-body THg concentration) = log(Toe THg concentration)*slope + intercept. Toes with a * following indicate equations after two outlier frogs were removed from the data set.

Toe	Slope	Intercept	R^2	P-value	Toe	Slope	Intercept	R^2	P-value
A_L1	0.07	-0.69	-0.02	0.520	A_L1*	0.59	-0.17	0.47	<0.0001
A_L2	0.33	-0.44	0.14	0.027	A_L2*	0.67	-0.08	0.48	<0.0001
A_L3	0.27	-0.51	0.07	0.097	A_L3*	0.50	-0.27	0.27	0.0038
A_R1	0.18	-0.59	0.03	0.184	A_R1*	0.47	-0.31	0.26	0.0031
A_R2	0.40	-0.37	0.17	0.013	A_R2*	0.82	0.07	0.60	<0.0001
A_R3	0.48	-0.30	0.20	0.010	A_R3*	0.78	0.01	0.53	<0.0001
P_L1	0.49	-0.30	0.32	0.001	P_L1*	0.77	-0.03	0.65	<0.0001
P_L2	0.31	-0.46	0.10	0.054	P_L2*	0.76	-0.01	0.48	<0.0001
P_L3	0.72	-0.05	0.43	<0.0001	P_L3*	0.79	0.05	0.58	<0.0001
P_L4	0.90	0.15	0.62	<0.0001	P_L4*	0.90	0.15	0.62	<0.0001
P_L5	0.66	-0.14	0.40	<0.001	P_L5*	0.98	0.24	0.73	<0.0001
P_R1	0.18	-0.60	0.04	0.158	P_R1*	0.66	-0.15	0.52	<0.0001
P_R2	0.69	-0.09	0.39	<0.001	P_R2*	0.90	0.13	0.65	<0.0001
P_R3	0.80	0.02	0.49	<0.0001	P_R3*	1.01	0.25	0.79	<0.0001
P_R4	1.02	0.15	0.76	<0.0001	P_R4*	0.98	0.24	0.73	<0.0001
P_R5	1.05	0.24	0.72	<0.0001	P_R5*	1.01	0.21	0.75	<0.0001

CHAPTER 3 – The effects of non-native trout on food web structure and mercury bioaccumulation in remote subalpine lakes

Abstract:

Mercury (Hg) contamination is a global conservation threat to freshwater ecosystems, commonly detected in even the most pristine remote lakes of western North America. Mercury contamination in lakes can negatively affect the health of sensitive ecological communities already subjected to multiple stressors such as climate change and non-native fish introductions. Multiple factors determine the pathway of Hg methylation and bioaccumulation in lake systems, including atmospheric deposition, wetting-drying cycles, primary productivity, and food web structure. The latter is particularly important in systems that have been affected by introduced species, due to induced changes in energy flow and trophic linkages. In order to examine the influence of introduced fish on the bioaccumulation of Hg in food webs, we sampled organisms for Hg and stable isotopes of $\delta^{13}C$ and $\delta^{15}N$ in 18 subalpine lakes in Olympic National Park (Washington, USA). Lab results revealed that Hg concentrations in fish and salamanders varied widely across lakes. Utilizing stable isotopes we derived food web components of percent pelagic reliance and trophic position for salamanders. We found that trophic position was slightly lower and Hg concentrations were slightly higher in salamanders from fish containing lakes compared to fishless lake, yet no food web indices nor Hg concentration were significantly different between lake types. We found higher rates of Hg biomagnification in the food webs of lakes containing stocked fish versus fishless lakes. Historically stocked fish are accumulating higher levels Hg compared to their native counterpart the salamander.

Introduction:

Aquatic ecosystems worldwide are stressed by multiple anthropogenic disturbances, such as habitat loss, invasive species, pollution, and climate change (Moyle and Leidy 1992, Vitousek et al. 1997, Meyer et al. 1999). Non-native introduced fish have been particularly wide spread, resulting in dramatic negative

effects on native communities, such as altering zooplankton community composition, shifting forging strategies and complete extirpation of amphibians (Bradford et al. 1998, Tyler et al. 1998b, Knapp and Matthews 2000). In some cases, stressors can interact with each other to further exacerbate risk to certain organisms. For example, non-native species invasions have been shown to shift trophic dynamics in aquatic ecosystems, resulting in altered mercury exposure and bioaccumulation through the fish community (Swanson et al. 2003, Eagles-Smith et al. 2008). However, it is unclear what the impact of fish introductions to historically fishless lakes might have on contaminant cycling.

Mercury (Hg) is a contaminant of serious concern to aquatic ecosystems, even in the most pristine locations (Fitzgerald et al. 1998). This concern has grown due to a threefold increase in global atmospheric Hg levels since the industrial revolution (Swain et al. 1992). Whereas some contaminants are transferred directly from the surrounding water to an organism, Hg is predominantly accumulated through food webs by trophic biomagnification (Mason et al. 1995). Organisms with high trophic status and higher contaminant concentrations can increase trophic magnification slopes (TMS) (Borag et al. 2011). Additionally, mercury concentrations have been documented to increase in lakes with longer food chains, due to the presence of forage fish (Cabana et al. 1994). Accordingly, fish introductions in previously fishless lakes would likely increase the trophic length of the food web and thus increase TMS.

Contaminant exposure and predation pressures from fish introductions may interact, exacerbating negative effects to sensitive native amphibians. Mortality of amphibian larvae exposed to both sublethal pesticide concentrations and predation pressure was far greater than the summed mortality associated with either stressor alone, suggesting that contaminant exposure can either facilitate susceptibility to predation (Sih and Kerby 2004), or that stress of potential predation can enhance the sensitivity to pesticides. Similarly, both pesticide and introduced fish influenced the probability of yellow-legged frog (*Rana muscosa*) occupancy in the Sierra Nevada Mountains of California (Davidson and Knapp 2007). Long-toed salamanders

(*Ambystoma macrodactlym*) from lakes with fish had smaller body size and a reduced likelihood that they would metamorphose to adults in comparison to long-toed salamanders from fishless lakes (Kenison, 2014). Physiological changes in salamanders, such as reduced size, likely are a consequence of reduced activity and feeding to avoid predator attacks (Tyler et al.1998a, Yurewicz 2004). A fish-induced shift in salamander foraging ecology could correspond with changes in bioaccumulation and biomagnification of contaminants such as mercury (Hg). Amphibians in particular are of concern because of their complex life history strategies, aquatic dependence, sensitivity to environmental change, and unprecedented global decline (Stuart et al. 2004). Additionally, recent work has shown that they are extremely sensitive to toxic effects of Hg exposure (Bergeron et al. 2011). Amphibian declines have been documented in even the most protected ecosystems (Adams et al. 2014). At the same time historically stocked fish populations have persisted in many previously naturally fishless mountain lakes in the western US (Donald 1987, Boiano 1999).

In this study, we investigated food web indices and mercury bioaccumulation in 18 remote subalpine lakes in order to assess how foraging ecology and Hg bioaccumulation in salamanders differ among subalpine lakes with- and without historic fish introductions. Specifically, we evaluated the variation in Hg concentrations of a range of food web components among lakes. We also assessed whether indices of food web structure (trophic position and pelagic energy reliance) differed in salamanders from lakes where they coexist with fish in comparison to those from lakes that are fishless. Additionally, we examined how the trophic magnification slope (TMS) of Hg changed with food web composition, comparing slopes among (salamander-only lakes, salamander-and-fish lakes, and fish-only lakes). Lastly, we assess whether Hg in salamanders differed in lakes with- and without fish.

Methods:

Study area

We sampled the food webs of 18 remote subalpine lakes in Olympic National Park in 2012 (n=11) and 2013 (n=18), ten of which were sampled in both years. All lakes were sampled in the narrow ice-out window between mid-July to early-October. Lake elevation averaged 1,351 meters, ranging between 1,074 and 1,642 meters. Lakes were located across a wide geographic range of Olympic National Park subalpine area (Figure 3.1; Table 3.1).

Field Collections

We sampled a range of nodes from the food webs of each lake, including zooplankton, littoral macroinvertebrates, salamanders, and fish. We determined the presence or absence of fish using a combination of National Park Service databases, historical introduction records, and physical sampling. We validated record-based accounts at every lake and collected fish when present using experimental variable mesh gill nets, which were set from the shoreline towards the center of each lake. Gill net set times ranged from 8 to 24 hours. Upon retrieval, fish were immediately removed from the net and stored on snow or ice until they could be transported to the laboratory. Thirteen of the 18 lakes contained fish, and 12 of those lakes contained a single fish species, whereas a single lake contained two different fish species (Table 3.1). Across the 12 fish containing lakes the fish species sampled included, cutthroat trout (*Oncorhynchus clarkii*), rainbow trout (*Oncorhynchus mykiss*), and brook trout (*Salvelinus fontinalis*).

We searched for and attempted to sample salamanders at each lake by conducting an intensive parameter survey of each lake. Capture methods included dip nets, and hand sampling. The Northwestern salamander (*Ambystoma gracile*) was the dominant species encountered (n=6 lakes), but we also found long-toed salamanders (*Ambystoma macrodactylum*) in two lakes. Upon capture we measured wet weight to the nearest 0.1 g using a spring balance (Pesola®), total length (TL), and snout-to-

vent length (SVL) to the nearest mm. We excised a 2cm clip from the tip of each salamander's tail using a clean and sterilized stainless steel blade, and placed each clip in individually labeled polyethylene cryotubes. To help prevent subsequent infection, we applied benzalkonium chloride (Bactine ®) solution to the clipped tail of each salamander prior to release at the capture site. Upon collection, all samples were kept cool on snow or ice while in transport to the laboratory and then frozen at -20C until analysis.

Bulk zooplankton samples were qualitatively collected using a 0.5 meter wide 156μm mesh plankton net, towed behind an inflatable boat at a depth of 1 to 3 meters. Zooplankton and free floating insects were concentrated into a slurry with lake water in a Nalgene 250ml bottle and transported on snow until frozen at the laboratory at -20C. Benthic macroinvertebrates were sampled along lake perimeter using a D-frame kick net with 0.5mm mesh. Samples were field sorted to order, placed in 10ml polyethylene cryovials, and transported on snow to laboratory freezers. Water was collected at a depth of 1 meter near the deepest point in each lake using a 1.5 L Van Dorn sampler. Water samples were filtered in the field using a pre ashed 24mm Whatman glass microfiber filter (GF/F) attached to a clean 60ml syringe. Water was filtered into acid washed and ashed 40ml amber glass vials. One vial was preserved with concentrated HCl to stabilize dissolved organic carbon. Vials were then wrapped in aluminum foil to reduce potential photodegradation and packaged on snow until being placed in a refrigerator until water chemistry could be performed.

Laboratory analyses

In the laboratory, fish were identified to species and thawed to room temperature prior to measuring standard length (SL) to the nearest mm using a fish board. Wet mass was recorded to the nearest 0.01 g on a digital balance. From each fish, we dissected 0.5g-10g of skinless axial muscle using scalpels cleaned with nitric acid and deionized water. Muscle tissue was placed on a clean aluminum dish and

weighed on an analytical balance (0.0001 g), then placed in a drying oven at 50˚C for 48h or until they achieved a constant mass. Salamander tail clips were rinsed in deionized water, blotted dry then weighed and dried as described above for fish muscle tissue. Samples were removed from the drying oven, allowed to cool to room temperature in a desiccator. Once samples cooled we measured the dry mass of each sample to the nearest 0.0001 g on an analytical balance. Samples were then homogenized using stainless steel scissors and ceramic mortar and pestle, then stored in a desiccator until analysis.

Zooplankton samples were thawed to room temperature and size separated using nitex® mesh sieves. Size fractions included: >500um, 500-210um, 210-153um, and 153-63um. Each size category was then hand-cleaned of residual organic material in a glass petri dish, and a small subset was removed for later taxonomic identification. Zooplankton samples were then either dried directly into clean glass containers at 50˚C, or if low in biomass they were first filtered onto a pre-weighed 24mm GF/F filter and prior to drying in the same fashion as above. Benthic macroinvertebrates were thawed to room temperature, rinsed with deionized water to remove surface contaminants, and sorted and composited to the family level using a dissecting microscope (Merrit and Cummings 2008). Benthic macroinvertebrate samples were then weighed on an analytical balance (0.0001 g) and dried at 50˚C for 48h or until they achieved a constant mass. Both benthic macroinvertebrate and zooplankton samples were subsequently removed from the drying oven, allowed to cool to room temperature in a desiccator before dry mass was measured to the nearest 0.0001 g. All zooplankton samples other than samples dried on GF/F and all benthic macroinvertebrates were homogenized using a glass rod or a ceramic mortar and pestle then stored in a desiccator until analysis.

Total mercury (THg) concentrations were determined in all individual fish muscle tissue and all salamander tail clips. Total mercury concentration is used as a

surrogate for methylmercury concentration in these matrices because (>95%) of mercury in fish muscle and approximately 70% of THg in salamander tissue is in the methylmercury form (Bloom 1992, Townsend and Driscoll, 2013). Total mercury was determined using thermal combustion, amalgamation, and cold vapor atomic absorption spectrophotometry, on a tricell DMA-80 Direct Mercury Analyzer (Milestone Inc., Monroe, CT USA), following U.S. Environmental Protection Agency method 7473 (U.S. Environmental Protection Agency, 2000). Certified reference material (dogfish muscle tissue [DORM-4; National Research Council of Canada, Ottawa Canada] and lobster hepatopancreas tissue [TORT-2; National Research Council of Canada, Ottawa Canada]), calibration verification (liquid standards), duplicates and blanks were included with each run. Recoveries were 101.4±1.11% (n=52) and 100.1±1.12 % (n=55) for certified reference material and calibration verification, respectively. Absolute relative percent difference for all duplicates averaged 2.63%±0.71% (n=25).

Methylmercury (MeHg) concentrations were determined for zooplankton (n=55) and benthic macroinvertebrate samples (n=102) because the percent of THg that is comprised of MeHg in these taxa can vary widely (Becker and Bigham 1995), and THg measurements alone would be poor predictors of bioavailable Hg at the base of the food web. Prior to MeHg analysis, samples were digested in 5M trace metal grade nitric acid following Hammerschmidt and Fitzgerald (2005). Methylmercury was then determined on a MERX 04400 automated methylmercury system (Brooks Rand Instruments, Seattle, WA) following US EPA Method 1630 (U.S. Environmental Protection Agency 2001 and Taylor et al. 2011). Certified reference material recoveries were (85.3±1.2%, n=18) and absolute relative percent difference for all duplicates averaged 2.51%±1.04% (n=9).

Stable isotopes ratios of carbon and nitrogen were measured from samples collected at lakes in Olympic National Park. Subsamples of dried fish muscle tissue,

salamander tissue, zooplankton, and benthic macroinvertebrates were packed in tin capsules and analyzed using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) at the University of California, Davis Stable Isotope Facility. Nitrogen isotope samples were standardized against N_2 in air by the following equation: $\delta^{15}N$ (‰) = $[(R_{sample} / R_{standard}) -1] * 1000$, where R = $^{15}N/^{14}N$. Carbon isotope samples were standardized against Vienna PeeDee Belemnite in a similar fashion as above. Based on replicate analyses of standard reference materials, instruments standard deviation was 0.2‰ for ^{13}C and 0.3‰ for ^{15}N.

Water chemistry was conducted at the Institute for Water and Watersheds Collaboratory (IWW) at Oregon State University for dissolved organic carbon (DOC), sulfate, and specific UV absorbance at 245nm ($SUVA_{254}$). Dissolved organic carbon was measured using a Shimadzu TOC-VSCH Combustion Carbon Analyzer, sulfate on a Dionex ICS-1500 Ion Chromatograph, and $SUVA_{254}$ on a Shimadzu UV-1700 UV-VIS Spectrophotometer.

Statistical Analysis

We first normalized stable isotope data across lakes by calculating percent pelagic reliance and trophic position from $\delta^{13}C$ and $\delta^{15}N$ data, respectively. We derived percent pelagic reliance and trophic position for consumers in lake food webs using a two-end-member mixing model (Post 2002): trophic position = $\lambda + (\delta^{15}N_{consumer} - [\delta^{15}N_{benthic\ invert} * \alpha + \delta^{15}N_{zooplankton} * (1- \alpha)] / \Delta$. Where λ is the trophic position of the organism used to estimate $\delta^{15}N_{benthic\ invert\ or\ zooplankton}$ ($\lambda = 2$ for secondary consumers), Δ = enrichment factor in $\delta^{15}N$, per a trophic level, and α is the portion of carbon in the consumer ultimately derived from the base of food web ($\alpha = \delta^{13}C_{consumer} - \delta^{13}C_{benthic\ invert}) / (\delta^{13}C_{zooplankton} - \delta^{13}C_{benthic\ invert}$). We used an

enrichment factor of 3.4‰ $\delta^{15}N$ per trophic step and did not correct for $\delta^{13}C$ fraction as it was considered negligible (Post 2002). We used α as an indicator of the proportion of pelagic energy (percent pelagic reliance) in fish and salamander diets. We used non-predatory invertebrates such as Ephemeroptera and Tricoptera as isotopic baseline end-members to calculate the littoral input to a consumer's diet using site-averaged $\delta^{15}N$ and $\delta^{13}C$ values. Similarly for the pelagic input, zooplankton $\delta^{15}N$ and $\delta^{13}C$ values were averaged by site. In one lake (Moose Lake), we did not adequately characterize the benthic invertebrate baseline and subsequently removed Moose Lake from the food web analyses.

We conducted statistical analyses using R 2.15.1 (R Core Team, 2013). We used general linear models to test for factors influencing Hg concentrations and bioaccumulation through lake food webs, as well as to test whether lake type (salamander-only and salamanders with fish) influenced the foraging ecology and Hg bioaccumulation in historic top predator. Prior to analysis we log-10 transformed all Hg concentrations in order to meet the assumptions of general linear models. Residuals were assessed for normality during model fitting, and data were assessed for homogeneity of variance.

We first characterized variation in Hg concentrations among lakes using analysis of covariance (ANCOVA). We ran separate models for fish, salamanders, zooplankton, and benthic invertebrates. Each model included site and either standard length or snout to vent length as covariates (fish or salamanders) or (size fraction for zooplankton or order for benthic invertebrates) as categorical factors. We then used linear regression models to correlate lake-specific mean zooplankton and benthic invertebrate MeHg concentrations with salamander and fish THg concentrations to determine if Hg at the base of the food web correlated to top predator Hg concentrations.

We assessed whether indices of food web structure (trophic position and percent pelagic reliance) differed in salamanders from lakes where they coexisted with fish as compared to those from lakes without fish. To do so, we ran ANOVA for salamanders to assess whether trophic position or percent pelagic reliance differed in salamanders from lakes with and without fish. Because trophic position and snout to vent length were auto-correlated we removed size from the food web models. For the model examining trophic position as the dependent variable we included lake type, individual lake (nested within lake type), pelagic reliance and the interaction between lake type and pelagic reliance. We subsequently removed the non-significant interaction term after using a likelihood-ratio test. For the model examining pelagic reliance as the dependent variable we included lake type, lake name (nested within lake type), and trophic position.

We then examined how trophic structure in the different lake types influenced Hg biomagnification through the food web. To do so, we developed separate linear regression models for each lake, correlating THg (or MeHg concentrations for invertebrates) with trophic position through the food web. We developed additional regression models that excluded fish in the salamander and fish lakes in order to compare with the regression lines from lakes that contain only salamanders. We then tested if slopes (trophic magnification slopes, TMS), derived from lake specific linear regression models, differed by lake type (salamander-only, salamanders and fish, fish-only). We also used the slopes of each lake type regression equation to calculate trophic magnification factor (TMF) (10^ß).

Lastly, we ran an ANOVA model to assess whether Hg in salamanders differed in lakes with and without fish. For the model, we included lake type (salamander only and salamanders with fish), individual lake (nested within lake type), trophic position, percent pelagic reliance and all two-way interactions. We then systematically removed non-significant interactions using likelihood-ratio tests, resulting in a salamander Hg model that contained all the main-effects parameters above, and no interactions terms.

Results:

Mercury concentrations (ng/g dry weight) in Olympic National Park lakes ranged 50-fold (127-6380 ng/g), 31-fold (24-733 ng/g), 32-fold (7-226 ng/g), and 31-fold (6-173 ng/g), in fish muscle, salamander tail clips, zooplankton seston, and benthic invertebrates, respectively. Across all lakes, the mean mercury concentration was highest in fish (544 ng/g), followed by salamanders (144 ng/g), and MeHg concentrations were lowest and comparable in zooplankton (60 ng/g) and benthic invertebrates (57 ng/g). Lake specific mercury concentrations varied significantly among lakes in all organism types (fish: $F_{11,240}$ =24.0, P<0.0001; salamanders: $F_{7,148}$ =14.0, P<0.0001; zooplankton: $F_{16,36}$ =7.2, P<0.0001; and benthic invertebrates: $F_{16,70}$ =1.9, P=0.03; Figure 3.2). The two highest lake-specific mercury concentrations in fish were found in Lunch Lake (901 ± 29 ng/g) and Hoh Lake (701 ± 19 ng/g). The five highest lake-specific fish mercury concentrations happened to occur in lakes from which salamanders were not found. The highest lake-specific mercury concentration in salamanders was in Lake Sun-Up (184 ± 7 ng/g), which also contained introduced fish. However, Hg concentrations in Lacrosse Lake (160 ± 6 ng/g) and Ferry Lake (156 ± 8 ng/g), both fishless lakes, were lower and similar to one another. Interestingly, Lacrosse and Ferry Lakes were the only two lakes that long-toed salamanders were found. Zooplankton Hg concentrations were highest in Lake Sun Up (112 ± 9 ng/g) and Deer Lake (111± 10 ng/g) and were lowest in Round Lake (12 ± 2 ng/g). Lake-specific benthic invertebrate Hg concentrations were highest in Long Lake (119 ± 16 ng/g), followed by Deer Lake (97.4 ± 13 ng/g), and the lowest concentrations were found in Crazy Lake (36 ± 3 ng/g). We found no relationship between lake-specific mean Hg concentrations in salamanders in

comparison to Hg in zooplankton ($F_{1,6}$ =0.04, P=0.85) or benthic invertebrates ($F_{1,6}$ = 3.76, P=0.10). We also found no relationship between lake-specific mean Hg concentration in fish in comparison to zooplankton ($F_{1,11}$ =0.97, P=0.35) or benthic invertebrates ($F_{1,11}$ = 3.15, P=0.10).

The mean trophic position of salamanders was lower in lakes that contained fish in comparison to salamander-only lakes (Figure 3). Salamander trophic position differed among lakes ($F_{6,153}$ = 22.08, P<0.0001), but was not influenced by pelagic energy reliance ($F_{1,153}$ = 1.98, P=0.16). Salamander trophic position was slightly lower in lakes that contained fish, but not significantly ($F_{1,153}$ = 3.25, P=0.07; Figure 3.4). Pelagic energy reliance of salamanders differed among lakes ($F_{6,153}$ = 47.8, P<0.001), but was not significantly related to trophic position ($F_{1,153}$ = 1.98, P=0.16; Figure 3.4) nor lake type ($F_{1,153}$ = 2.54, P=0.11).

We assessed the biomagnification rate of Hg through the food webs of the three types of lakes (salamander-only, fish and salamanders, fish-only). Trophic magnification slopes (TMS) ranged from 0.28±0.03 in salamander lakes, 0.57±0.02 in fish-only lakes, and 0.55±0.03 salamander and fish lakes (Figure 3.5). When we removed fish Hg from the salamander and fish lakes model, trophic magnification slopes lowered slightly, 0.44±0.06. Trophic magnification slopes differed by lake type ($F_{2,16}$ = 7.92, P=0.001; Figure 3.6. Using a bonferroni adjusted pairwise comparison test, we found that TMS were lower in salamander-only lakes compared to salamander and fish lakes (P=0.03) and fish-only lakes (P=0.002). Additionally, salamander and fish lakes did not differ in TMS from fish-only lakes (P=1.0) nor did the TMS of salamander-only lakes differ from TMS values salamander and fish lakes when fish were removed from the regression model (P=1.0).

Lastly, we assessed the food-web parameters between salamander Hg concentrations in the different lake types. Mercury concentrations in salamanders differed among lakes ($F_{6,147}$ = 7.88, P<0.0001), but not lake type ($F_{1,147}$ =2.33,

P=0.13), or by pelagic reliance ($F_{1,147}$ = 2.78, P=0.10), nor trophic position ($F_{1,147}$ = 2.84, P=0.09; Figure 3.7).

Discussion:

Mercury

Mercury concentrations varied widely in all taxa among lakes in our study area. Fish consistently had the highest mercury concentrations compared to salamanders. In fact, only four fish had THg concentrations lower than the highest THg concentration found in salamanders. Two of the 13 fish lakes (Hoh and Long) had fish THg concentrations in the muscle tissue that meet or exceed EPA consumption advisory of 300 ng/g wet weight (USAEPA, 1997). Two fish in Hoh lake exceeded the Great Lakes Advisory Group for no consumption (950 ng/g wet weight) (GLAG, 2007). Fish exceeding EPA consumption advisory represent 6% of the fish caught in Hoh Lake and 4% of the fish caught in Long Lake. These results concur with finding from other studies of Hg in subalpine lakes in Western National Parks and recognize additional lakes with high fish mercury concentrations in Olympic National Park (Eagles-Smith et al 2014, Lander et al. 2008).

Whole food-web mercury magnification slopes averaged (0.48 ± 0.15), but were consistently lower in salamander-only lakes when compared to salamander and fish lakes and fish-only lakes. This indicates that mercury is moving through the food web at a faster rate in lakes that contain fish verse salamander-only lakes. This reduction in TMS in fishless lakes contrasts with the Lavoie et al. (2013) finding that fish lakes, fish with other species, and fishless lakes did not differ in TMS. The high TMS found in the fish containing lakes could be related to slow growth rates of old fish. Fish in high elevation oligotrophic lakes, similar to our study lakes, exhibited slow growth and long life spans (Reimer, 1958). Additionally, elevated Hg concentrations have been found in older slow-growing fish (Bache et al. 1971). These combined attributes could be related to the higher TMS we found in the fish lakes.

When fish are removed from the TMS calculation in lakes with salamanders and fish, those TMS compared to TMS of salamander-only lakes are still higher (0.40) then in true fishless lakes (0.30). Although these slope differences were not significantly different, this finding indicates that top predators, such as salamanders, living in lakes with introduced fish could be obtaining mercury at higher rates compared to fishless lakes.

Although we did not find significant differences in the food web structure of salamanders in lakes containing salamanders only versus lakes with both salamanders and fish, we still found some limited evidence that fish presence was affecting salamanders. For example, there was a slight reduction in trophic position in salamanders from fish-containing lakes in comparison to salamander-only lakes. This slight reduction in trophic position could be related to salamanders being out competed by fish for higher trophic level prey items. We found that the salamanders and fish sampled in our study lakes obtained a majority of their energy resource from benthic prey sources (>90%). Thus, the slight decline in trophic position in salamanders when co-existing with fish would be likely attributed to a resource shift to lower trophic position prey items within the benthos. If salamanders were feeding on lower trophic level prey items in fish lakes, we were unable to relate those differences to changes in Hg concentration. After controlling for food web metrics, mercury concentrations in salamanders were slightly higher in fish containing lakes then fishless lakes. Higher salamander Hg concentrations in fish containing lakes did correspond with our original hypothesis, that the consumption of lower quality food resources could be related to higher Hg exposure, as Hg is linked to energy flow in food webs. Salamanders proved exceedingly challenging to find and capture in fish containing lakes compared to fishless lakes, we found only 3 lakes with fish and salamanders over the 2 collection years. Our small sample size of lakes with fish and salamanders in coexistence likely reduced our ability to detect differences in salamander food web indices and Hg concentrations between lake types. It is likely that our inability to detect salamanders in fish containing lakes is attributable to the presence of fish. Larson and Hoffman (2002) found significantly lower abundances of

Northwestern salamander larvae in lakes with fish compared to fishless lakes. Additionally, we never observed or captured northwestern salamanders in lakes that contained brook trout, nor did we find long-toed salamanders in any of the fish containing lakes. These observations are consistent with current understanding that long-toed salamanders are very sensitive to fish invasions (Funk and Dunlap, 1999). Fish populations from historical stocking events have continued to cause stress to native aquatic communities and will likely persist well into the future.

Similar to introduced fish, high levels of mercury will continue to be an ongoing issue in remote aquatic ecosystems. Although we did not find significant evidence of foraging shifts or Hg concentration in amphibians due to the presence of fish, we found some lines of evidence that salamanders in fish containing lakes are under additional stress. In the wake of growing stressors such as climate change, Hg may further be bio-available to food webs by increased methylation rates (Bodaly et al. 1993). Our research provides evidence that fish in historically fishless lakes can increase Hg concentrations to much higher levels than salamanders, the native top predator.

Table 3.1. Physical and water chemistry characteristics of sampled lakes from Olympic National Park. All water chemistry analysis was conducted in sampling year 2013. BRTR = brook trout, RATR= rainbow trout, CUTR = cutthroat trout, NOSA = northwestern salamander, LTSA= long-toed salamander.

Olympic National Park Sampled Lakes

Lake Name	Sample Year	UTM X	UTM Y	Elevation (m)	Salamander Species	Fish Species	Depth (m)	Lake Area (ha)	Lake Perimeter (m)	DOC (C mg/L)	UVA-254 (cm-1)	SUVA (L/mg-M)	Sulfate_ppm
Connie	2012 / 2013	455347	5263487	1300	NOSA		9.1	2.11	843	1.34	0.00	0.00	0.97
Ferry	2012 /2013	455307	5300225	1434	LTSA		10.9	1.31	464	0.50	0.04	7.95	1.64
LaCrosse	2012 / 2013	472945	5279102	1447	LTSA		8.0	2.84	851	0.61	0.00	0.16	2.10
Crazy	2012 / 2013	478934	5274074	1457	NOSA		7.7	0.96	439	5.72	0.01	0.09	2.05
Clear	2013	441698	5307650	1290	NOSA			2.30	645	1.08	0.02	1.85	1.95
Sun Down	2013	461588	5264808	1165	NOSA	RATR		1.55	497	4.73	0.01	0.30	3.69
Upper Lenna	2012 / 2013	484371	5275546	1386	NOSA	RATR		9.17	1655	0.78	0.02	1.93	1.73
Sun Up	2012 / 2013	463406	5264336	1224	NOSA	CUTR	6.0	0.98	406	6.92	0.02	0.27	1.24
Hoh	2012 / 2013	441219	5305358	1383		BRTR	13.0	7.44	1059	0.44	0.00	0.46	3.25
Gladys	2012 / 2013	473243	5302663	1642		BRTR	4.2	0.49	316	0.52	0.01	1.15	6.94
Hagen	2012 / 2013	479526	5273915	1422		CUTR		1.46	569	3.37	0.01	0.24	2.42
No Name	2013	443072	5307142	1273		BRTR/ RATR		2.66	669	3.79	0.01	0.26	7.86
Deer	2013	438445	5308470	1074		BRTR		3.41	709	1.55	0.02	1.55	9.29
Grand	2013	474199	5304184	1446		BRTR		5.15	1126	5.18	0.01	0.14	3.65
Long Lake	2013	442217	5307991	1169		BRTR		5.73	1147	0.52	0.01	1.92	5.17
Lunch	2013	441485	5307195	1365		BRTR	21.0	1.14	438	0.27	0.00	0.37	3.94
Moose	2013	473792	5303298	1540		BRTR		3.29	1049	1.68	0.00	0.24	3.82
Round	2013	441035	5307241	1303		BRTR		1.10	439	0.47	0.01	1.71	9.56

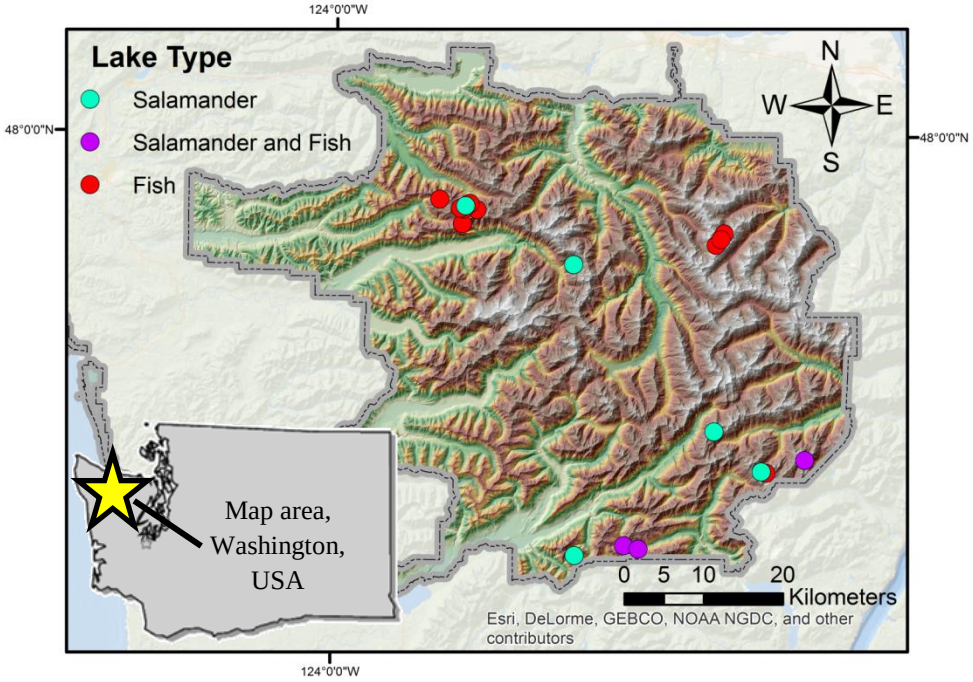

Figure 3.1. Study area map of Olympic National Park, Washington.

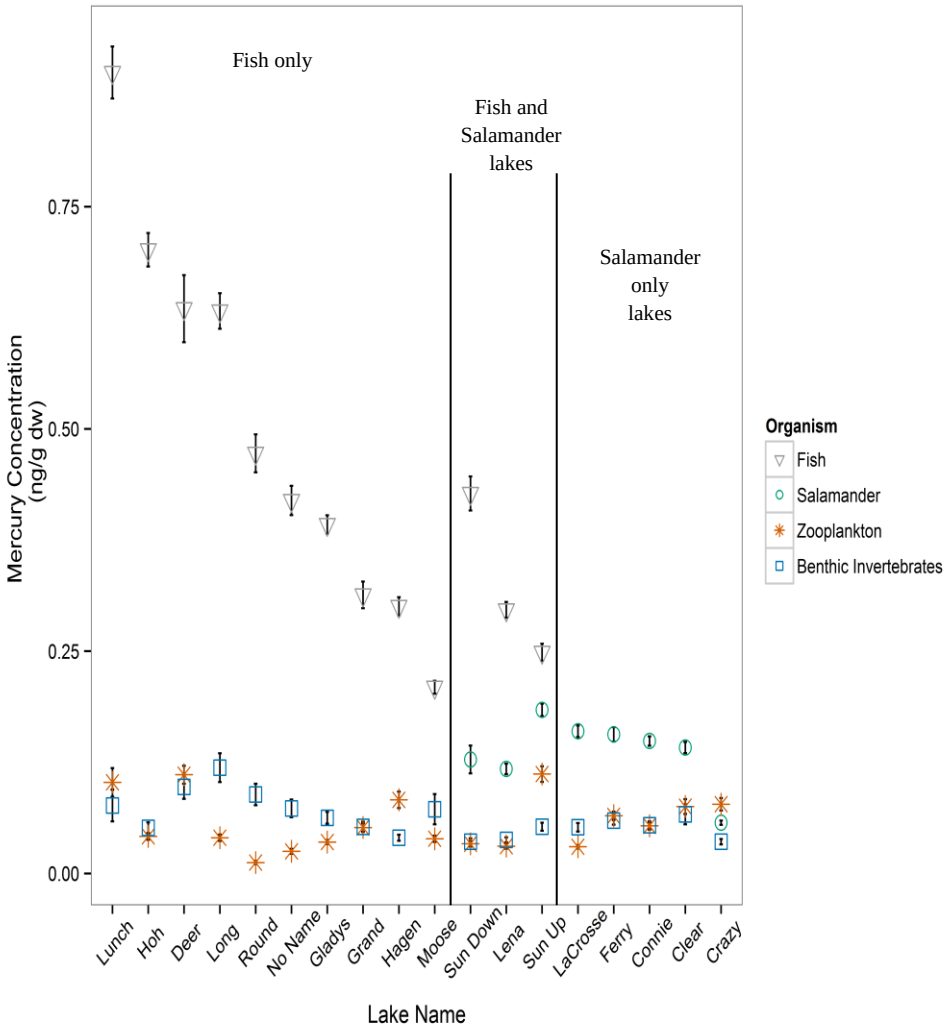

Figure 3.2. Back-transformed least square mean mercury concentrations (ng/g) dry weight (dw) of organisms composing the food web in lakes from Olympic National Park, collected in 2012 and 2013 after adjusting for standard length in fish, snout to vent length in salamanders, size in zooplankton, and order of benthic invertebrate. Error bars indicate standard error, calculated using the delta method.

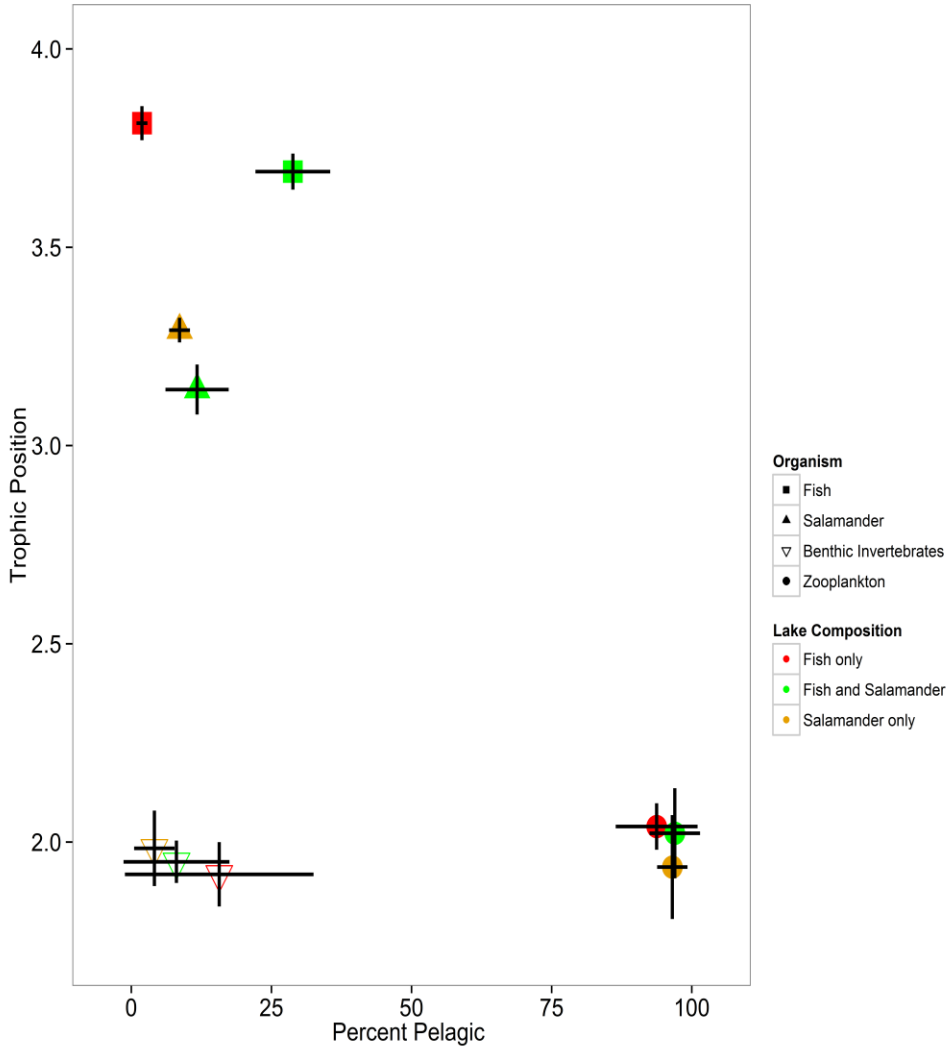

Figure 3.3. Trophic position and percent pelagic energy reliance of organisms from lake food-webs differing in top predator composition. Points represent mean values of organism type, and error bars represent 95% confident intervals. Benthic invertebrates not selected for the baseline food-web have been omitted.

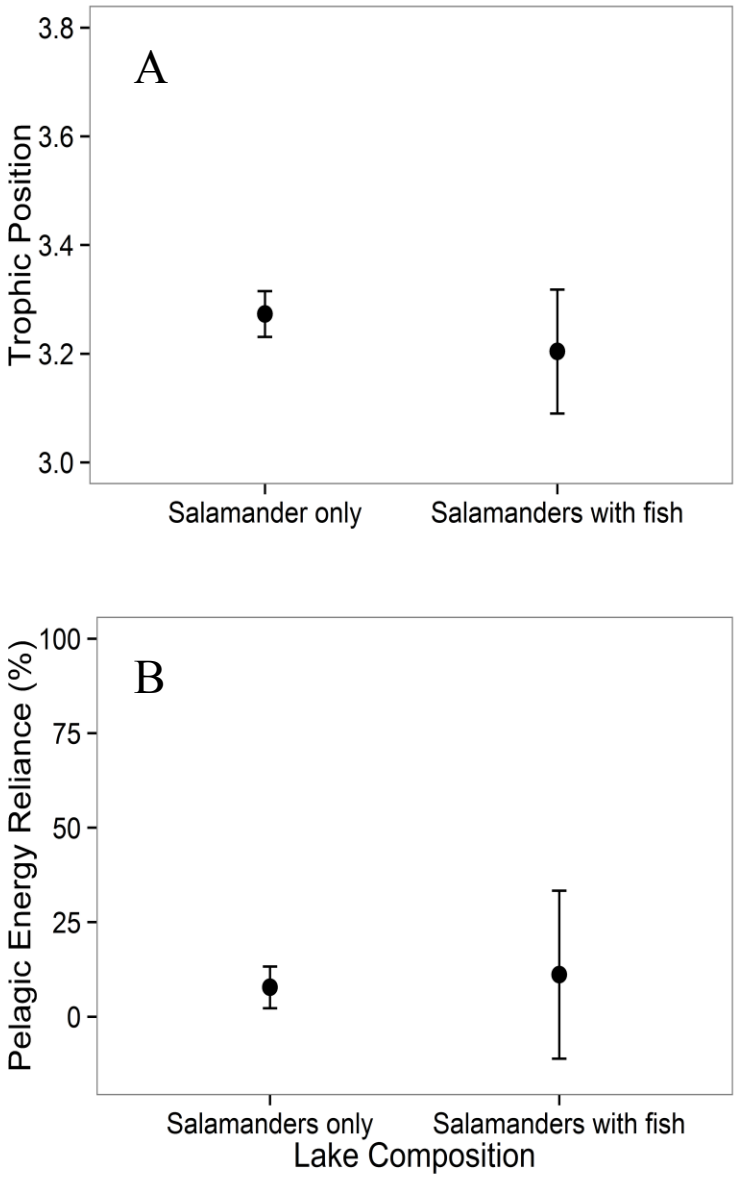

Figure 3.4. A. Trophic position of salamanders from salamander only lakes and salamanders with fish lakes, after adjusting for pelagic energy reliance and individual lake nested within lake type. B. Pelagic energy reliance of salamanders from salamander only lakes and salamanders with fish lakes, after adjusting for trophic position and individual lake nested within lake type.

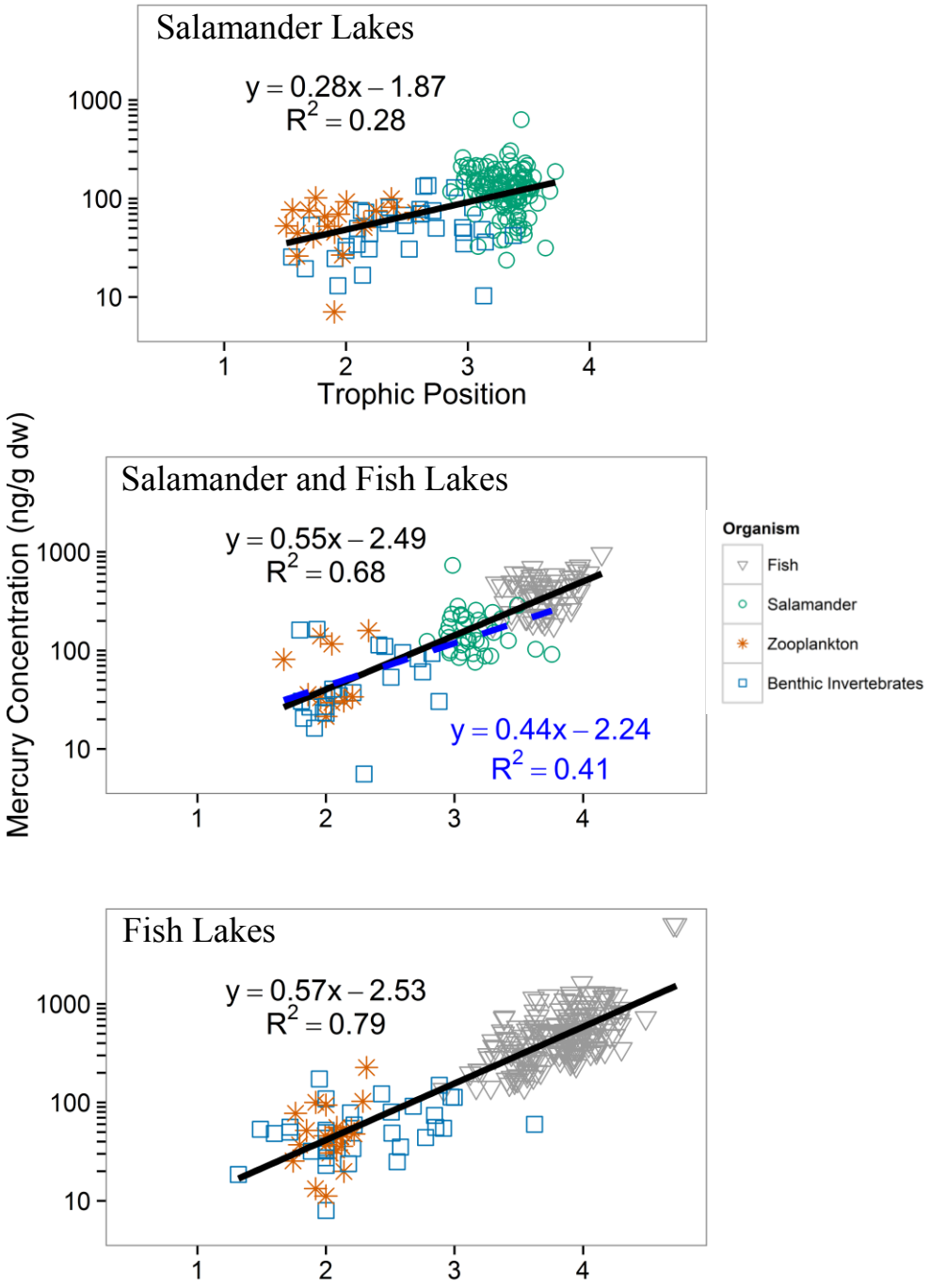

Figure 3.5. Rates of mercury trophic magnification slopes (TMS) through food-webs of lakes with different top predator compositions. Blue dashed line and text are regression equation and slopes of TMS when fish Hg data is omitted in salamander with fish lakes.

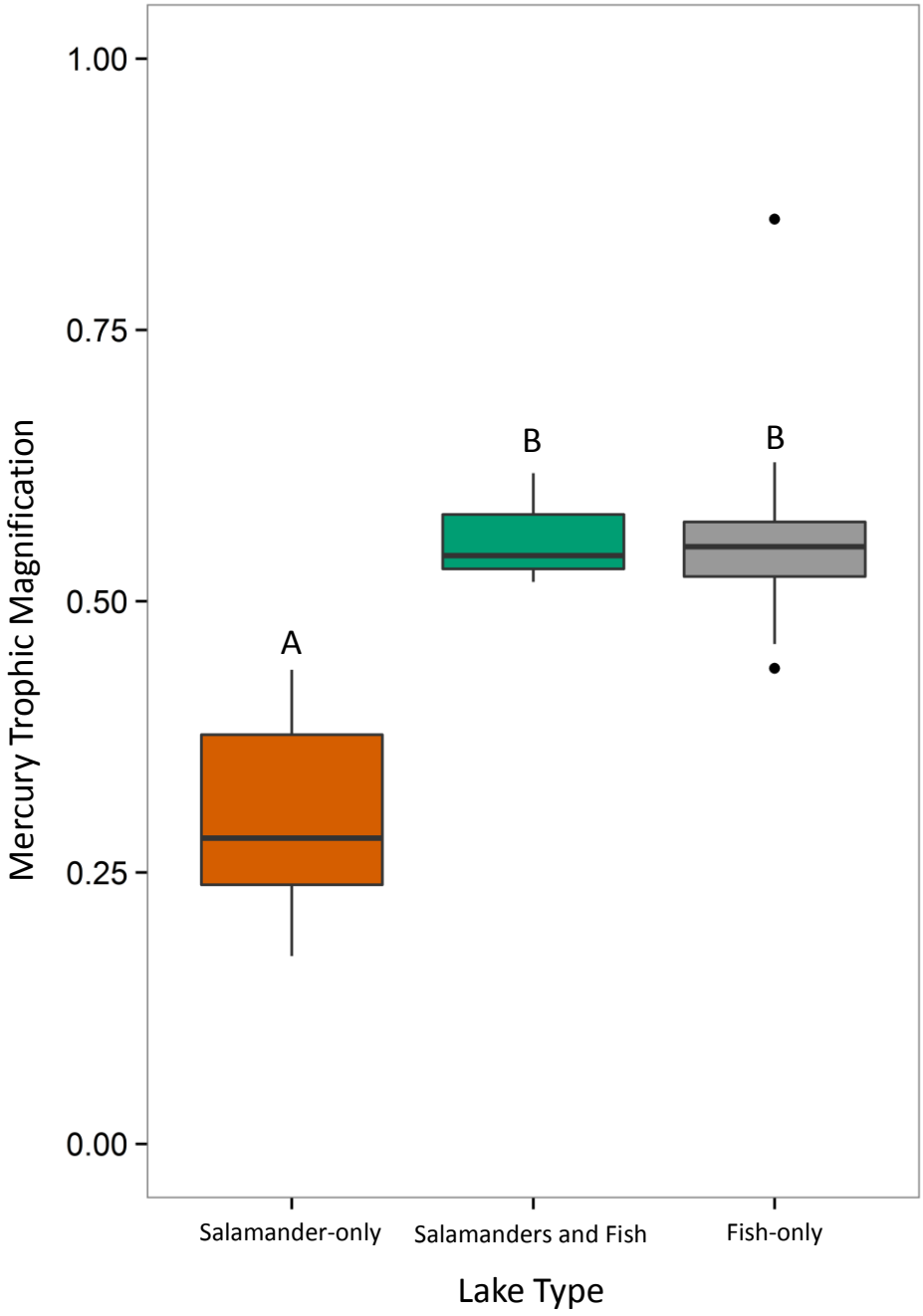

Figure 3.6. Mercury trophic magnification slopes (TMS) of whole food webs of lakes with different top predator compositions. Letters above the error bars indicate pairwise differences.

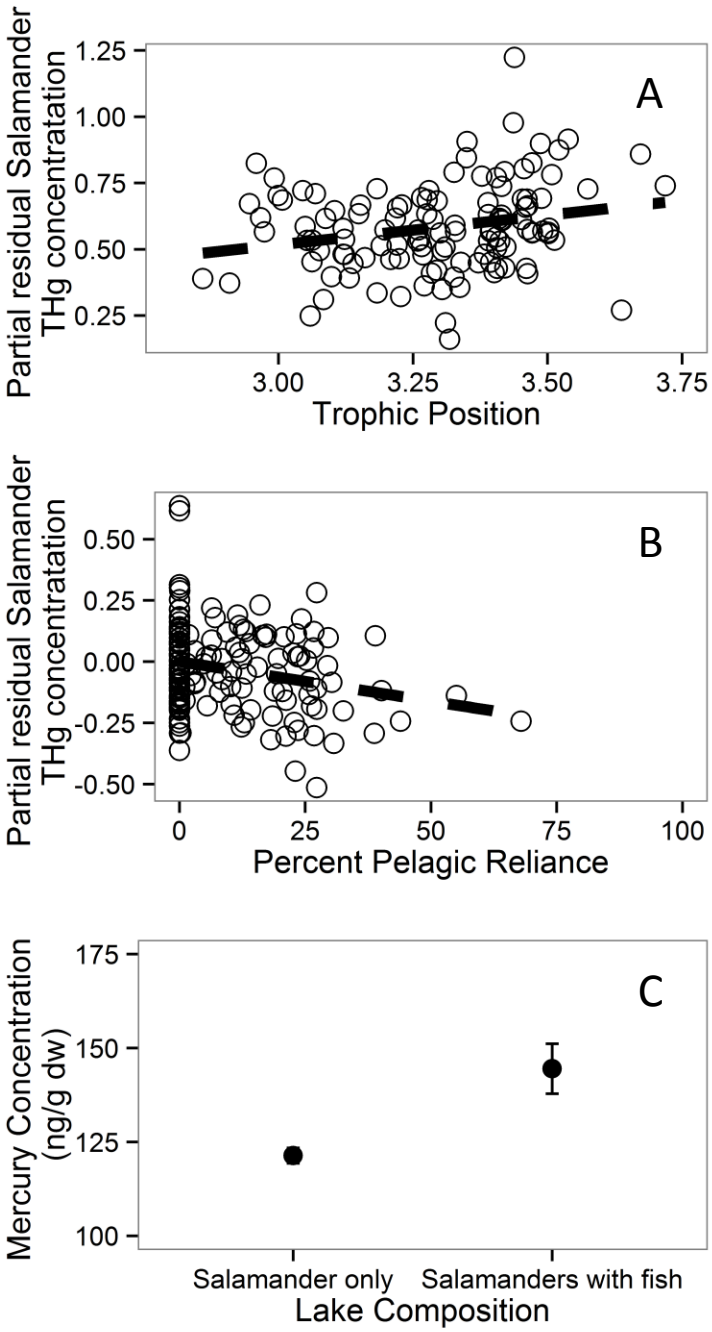

Figure 3.7. Salamander THg concentration partial residuals by trophic position (A) and percent pelagic energy reliance (B). C. Back transformed least square means total mercury concentrations (ng/g dry weight) of salamanders from differing lake compositions (salamander only, salamanders and fish).

CHAPTER 4 – Conclusions

We demonstrated that commonly practiced non-lethal tissue sampling techniques of tail and toe clipping can be used as a non-lethal alternative to whole body collections to determine THg concentrations in five species in the Pacific Northwest. Furthermore, we compared the correlation of tail clip THg to whole body THg across three size classes of tail, in four species of salamander. Our results indicate a 0-2cm tail clip THg concentration has the best predictive relationship across all species, while minimizing the impact of on the species. Because the tail THg concentration to whole body relationship varied negligibly between species there would be some justification to utilize the regression equations we provide ubiquitously among salamander species. Additionally, we examined the differences of THg concentrations between frog toes and found significant differences between toes. These toe THg differences maybe be related to the developmental process of toes on a frog, but additional research will need to be conducted to determine this. Importantly we still found high correlations between certain toe THg concentrations and the whole body THg concentration, particularly for outer toes in the hind feet. Additional frog species would need to be tested to determine if toe clip regression equations are similar across species. We used these non-lethal sampling techniques to collect tissue samples from salamanders in subalpine lakes to study the influence of introduced fish on salamander THg concentration and foraging ecology.

We studied food web components for mercury and stable isotopes of ^{13}Carbon and ^{15}Nitrogen from 18 subalpine lakes in Olympic National Park to determine if introduced fish changed salamander feeding ecology and mercury concentration. We found mercury concentrations within all the organism types (fish, salamanders, zooplankton, and benthic invertebrates) to vary widely among lakes. Utilizing stable isotopes, we found that salamander and fish exhibited some similar patterns in terms of resource use. Both salamanders and fish consumed a diet composed predominantly (>90%) of benthic derived nutrients. Salamanders in fish containing lakes had slightly lower trophic positions then in salamander only lakes. Salamander Hg concentration was not significantly correlated to trophic position, energy reliance on pelagic

resources, nor by the presence of fish. Our inability to detect differences may have been limited by our sample size of salamanders in fish invaded lakes. Mercury levels in 3 fish from 2 lakes exceed EPA's human consumption advisory (300 ng/g wet weight) and two of those fish exceeded the Great Lakes Advisory Group for no consumption (950 ng/g wet weight). This research provides evidence that introduced fish have high Hg concentrations compared to native salamanders in protected and pristine mountain lakes.

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