12-2013

Concentrations of Heavy Metals in Scute Samples from Nesting Female Olive Ridley, Lepidochelys olivacea, and Eastern Pacific Green, Chelonia mydas agassizii, Sea Turtles in Costa Rica

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Graduate School ETD Form 9
(Revised 12/07)

PURDUE UNIVERSITY
GRADUATE SCHOOL
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By Jacob M. Bryan

Entitled Concentrations of Heavy Metals in Scute Samples from Nesting Female Olive Ridley, Lepidochelys olivacea, and Eastern Pacific Green, Chelonia mydas agassizii, Sea Turtles in Costa Rica

For the degree of Master of Science

Is approved by the final examining committee:

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CONCENTRATIONS OF HEAVY METALS IN SCUTE SAMPLES FROM NESTING FEMALE OLIVE RIDLEY, *LEPIDOCHELYS OLIVACEA*, AND EASTERN PACIFIC GREEN, *CHELONIA MYDAS AGASSIZII*, SEA TURTLES IN COSTA RICA

A Thesis

Submitted to the Faculty

of

Purdue University

by

Jacob M. Bryan

In Partial Fulfillment of the

Requirements for the Degree

of

Master of Science

December 2013

Purdue University

Fort Wayne, Indiana

To my grandpa Hank, Gramps, and to Watson,
the best friend a man could ask for.

ACKNOWLEDGEMENTS

I would like to begin by thanking my academic advisor, Dr. Frank Paladino. His direction and guidance in the field of sea turtle biology and scientific research has led me down avenues I never once thought I would travel. He took me under his wing without any prior knowledge of who I was or what I could do. I have gained valuable insight from him on not only science, but also life, that will take me far beyond the third floor of IPFW. To my committee members, Dr. Robert Gillespie and Dr. William Demott, for their continuous, helpful revisions and for their interest not only my thesis, but as graduate student from the moment I stepped into their classroom. And to the rest of the IPFW faculty, staff and graduate students, even though I did not personally know most of them, their support and presence has been felt and gratefully received.

To a particular individual, Nathan Robinson, who has known me from day one of this pursuit of a master's degree. If it were not for his genuineness, I would not be where I am today, in a position to complete a master's program and submit this thesis. The long, thought-provoking conversations we have had in the laboratory, about everything sea turtles, were truly beneficial to my project and my growth as a research scientist. There are countless things I could thank him for, so I can only hope this does him justice.

To The Leatherback Trust and everyone affiliated, especially Maria Pilar Santidrian Tomillo, better known as Bibi. The assistance I received from her in the field and via the Internet has been invaluable; from learning how to PIT tag a leatherback sea turtle to translating permits due to my broken Spanish. Her faith in me as a field biologist has given me the confidence and encouraged me to pursue goals beyond a master's that can hopefully prove beneficial to conservation. To Jennifer Swiggs and Spencer Roberts, whom were there when I could not be, helping to collect samples at any cost. Jacob Hill, who came through and swooped me out of what felt like a never-ending hole. Ocean

plays, beach plays, and nighttime jungle tours kept me out and about and kept my mind off of things, instead of being pulled into the wasteful grasp of a computer screen. And to the Earthwatch volunteers, who not only took an interest in the work I was doing, but continue to do so and see that I follow through and finish my degree.

Lastly, I need to thank my family. No matter where I go, no matter what I do, even when they cannot find it on the map, they are there to support me. Mom and Dad were right there to pick me up after the worst and continued to do so when I went back to Costa Rica to finish what I thought was an improbable three months. Everyday, their thoughts, prayers, and kind words kept me going forward to finish the job I had started. To Natalie, Cole, and Tristan for their continued love and support, giving me a roof over my head, food in my stomach, and a smile on my face when I needed it most. And last of all, the one who got me to where I am today, my Grandpa Hank. Pursuing a master's degree was not even a thought until I had him push me into a field that I truly wanted to be in. He taught me how to persevere and continue further down the rabbit hole, no matter how deep it went. Gramps, there is still no grass growing under my feet.

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ABSTRACT

Bryan, Jacob M. M.S., Purdue University, December 2013. Concentrations of Heavy Metals in Scute Samples from Nesting Female Olive Ridley, *Lepidochelys olivacea*, and Eastern Pacific Green, *Chelonia mydas agassizii*, Sea Turtles in Costa Rica.
Major Professor: Frank V. Paladino.

Sea turtle populations are declining worldwide due to a number of anthropogenic factors: fishery bycatch, habitat destruction, illegal egg harvesting, and marine pollution. Marine pollution is the result of industrial processes or waste disposal that produce by-products that are either discharged into the atmosphere or directly into oceans. Sea turtles undertake extensive migrations; therefore, they can readily serve as biomonitors for the overall health of the aquatic ecosystem, especially for the evaluation of heavy metals that biomagnify across trophic levels. Heavy metals are known to have endocrine-disrupting properties, and at high concentrations, can be lethal. The objective of this study was to provide baseline toxicological information on heavy metal concentrations in nesting female sea turtles, specifically, the olive ridley (*Lepidocheyls olivacea*), a pelagic omnivore, and Eastern Pacific green (*Chelonia mydas agassizii*), a neritic herbivore, that migrate to Playa Grande and Playa Cabuyal in Guanacaste, Costa Rica.

Tissue samples were collected along the posterior marginal scutes located on the carapace and analyzed for the following metals: Cd, Co, Cr, Mn, Ni, and Pb. Concentrations were analyzed using inductively-coupled plasma mass spectrometry (ICP-MS) at the level of parts per billion (ppb). Scute tissue was sampled as it has been shown to yield higher metal concentrations than blood and provides a more accurate estimation of long-term metal accumulation. Other studies have demonstrated that metals have a high affinity for keratin, which is the tissue that makes up the outer layer of a turtle's

scute. For this reason, sea turtle scutes serve as an accurate storehouse of exposure to marine metal exposure. Since green turtles are herbivorous and remain in neritic, nearshore environments and olive ridley turtles are open-ocean pelagic omnivores these results should provide a good comparison of possible differences in exposure and accumulation in two distinct habitats and lifestyles.

A total of 34 scute samples were collected from olive ridley (n=17) and Eastern Pacific green (n=17) nesting females. Olive ridleys were sampled from Playa Grande, while Eastern Pacific green samples were collected from Playa Cabuyal. Concentrations (mean ± SE) for olive ridleys and Eastern Pacific greens are reported in ppb, respectively: Cd – 26.9 ± 1.5; 30.9 ± 1.6, Co – 26.7 ± 8.0; 69.8 ± 6.8, Cr – 106.0 ± 6.6; 123.5 ± 6.9, Mn – 524.2 ± 52.0; 485.8 ± 68.7, Ni – 216.8 ± 75.8; 123.7 ± 20.2, Pb – 90.5 ± 25.1; 94.8 ± 25.1. Cd and Co were significantly different ($p < 0.05$) between the two species with Eastern Pacific green turtles having higher concentrations. Relative size comparison using curved carapace length, a rough estimate of turtle age, was not correlated with metal concentration within the sea turtles sampled ($p > 0.05$).

The reported concentrations found in these two marine turtle species are lower than other studies that used scute tissue samples as indicators of heavy metal pollution. However, study site locations were associated with major industrialized areas whereas this study was along the somewhat undeveloped Central American Pacific coast where there may be differing concentrations of pollution, explaining these differences. Furthermore, the results of this study suggest that Eastern Pacific green turtles, a neritic herbivore, may be at a higher risk of contamination by specific metals than a pelagic omnivore like the olive ridley. Sea grasses and red algae, the main staple of the Eastern Pacific green's diet, have unique uptake pathways where some metals are utilized in the plant's metabolic processes, while others accumulate as free ions. Accompanied with their inshore, neritic habitat utilization, the Eastern Pacific green turtle's risk of run-off and land-based contamination will increase if it happens to reside in a heavily polluted area.

Determining a baseline for the amount of heavy metal contamination in ocean habitats using long-lived species like sea turtles from both pelagic and neritic

environments is important for understanding anthropogenic influences on aquatic ecosystems. Sea turtle species spend the majority of their lives in the ocean, only coming ashore for brief periods during nesting events. During their lifetimes, they traverse waters with varying levels of pollution that can provide us with important insight into the health of the environment that they inhabit.

INTRODUCTION

Sea turtle populations are in decline all over the world mainly due to anthropogenic factors such as marine pollution (Bjorndal 1997). As defined by the United Nations Convention of the Law of the Sea, marine pollution consists of any substance produced or emitted into a marine environment that could potentially be hazardous, resulting in noxious effects on an aquatic species. Some of the major marine pollutants are agrochemicals, municipal wastes, oils, organic compounds, and heavy metals (Islam et al. 2004), all of which are a major concern for aquatic life.

Metals are mostly derived as by-products of industrial processes that are often discharged into the atmosphere or directly into the ocean (Islam et al 2004); however, they can be found as natural components in marine environments. Heavy metals are known to create a variety of toxicological problems due to their highly reactive chemical properties and origins, both natural and anthropogenic. Since they can naturally occur in the environment or be added via waste and not be degraded, biomagnification of heavy metals in food webs exacerbates their effects on organisms. It is important to note that heavy metals are not the only concern, but the metal's speciation becomes problematic. Specific heavy metals are essential for many different physiological processes (Yaclin-Ozdilek et al 2011), yet can be toxic at elevated concentrations, while others have the ability to biomagnify as the trophic level increases. Deleterious and toxic effects can occur at any concentration, depending on the type of metal, the organism it's exposed to, and the physiological endpoint being studied (i.e. reproduction vs. growth vs. mortality). Lower persistent concentrations have been shown to generate significant effects inhibiting normal physiological processes, such as hormone function and neural humor signaling (Clotfelter et al 2004). As a result, heavy metals are categorized as endocrine-disrupting chemicals because of their abilities to act agonistic-and-antagonistically on

hormone receptors that can cause disruptions from molecular to organismal levels. As pollution bearing effluents become increasingly pervasive in marine ecosystems, the need for developing baseline information using a long-lived readily available aquatic organism as an indicator of pollution is essential.

Sea turtles undertake extensive migrations utilizing different marine ecosystems, thus they can serve as important biomonitors for assessment of the overall health of the ocean ecosystem, and since sea turtles are long lived they can be especially important for the evaluation of heavy metals and bioaccumulation (Burger 1998; Sakai 1995). Each species of sea turtle has a unique migration pattern that starts as soon they reach the ocean as a hatchling. Olive ridley (*Lepidocheyls olivacea*) sea turtles maintain and utilize pelagic, oceanic zones (Musick and Limpus 1997) in circumtropical waters throughout their lives, making migrations across the Atlantic, Pacific, and Indian Oceans (Pritchard 1969). These migrations are considered intermediate compared to short migration distances of neritic green sea turtles (*Chelonia mydas*) and extensive ones taken by pelagic leatherback (*Dermocheyls coriacea*) sea turtles (Morreale 2007). Eastern Pacific green sea turtles (*Chelonia mydas agassizii*) are herbivores that occupy coastal zones in the Pacific Ocean (Alvarado and Figueroa 1998) and in the Pacific do not migrate vast distances, as is seen in leatherbacks.

Foraging behaviors also need to be taken into consideration as metals can increase through trophic levels. Olive ridleys are pelagic omnivores that feed on a number of benthic and pelagic invertebrates (Richardson 1997; Bjorndal 1997) that occupy different levels of the food web. The uptake of heavy metals in invertebrates can occur through the ingestion of contaminated water, sediments, or small animals (Rainbow 2002). Eastern Pacific green sea turtles are primarily herbivorous and feed on sea grasses and red algae (Seminoff et al 1998; Presti et al 1999). The uptake mechanisms in plants begin in the root, where accumulation is usually found at its highest, and metals can either be distributed to the shoots or remains in the root (Kamal et al 2004). Algae typically uptake metals that attach to ligands located in the cellular wall matrix before transport into the cell (Gadd 1990). While invertebrate species tend to have more uptake pathways than

those of plants, metal concentrations are dependent on the bioavailability and the chemical form of the metal.

The use of sea turtles, both live and dead, as specimens in toxicological assessments is a field that is gaining interest in scientific research. Dead specimens, usually found stranded, are sampled and analyzed with the availability of every tissue type, except blood. Live specimens are more problematic due to complications of locating sea turtles in the open ocean and all seven species are considered as endangered by CITES (Convention on International Trade of Endangered Species Appendix I). Knowledge of an animal's foraging grounds and migration routes that have been previously determined using satellite telemetry can help identify where these turtles live in the ocean. From studies of the life histories of female sea turtles, we know that they return to their natal beach in order to nest. Male sea turtles are rarely seen after they hatch and leave the beach, because, unlike females, they do not emerge onto their natal beaches. The chances of encountering a turtle out in the wild is greater when patrolling a known nesting beach, rather than searching out in the ocean's open waters. Researchers have been limited to egg, blood, and more recently, scute tissue sampling for analysis, all of which can be collected from females on a nesting beach.

Egg sampling allows for large sample sizes, but only gives limited information because metal concentrations must be maternally transferred, during ovulation, and the concentrations between the various layers of an egg and the actual female heavy metal loads can be significantly different. Paez-Osuna et al (2010, 2010a, 2011) reported that oviposition is not a practical route of elimination for heavy metals after examining the concentrations of heavy metals in both blood and egg samples from the same females. However, depending on the metal, they did observe that some metals are maternally transferred and distributed amongst clutches of eggs. Certain essential heavy metals are needed for embryonic development in trace amounts (Yaclin-Ozdilek et al 2011); yet heavy metals that are nonessential are not as readily mobile and can become problematic in a developing embryo (Jakimska et al 2011). In freshwater turtles, Burger et al (1998) demonstrated that high concentrations of lead have an effect on hatchling survival and

behavior, which becomes a potential concern considering that hatchling success and survival in sea turtles is relatively low.

Blood samples can provide indications of what metal concentrations are currently circulating throughout the animal as a result of a recent ingestion and absorption (Bergeron et al 2007). This method of sampling is most commonly practiced, yet it can be problematic since it can create a stressful environment for the animal. An additional complication is that once a metal enters the bloodstream it can either bind to a receptor or may circulate as a free ion or complex compound. In a number of different forms and states a metal may then be transported and taken up by other tissues at different rates. Metals can then be sequestered and accumulated in other tissues that are not readily accessible for sampling from a live animal. Thus the absorption, metabolism, movement and accumulation of metals in animals can create a challenge when using only blood as a bioindicator of heavy metal content.

In humans, scientists have used fingernails and hair as indicators of heavy metal exposure (Jenkins 1979) because many metals have an affinity and easily bind to keratin, the major protein in nail and hair structures. The top tissue layer of a sea turtle's carapace also is made up of layers of keratin (Fig. 1) and has recently been used in contamination studies (Presti et al 1999, 2000; Sakai et al 2000; Day et al 2005; Wang et al 2005). The sampling of scute tissue involves using a sterilized metal blade in a sampling punch for scraping off the top layer of a turtle's shell scute. Minimal restraint of the animal is required because the animal is not as responsive to a blade slicing thinly into the surface of its shell as it is to a needle that penetrates its skin. This technique is also considered less invasive and less risky for the animal's well-being.

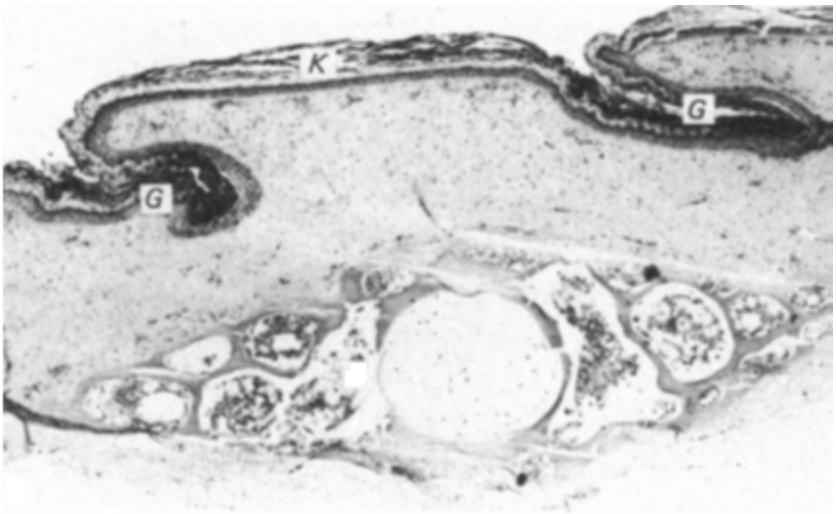

Figure 1. Cross-section of a green sea turtle scute using transmission electron microscopy. K denotes keratin layers and G denotes the growing keratin layers. Modified from Solomon et al (1986).

Studies have also shown that scute tissue samples yield anywhere from 10-35 times higher metal concentrations than blood when sampled from the same turtle (Presti et al 1999, 2000; Day et al 2005; Wang et al 2005). The shell is thus thought to serve as a reservoir for metals and can be used as a good indicator of long-term exposure. Concentrations in scute tissue can be correlated with contamination found within internal organs, signifying its usefulness as an indicator for heavy metal pollution in sea turtles (Sakai et al. 2000).

It is important that more baseline data be gathered on marine organisms as pollution and excessive dumping into the ocean continues to increase. Studies have demonstrated that heavy metals are accumulated by sea turtles (Jakimska et al 2011), and detectable in varying levels within different tissues (Storelli and Marcotrigiano 2003). A sea turtle's distinct habitat utilization, migrating patterns, and foraging behaviors enable researchers to infer the health of specific areas of the ocean since the turtle can function as unique indicators of marine pollution in different marine ecosystems.

Background

Playa Grande

Playa Grande (10°20’N, 85°51’W) is a crescent-shaped beach spanning 3.6 km located in the Guanacaste province in Costa Rica. It hosts the largest nesting population of leatherback sea turtles in the eastern Pacific Ocean (Spotila et al 1996; Steyermark et al 1996). This site, situated within the Parque Nacional Marino Las Baulas (PNMB), has been the focus of conservation research efforts in order to protect the critically endangered leatherback from extinction for the past 25 years under the direction of The Leatherback Trust and the Ministerio de Ambiente, Energia, y Mares de Costa Rica (MINAET). However, this beach has only recently become recognized as an important nesting site for the olive ridley (Dornfeld 2012). Since data collection began in 2009, 239 different individuals have been identified and tagged (not including this current season) nesting on Playa Grande. Normally, females of this species are known to nest only once at this beach per season; however, about 5% of the females have been identified to return and re-nest in the same season and also return to PNMB to nest again in subsequent seasons.

Although development in Playa Grande has ceased on its shoreline, beach development continues to increase in surrounding and adjacent areas to keep up with the demand of tourism. The increase in human activity and development can negatively impact local sea turtle nesting populations, especially with regards to marine pollution. Because there is evidence that this species is facing a decline in population along the Pacific coast of Costa Rica (Fonseca et al 2009). The continued protection and management of this beach, and those surrounding, is critical to manage and maintain their populations.

Playa Cabuyal

Playa Cabuyal (10°40’N, 85°40’W) is a moderately sloped beach that extends 1 km located in the Guanacaste province in Costa Rica. It is primarily a nesting beach for Eastern Pacific green sea turtles (*Chelonia mydas agassizii*), but has recently seen a few leatherback sea turtles. Research and conservation efforts have only been in place for

three years, but the number of nesting females each season has proven to be worthy for further research undertakings. Playa Cabuyal has anywhere from 70-80 turtles that average around four nests. In contrast to olive ridley turtles at Playa Grande, these turtles have high nest site fidelity and return to lay a clutch every two weeks until they complete their nesting season.

Unlike Playa Grande, Playa Cabuyal is a remote beach with no major human development in the immediate beach area. This provides a less impacted habitat for females to nest without as much anthropogenic influences. Satellite telemetry data of post-nesting migration and interesting movements for Eastern Pacific greens on nearby nesting beaches show that while they remain in costal habitats throughout these life stages, they did travel great distances and were observed as far north as El Salvador and as far south as Panama (Blanco et al 2012). While traversing along these migration routes to their foraging grounds, these females pass through coastal areas with increased urbanization that may increase the turtle's exposure to heavy metal pollution.

Objectives

This study was designed to determine baseline metal concentrations from sea turtles nesting in Costa Rica. Secondly, this study is designed to assess the use of scute tissue sampling as a reliable technique for determining heavy metal loads in marine turtles. The methods used for extracting scute samples are not only less invasive, but also very simple and are considered less stressful on the nesting females when compared to blood sampling. During oviposition, females are stimulated by high levels of sex hormones that their sole focus is to lay eggs, which becomes important to not put the turtle under any addition stress or she may fail to nest. Green turtles tend to be more susceptible to disturbance while nesting which could result in a female aborting her nesting behavior and subsequently dumping the clutch of eggs in the water. If researchers are not using nesting females as specimens and capturing turtles in open-ocean instead, techniques such as blood collection, could add additional stress to an animal that is already experiencing stress from being captured. Scute sampling has been proven, in

other studies (Presti et al 1999, 2000; Sakai et al 2000) to be a non-lethal, less stressful technique that should be used when conducting metal analyses.

This study aims to test three hypotheses, (1) Scute tissue will indicate a detectable level of heavy metal contamination from sea turtles nesting at Playa Grande and Playa Cabuyal, Costa Rica; (2) Pelagic, omnivorous olive ridley turtles will contain higher metal concentrations due to their habitat utilization, migration behavior, and foraging habits and possible biomagnification, due to their position in the food chain, than the neritic, herbivorous Eastern Pacific green turtle; (3) Biometric measurements will show that levels of metal contamination will increase as size increases, a consequence of size being a possible indicator of age and duration of exposure

MATERIALS AND METHODS

Study Area

This study took place at two different beaches in Guanacaste Province, Costa Rica: Site 1, Playa Grande, and Site 2, Playa Cabuyal (Fig. 2).

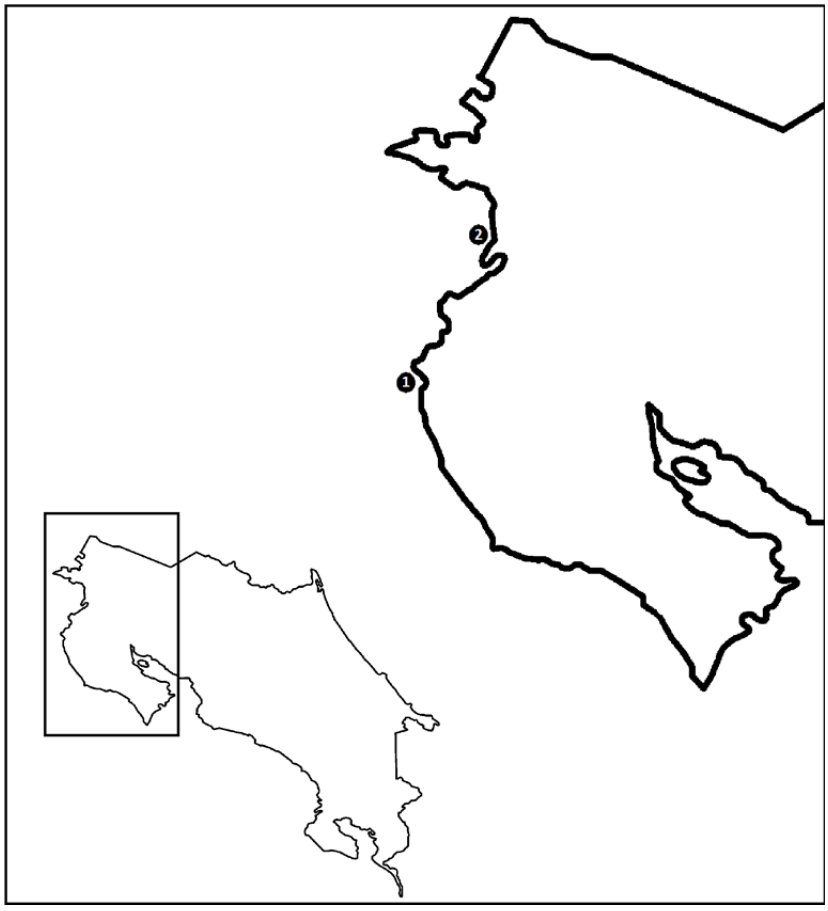

Figure 2. Map of study sites in Costa Rica: (1) PMNB (2) Playa Cabuyal.

Data Collection

A team of researchers conducted nightly beach patrols at Playa Grande to locate nesting olive ridley females during the 2012-2013 nesting season from 1 October to 1 March. Adhering strictly to protocols set aside by Purdue PACUC (#1210000742), USFWS, The Leatherback Trust and Ministry of Environment, Energy and Technology of Costa Rica, the beach was patrolled three hours prior to and after high tide. Once a turtle was encountered, it was not disturbed; allowing it to begin the nesting process. Only after the female had deposited 30-40 eggs from an average clutch of 100, which was about midway through the process of oviposition, did scute sampling begin. At this point, in oviposition less than 1% of female turtle abort their nesting process and for this study there were no aborted nests. The sampling procedure included, first, measurement of the curved carapace length and curved carapace width. (Gardener et al 2006). After biometric measurements were taken, the carapace was cleaned with sterile alcohol swabs to remove any debris, bacteria or algae from the scute to be sampled. Subsequently, a small biopsy punch was used to remove a 4-6 mm surface piece of scute tissue from the posterior marginal scute of each individual in the study. A sterile biopsy punch removes the top layer of the keratin was cut and scraped off, yielding a sterile biopsy sample. Clean sterile examination gloves were worn during the sampling procedure to reduce unwanted contamination. Each sample collected was immediately stored in a sterile cryo-vial and kept at room temperature until further analysis (Presti, personal communication).

These same methods were applied and carried out when collecting data at Playa Cabuyal, where only Eastern Pacific green turtles were sampled. The collection took place during the same time frame as that at Playa Grande.

Sample Preparation

The preparation of each sample followed the protocol found in Day et al 2005, but was slightly modified in order to meet the requirements of the protocol supplied by the chemistry department at Purdue University (Appendix).

Sample Digestion

The 4-6 mm biopsy samples yielded approximately 20 milligrams of scute tissue sample which was added to a 5 mL of 70% nitric acid (Fisher Chemical) solution in a 15 mL polypropylene conical tube (BD Falcon). Samples were then digested at room temperature under a fume hood for at least 24 hours or until the tissue was completely digested. Each sample was then diluted to 2% nitric acid using nanopure water to meet the range requirements of the analysis instrumentation. This diluted sample was prepared to a uniform 5 mL. A 5 mL blank of 2% nitric acid was also prepared from the stock nitric acid used to compare with the original sample, along with two known and standard samples (+10 ppb and +20 ppb), to determine the concentration of the original sample.

Metal Analysis

The study samples prepared were analyzed for concentrations in parts per billion of metals using inductively coupled plasma mass spectrometry (ICP-MS). The chemistry department at Purdue University served as the location for the analysis, working under the direction of Dr. Karl Wood. The metals of interest include cadmium (Cd), chromium (Cr), cobalt (Co), manganese (Mn), nickel (Ni), and lead (Pb). These metals were chosen in order to compare with studies that reported concentrations for the same metals using different tissues. They were also chosen to provide baseline contaminant information for metals that have yet to be analyzed in scute tissue.

Samples were analyzed using an ELEMENT-2 (ThermoFinnigan, Bremen, Germany) mass spectrometer in the medium resolution mode. The samples were introduced into the plasma using an Aridus desolvating system with a T1H nebulizer (Cetac Technologies, Omaha NE), which is used to enhance sensitivity and reduce oxide and hydride interferences. The argon sweep gas and nitrogen of the Aridus is adjusted for maximum peak height and stability using (7)Lithium, (115)Indium and (238)Uranium peaks obtained from a Merck multi-element standard (1ng/ml, Merck & Co.).

ICP-MS results when a high temperature argon plasma (attaining temperatures on the order of 10,000K) is produced. The sample is introduced into the plasma through a nebulizer into a stream of flowing argon as a finely divided aerosol. At these high

temperatures the sample is rapidly desolvated, vaporized and ultimately atomized. The resulting atoms are then ionized (often with yields near 100%), typically as singly charged ions, then mass is detected and analyzed. The detection limits for Cd, Cr, Co, Mn, and Ni range from 1-10 parts per trillion (ppt), while that for Pb was 1 ppt.

During the analysis, the instrument undergoes three separate runs, scanning the sample ten different times (for a total of 30 scans per sample), before taking the average metal count found each sample (the "count" represents the amount of times the element was detected by the instrument). This is also done for samples only containing nitric acid (blank). For statistical analysis and comparison, samples from individuals were run in duplicates. Once the analysis was finished, results were tabulated and concentrations were determined by subtracting the standard acid blank levels detected from the individuals sample counts. By setting up a ratio between the standard +10 ppb sample and its metal counts versus the sample metal counts, the concentration of the sample could be calculated. Sample dilution was also taken into consideration for the final concentration value because samples were originally diluted by a factor of 35 from the original sampled obtained and digested. The formula for this entire calculation is as follows:

[sample metal count – blank metal count = metal count

$$\frac{\text{metal count}}{\text{x ppb}} = \frac{\text{10 ppb metal count}}{\text{10 ppb}}$$

x ppb * 35 = concentration of metal]

In the instance a count value for a metal in a sample was lower than the metal count value in the blank, ion suppression was known to have occurred. For reasons unknown, metals were still being detected, but at concentrations lower than those of the blank. As a result, the formula previously mentioned would no longer apply. When ion suppression had occurred, a modified calculation had to be used in order to obtain the proper concentration value.

Statistical Analysis

For statistical purposes, SPSS 21 software (IBM Corp. Armonk, NY) was used to determine any significance among concentrations within and between species, as well as correlations between biometric measurements (CCL) and concentrations. A Spearman's rank correlation was used to investigate the intraspecific significance between an increase in metal concentration and an increase in CCL. Nonparametric statistics were run using a Kruskal-Wallis test to test for interspecific differences in metal concentrations.

RESULTS

A total of 33 different individual female turtles were sampled during this study (n=16 olive ridleys; n=17 Eastern Pacific green), however, 34 samples were collected. There was one olive ridley turtle that was resampled during a subsequent nesting after having returned to nest in Playa Grande three weeks after its first nesting encounter. This clearly indicated that these turtles were probably not affected by this biopsy punch sampling method since this was about the same rate of reencountering nesting females from previous studies on this beach (Dornfeld 2012) Biometric information for the turtles by species can be found in Table 1. There were instances for both species where biometric measurements were not taken; however, a scute tissue sample was obtained nonetheless. Carapace measurements for both species indicated that all females sampled were in the adult size range and since eggs were deposited all were considered to be sexually mature adults (Lutz and Musick 1997).

Table 1. Biometric measurements; curved carapace length (CCL) and curved carapace width (CCW).

Species	*N*	CCL (cm)		CCW (cm)	
		Mean ± SD	Range	Mean ± SD	Range
L. olivacea	15	66.1±4.1	59.5-74.3	70.2±3.0	65.2-76.6
C. mydas agassizii	16	85.7±5.0	78.6-98.5	79.8±5.4	73.0-90.7

Mean concentrations for all metals studied in both species of turtles (n=15 for olive ridleys and n= 16 for Pacific greens) are reported in Table 2. The data indicate that all turtles have some level of contamination for every heavy metal analyzed (Min. 8.0

ppb (Co); Max. 1364.6 ppb (Ni)). Results demonstrate that olive ridleys had higher concentrations than Eastern Pacific green turtles for two metals (Mn and Ni); while they had lower concentrations for all the other metals tested (Cd, Co, Cr, and Pb). The ranking order of metal concentration in olive ridleys starting with the highest is: Mn>Ni>Pb>Cr>Cd>Co. There was a similar ranking order for the metals found in Eastern Pacific green turtles with: Mn>Ni>Pb>Cr>Co>Cd. When examined individually in scute tissue, Mn was found in the highest concentration in both species, while Cd and Co were the two metals with the lowest concentrations with different orders for each species.

Table 2. Metal concentrations (mean ppb ± SE) from olive ridley and Eastern Pacific green turtles.

Species	*L. olivacea* (n=17)	*C. mydas agassizii* (n=17)
Metal	Mean ± SE (Range)	Mean ± SE (Range)
Cd	26.8 ± 1.5 (21.0 - 38.0)	30.9 ± 1.6 (18.2 - 46.1)
Co	26.7 ± 8.0 (8.0 - 100.6)	69.8 ± 6.8 (10.7 - 102.4)
Cr	106.0 ± 6.6 (79.1 - 153.7)	123.5 ± 6.9 (96.5 - 218.1)
Mn	528.3 ± 55.2 (91.3 - 770.8)	485.8 ± 68.7 (95.4 - 1133.0)
Ni	216.6 ± 75.8 (49.6 - 1364.6)	123.7 ± 20.1 (50.4 - 304.7)
Pb	90.5 ± 25.1 (32.7 - 398.5)	94.8 ± 25.1 (40.5 - 383.1)

Table 3 shows the individual concentrations for each turtle sampled for both species. Some level of contamination was found in every turtle; however, as presented in the table, two metal concentrations were not analyzed for the individual turtle PG1. Analysis of the minimum values of each metal found in individual turtles PG8 (Co, Cr, Ni), PC12 (Cd, Ni), PC16 (Cd, Co), and PC17 (Cd, Co) were found to have lower concentrations than any other turtles. Conversely, turtles PC1 (Co, Cr, Mn, Ni, Pb) and PG3 (Co, Cr, Pb) yielded higher concentrations than any of the other turtles. These results indicate that these turtles may inhabit waters with higher metal concentrations, specifically Cr.

Table 3. Individual metal concentrations (ng/g wet weight) from turtles sampled at Playa Grande (PG) and Playa Cabuyal (PC). NA = Not Analyzed.

L. olivacea	Cd	Co	Cr	Mn	Ni	Pb
PG1	36.4	NA	NA	459.1	375.2	217.1
PG2	33.5	100.6	126.2	124.0	101.9	83.9
PG3	37.9	87.9	153.7	99.4	121.8	263.9
PG4	32.8	83.1	131.3	91.3	120.6	67.7
PG5	22.8	17.6	86.3	642.2	1364.6	57.0
PG6	25.0	10.7	121.9	770.8	212.3	36.9
PG7	22.6	11.1	118.3	724.0	380.1	41.2
PG8	37.7	8.0	79.0	628.6	45.3	398.5
PG9	23.2	9.1	82.8	705.4	78.2	32.7
PG10	23.0	9.2	83.8	643.3	118.7	37.6
PG11	21.0	19.6	127.5	590.3	89.6	32.7
PG12	21.9	20.3	151.7	643.9	243.1	37.6
PG13	23.4	9.3	85.5	614.5	49.5	35.5
PG14	23.8	9.4	88.5	544.0	67.9	37.4
PG15	23.7	9.7	82.8	528.8	105.3	37.3
PG16	24.1	9.5	82.0	520.8	98.8	46.2
PG17	24.2	12.0	95.3	581.5	112.6	75.7

C. mydas agassizii	Cd	Co	Cr	Mn	Ni	Pb
PC1	46.1	102.4	218.1	607.2	301.4	337.9
PC2	31.0	84.2	122.6	95.4	304.7	40.5
PC3	33.2	80.0	123.9	192.1	99.3	43.3
PC4	35.0	85.0	148.0	119.9	242.8	44.3
PC5	35.6	80.6	137.1	825.9	108.2	113.7
PC6	27.2	73.7	104.7	338.2	52.5	31.7
PC7	27.7	74.9	115.4	278.4	102.6	49.2
PC8	27.7	74.5	106.5	369.6	63.5	37.8
PC9	29.3	79.2	112.4	448.5	74.6	57.3
PC10	29.2	79.0	112.5	507.6	57.7	41.4
PC11	37.7	90.9	142.6	457.4	90.7	99.3
PC12	27.8	75.5	100.3	351.1	50.4	54.8
PC13	31.1	76.8	107.1	564.1	50.7	55.1
PC14	40.1	90.7	129.3	349.8	160.0	119.1
PC15	25.3	11.2	105.8	930.7	149.2	383.1
PC16	23.3	17.9	96.5	1133.0	94.0	45.6
PC17	18.2	10.7	116.4	689.0	101.0	58.4

To determine if there was an intraspecific correlation between increasing metal concentration and relative size as indicated by increasing CCL, correlation statistics were run. Spearman's rho data can be found in Table 4. Two-tailed p values showed that there was no significant correlation between the two variables ($p > 0.05$).

Table 4. Spearman correlation coefficients (CCL vs. metal).

	Cd	Co	Cr	Mn	Ni	Pb
L. olivacea	0.234	-0.138	-0.233	-0.274	0.24	0.508
C. mydas agassizii	0.085	0.168	-0.2	-0.284	-0.324	-0.162

Figure 3 displays a comparison between metal concentrations (mean ppb ± SE) for all the metals of interest found in both olive ridleys and Eastern Pacific green turtles. There is a significant difference between species in Co ($p < 0.05$). Since metal concentrations were not normally distributed, further analyses were run to determine any interspecific difference using nonparametric statistics. A Kruskal-Wallis test still showed a significant difference ($p < 0.05$) in Co, but also showed a difference in Cd. Both ranked means were greater in Eastern Pacific green turtles. However, the rest of the metals failed to reject the null hypothesis of similar concentration loads ($p > 0.05$).

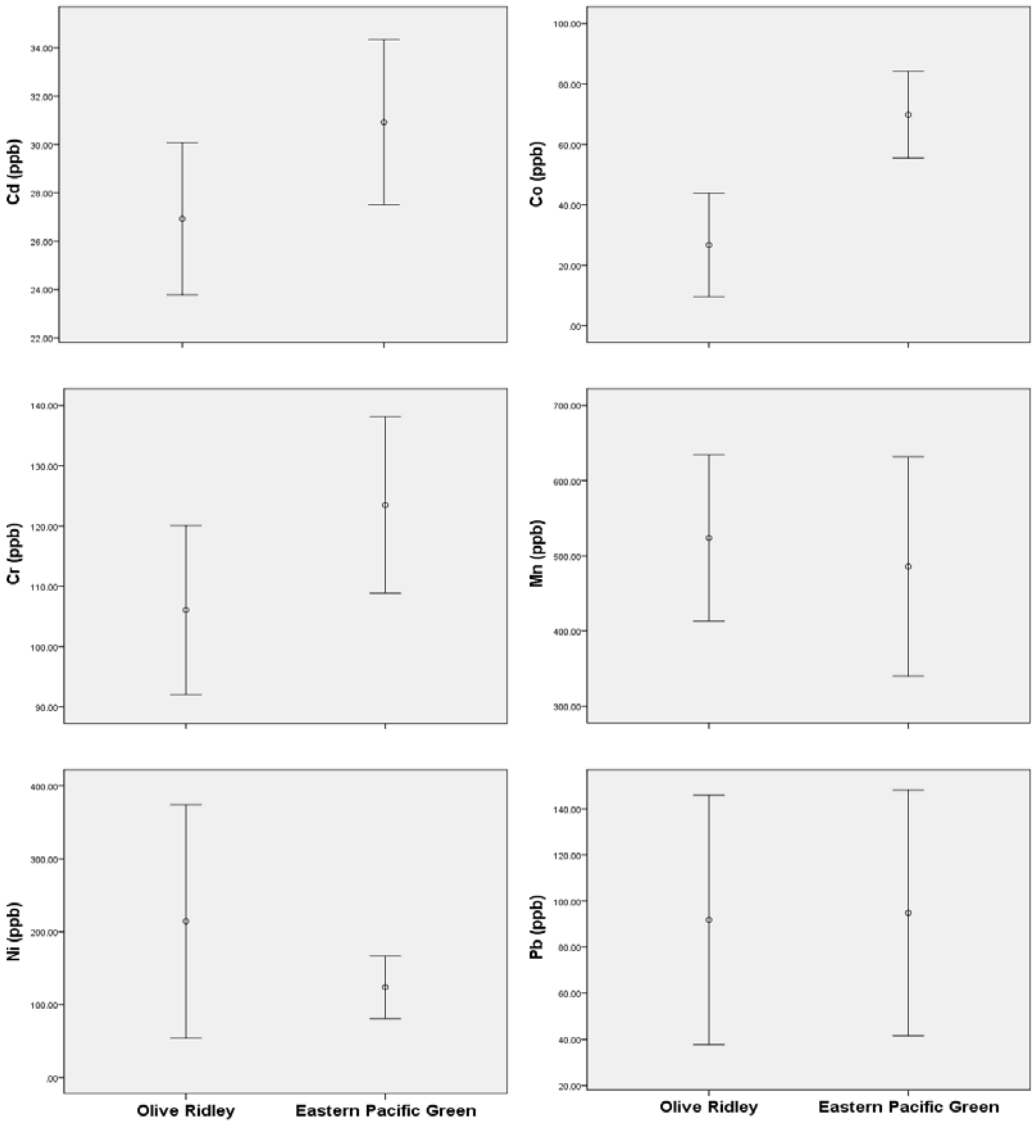

Figure 3. Metal concentrations (mean ppb ± SE) for olive ridleys (n=17) and Eastern Pacific green (n=17) turtles.

Table 5 shows concentrations from this study as well as concentrations from previous studies using scute samples from similar species. Comparisons were made between our results and other studies that had different site locations (Komoroske et al 2011, Sakai et al 2000, Wang et al 2005). The mean concentrations found in this study were relatively lower than most other concentrations from the other studies, with exception of Co and Ni when compared to data from green and loggerhead turtles.

Table 5. Comparison of mean metal concentrations within scute tissue from other studies. NA = Not Analyzed

Species	Cd	Co	Cr	Mn	Ni	Pb	Location	Reference
Chelonia mydas	54	<30	NA	3920	191	2300	Haha-Jima, Japan	Sakai et al 2000
Chelonia mydas agassizzi	443 ± 53.7	NA	NA	48700 ± 7040	NA	7230 ± 2330	San Diego Bay, USA	Komoroske et al 2011
Chelonia mydas agassizzi	30.9 ± 1.6	69.8 ± 6.8	123.5 ± 6.9	485.8 ± 68.7	123.7 ± 20.1	94.8 ± 25.1	Guanacaste, Costa Rica	This study
Caretta caretta	129 ± 34	NA	NA	7010 ± 3490	94±22	2420 ± 520	Cape Ashizuri, Japan	Sakai et al 2000
Lepidochelys kempii	139	NA	198	NA	NA	986	Texas, USA	Wang et al 2005
Lepidochelys olivacea	26.8 ± 1.5	26.7 ± 8.0	106.0 ± 6.6	528.3 ± 55.2	216.6 ± 75.8	90.5 ± 25.1	Guanacaste, Costa Rica	This study

DISCUSSION

This study tested three hypotheses, (1) Scute tissue will indicate a detectable level of heavy metal contamination from sea turtles nesting at Playa Grande and Playa Cabuyal, Costa Rica; (2) Pelagic, omnivorous olive ridley turtles will contain higher metal concentrations due to their habitat utilization, migration behavior, and foraging habits and possible biomagnification, due to their position in the food chain, than the neritic, herbivorous Eastern Pacific green turtle; (3) Biometric measurements will show that levels of metal contamination will increase as size increases, a consequence of size being a possible indicator of age and duration of exposure

Hypothesis (1) Scute tissue will indicate a detectable level of heavy metal contamination from sea turtles nesting at Playa Grande and Playa Cabuyal, Costa Rica

The data that was collected and analyzed in this study supported hypothesis (1). Although our turtles had a wide range for the metals analyzed, the presence of detectable concentrations indicates at least some level of contamination, regardless of the amount. Olive ridleys were shown to have a minimum and maximum contamination for Co (8.0 ppb) and Ni (1364.6 ppb). Both concentrations significantly differed from their means (26.7 ± 8.0 ppb Co; 216.8 ± 75.8 ppb Ni). Ni, while occurring naturally, is usually a product of metal smelting that is easily bioaccumulated by olive ridley prey (Cempel et al 2006). However, this same individual showed lower contamination in other metals (Co, Cr, Pb) relative to the concentration means and other turtles. The trend in which a turtle had high contamination in one metal, but low in another was noticeable throughout the rest of the individuals. This suggests that they all have unique migration routes and forage in waters separate from one another, which is consistent with the solitary behavior discussed in Morreale et al (2007). Out of all the individual olive ridleys that nest on Playa Grande, only 5% have been found to return to nest, indicating that the rest of the

nesting population travel to other beaches to nest, a similar trend found in Kalb (1999). This suggests that nest site fidelity in solitary olive ridley females is weak and that migrations to and from nesting beaches are complex. Figure 4 shows post-nesting migration movements from olive ridleys from a nearby beach in Costa Rica and the differences between individuals. It is possible that these differences are represented within the nesting populations at Playa Grande and, therefore, explain the varying levels of heavy metals found within those females.

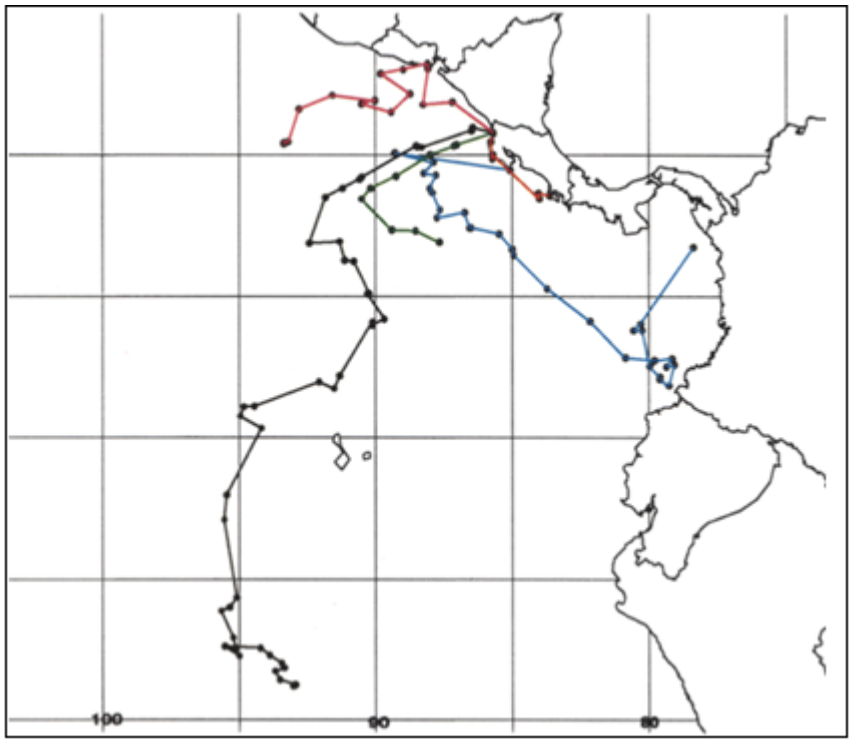

Figure 4. Migration routes of eight olive ridleys from Nancite, Costa Rica (Taken from Plotkin et al 1995).

Previous studies have shown that blood samples collected from olive ridleys in a similar region (just north in Mexico) identified similar metal contamination (Cd, Ni, and Pb), but at higher levels than this study reports: 450 ppb Cd vs. 26.9 ppb Cd; 2800 ppb Ni vs. 216.8 ppb Ni; 950 ppb Pb vs. 90.5 ppb Pb (Paez-Osuna 2010, 2010a). Since blood concentrations are lower than scute tissue, the turtles from these studies should have

higher contamination within scute tissue, which would prove to be even greater than the turtles from our study. This also means that if blood had been sampled in our study, those concentrations would have been considerably lower. This suggests that the turtles studied from Oaxaca, Mexico migrate to and forage in areas where exposure to metal contamination is greater and separate from the areas our turtles inhabit.

Kemp's ridleys (*Lepidochelys kempii*) showed a similar trend from turtles studied in the Gulf of Mexico (Wang et al 2005). This study suggested that turtles found with varying metal concentrations were due to the difference in foraging grounds. Turtles that were sampled in industrialized areas experienced higher metal concentrations than those that were found in areas with little to no industrialization. Heavy metal analyses of crab species, a staple in an olive ridley diet, in these areas exhibited this same trend.

In comparison, trace amounts of each metal tested was also found in Eastern Pacific green turtles. Similar to olive ridleys, these turtles showed a minimum concentration for Co (10.7 ppb), but had a maximum concentration for Mn (1133.0 ppb), both of which were significantly different from the mean (69.8 ± 28.0 ppb Co; 485.8 ± 283.4 ppb Mn). Even though one individual turtle accounted for the highest Mn contamination, Eastern Pacific green turtles still exhibited a lower Mn mean concentration when compared to olive ridleys.

Mn can be found in open-ocean sediments or nodules on the ocean floor (Cronan et al 1969). Since olive ridleys utilize the open-ocean as their habitat, they are more likely to come across these Mn sources, considering they feed on benthic invertebrates. However, Mn can also come from wastewater sources (Cempel et al 2006). Lizano et al (2012) show the presence of Mn in Culebra Bay (just south of Playa Cabuyal) in an unnatural distribution, implying that its source was not natural. It is possible that this source derived from an effluent as a result of development in the surrounding area. Since the location of this bay is in close proximity to one of our study sites, we determined that our turtles must occupy or navigate through these waters, and are therefore exposed to heavy metal contamination.

Komoroske et al (2011) conducted a study using scute tissue from Eastern Pacific green turtles from the San Diego, USA (Data summarized in Table 5). This particular

study site was an urbanized bay that reported high levels of pollution that were reflected in the metal levels found in the turtles sampled there (443 vs. 30.9 ppb Cd; 48700 vs. 485 ppb Mn; 7230 vs. 94.8 ppb Pb). Concentrations were more than ten times greater than those reported in this study. From this result we determined that the foraging areas our turtles are migrating to, while still contaminated, are not exposed to the pollution levels found in areas with high human development.

Outside of scute tissue sampling, a number of studies have been conducted using internal organs to determine if and where metals accumulate. Results have shown that metal concentrations are present in the kidneys, pancreas, liver, muscle, brain, bone and adipose (Aguirre et al 1994; Anan et al 2001; Andreani et al 2008; Barbieri et al 2009; Sakai et al 1995, 2000, 2000a; Witkowski 1982). Within those studies, trends have demonstrated that the kidneys and pancreas accumulate higher concentrations of Cd (Andreani et al 2008; Barbieri et al 2009; Gardner et al 2006; Storelli and Marcotrigiano 2003) than any other tissue; the kidneys also accumulate higher amounts of Co (Anan et al 2001). Bone tissue mainly accumulates Pb (Sakai et al 2000a; Witkowski 1982). Cr and Mn showed a homogenous distribution amongst the kidney, muscle, and tissue (Aguirre et al 1994; Anan et al 2001; Andreani et al 2008; Godley et al 1999; Sakai et al 2000, 2000a).

Hypothesis (2) Pelagic, omnivorous olive ridley turtles will contain higher metal concentrations due to their habitat utilization, migration behavior, and foraging habits and possible biomagnification due to their position in the food chain than the neritic, herbivorous Eastern Pacific green turtle

Evidence did not support hypothesis (2) even though olive ridleys showed higher concentrations in two of the six metals analyzed (Mn and Ni); however, these differences were not significant ($p > 0.05$). Since olive ridleys demonstrate pelagic migrations, I hypothesized that their diet and foraging behavior would expose them to higher metal concentrations through biomagnification. Olive ridleys have been known to feed in both open-ocean and coastal habitats feeding primarily on small invertebrates, mainly crustaceans. This omnivorous diet places these turtles as a top predator in the food web increasing its exposure to metal contamination. Crustaceans are able to uptake metals in

three ways: passive and active transport and by ingesting contaminated prey. Through passive facilitated diffusion, metals cross epithelial membranes via metal-binding proteins before entering the organisms' circulatory system, where they are subsequently sequestered and accumulated amongst tissues throughout the body (Rainbow and Dallinger 1993). Active transport occurs as a result of metals binding with other ions that are being pumped across membrane channels used in normal physiological processes. Once across the membrane, the distribution of the metals is comparable to those that have entered by facilitated diffusion. Lastly, metals can enter the body when an organism ingests a previously contaminated organism. This method of uptake shows that metal concentration will increase as it is magnified when moving up trophic levels, until the top predator is reached.

Metal concentrations will not only be dependent on bioavailability, but how the organism utilizes each metal. Organisms use specific essential metals in regulatory processes, thereby reducing concentrations. Plankton and bacteria have been found to transform metals via redox reactions that reduce their availability as free, circulating ions that become available to the next organism in the trophic food chain (Morel and Price 2003; Errecalde et al 1998). Metals can even be excreted, mainly through fecal wastes, adding further to their reduction. Some nonessential metals, however, persist in tissues with little to no excretion.

Sediment and invertebrate metal concentrations in the Nicoya Gulf, Costa Rica show a relationship indicating metal uptake by the invertebrates from the sediments (Dean et al 1986). Concentrations of Cd, Cr, and Pb found in three invertebrate species varied from those of sediment, showing both higher and lower concentrations than the sediments. Metal concentration seemed to be dependent of the sampling site, as well as the species sampled. This data suggests that metal uptake is more complex and that the certain invertebrate species not only bioaccumulate metals, but bioconcentrate them, or uptake them from the water column, which is another viable uptake pathway. The pollution in this area is mainly related to increases in urbanization, industrialization, and boat traffic throughout the bay (Whelan et al 1989). It is possible that the Tempisque River, a major river in Costa Rica, experiences anthropogenic-derived pollutants before it

empties into the bay. While metal contaminant levels in the olive ridleys in this study do not reflect the elevated levels found in the sediments and invertebrate species of the Gulf of Nicoya (26 ppb vs. 6620 vs. 560 ppb Cd; 106 ppb vs. 160 ppb 1470 ppb Cr; 90 ppb vs. 5900 ppb vs. 1630 ppb Pb), there is evidence to believe that they, along with the Eastern Pacific green turtles, have accumulated low amounts of the same metals from occupying these areas of the gulf.

Eastern Pacific green turtles had higher concentrations in four of the six metals, showing only one significant difference in Co. However, Co also showed a significant difference when using nonparametric statistics. These results indicate that Eastern Pacific greens may be at a greater risk to heavy metal contamination because of their preference to coastal waters and herbivorous feeding habits. Using coastal regions as foraging grounds, these turtles take advantage of feeding on the various sea grasses and red algae. Uptake pathways in these food sources are similar to those in terrestrial plants. Metals can accumulate in the roots from sediments, which can be transported throughout the rest of the plant; or metals can be absorbed directly from the water into the algal structure as well as sea grass fronds. Levels are contributed to the availability of the metal around and within the plant. Sea grasses in the Eastern Pacific have shown patterns of fluctuation in metal concentrations both temporally and spatially, resulting in varying concentrations throughout the year (Riosmena-Rodríguez et al 2010). Once accumulated, the fate of a metal is important; essential metals are involved in metabolic pathways, while nonessential metals, like Cd, can be compartmentalized and stored within the plant (Debusk et al 1996). Talavera-Saenz et al (2007) reported significantly higher levels of Cd within sea grasses than any other metal. The persistent uptake and compartmentalization of a metal could explain the significant differences in Cd and Co concentrations between these two turtle species.

Heavy metals in sediments have been assessed along the eastern Pacific coast of Costa Rica in Culebra Bay (Garcia-Céspedes et al 2004; Lizano et al 2012) showing low to high levels of contamination (302,000 ppb Cr; 0.094% Mn; 9,000 ppb Pb). Preliminary satellite telemetry data shows that Eastern Pacific green females that nest on Playa Cabuyal travel in and out of this bay during internesting and post-nesting movements

(*unpublished data*, Clyde-Brockway). While it is thought that sea turtles do not forage during internesting periods, the females that are shown to inhabit this bay are still be exposed to heavy metal contamination via bioaccumulation and bioconcentration. Since duration of exposure is indicative of contamination, we can infer that Eastern Pacific green turtles sampled from this study do not occupy these waters for long periods of time because of the low levels detected. The same inference can be made for olive ridleys, as they did not experience contamination levels as reported in the sediments. This information can also be significant in determining the health status of coastal zones in Costa Rica, reassuring that many of these areas can still be considered pristine, while those that experience high metal concentrations warrant closer monitoring.

(3) Biometric measurements will show that levels of metal contamination will increase as size increases, a consequence of size being a possible indicator of age and duration of exposure

Bishop et al (1994) demonstrated that body size could be an indicator of the level of contamination concluding that as body size increased, so do levels of contaminants. Our results suggest that while metal concentrations were shown to increase with size, it is not at any level of significance. We found that smaller turtles within each species did not have significantly less contamination than the larger turtles in this study. In some instances, smaller turtles even displayed higher concentrations, especially in Eastern Pacific green turtles. This could be due to a number of reasons such as the bioavailability of the metal, in which a turtle would be exposed during foraging or migration movements, uptake pathways, utilization of the metal, including the excretion of the metal. Failure to correlate body size parameters with metal contamination is the case in most studies. Presti et al (1999) found that when they split their turtles into small and large groups, a correlation was easier to conclude; however, this study failed to find any correlation. We did manage to find a positive trend between both CCL and CCW measurements and metal concentrations; however, none were of significance.

Since our study, and those studies alike, failed to conclude that size is an indicator of contamination poses a problem for animals in all size classes, meaning smaller turtles may be at a risk for high metal contamination. This supports the conclusion that the age

of marine turtles based from size measurements should not be a determining factor when investigating metal contamination and body load concentrations, but instead, future studies should shift their focus to also determine the origin of the turtles in a nesting population and focus on contamination from that habitat and/or foraging grounds when comparing individuals from these species.

Conclusion

This is the first study believed to characterize this array of metals using scute samples from olive ridleys and only the second (Komoroske et al 2011) from Eastern Pacific green turtles in the Pacific Ocean, creating the need to further develop baseline information on heavy metal contamination along the Pacific coast from animals originating in highly contaminated areas as well as those habitats that are relatively undisturbed. It is also one of the first studies to compare heavy metal concentrations between two species that have different life histories; a neritic, herbivorous species vs. a pelagic, omnivorous species. While heavy metal levels detected were not alarming when compared to other studies, significant differences were found in metals between both species. This suggests that the differences in foraging habits and habitat utilization between species are a result of the varying heavy metal levels. It also suggests that each species may be exposed to certain metals more so than others due to the differences in life history and the metals fate when accumulated.

Sakai et al (2000) determined that concentrations found within scute tissue are correlated with whole body metal burden and are indicative of the levels of contamination within the body without having to analyze internal tissues. Scute tissue should be sampled at different locations on the carapace to appropriately determine heavy metal concentrations because the way each metal can be distributed throughout the entire shell (Day et al 2005), thereby giving a more appropriate indication of whole body burden. As a result of the low concentrations detected from scute tissue in this study, we can infer that the turtle's body burden is also low and that they must inhabit relatively undisturbed areas along the Pacific coast of Central America. Compared to other studies in more developed areas such as the San Diego Bay, who regarded their turtle specimens

as healthy, we can conclude that the metal contamination in our sampled turtles does not yet pose a threat to their well-being, but should still be monitored to ensure levels of contamination are not increasing.

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APPENDIX

APPENDIX

ICP-MS Sample Preparation

Bottom Line:
The samples submitted for ICP-MS need to be 2% nitric acid solutions. Each sample should contain 5-10 ml of solution. The samples should be free of solid particulates. The samples should be submitted in plastic vials (i.e. 15 ml Falcon tubes) to minimize contamination.

General Considerations:
The water used in sample preparation should be free of metals, nanopure is the best, but deionized or doubly distilled should be sufficient for many metals.

The nitric acid should ideally be free of the metal ion(s) being studied. Obviously differing elements might require different grades of nitric acid. A good nitric acid for trace metal analysis is Aristar Ultra (Catalog number 87003-226) available from VWR International. In some cases a lesser grade of nitric acid can be used for example, Fisher Chemical Nitric Acid trace metal (Catalog number A509-500).

Always be concerned about the quality of the containers you are using to prepare your standards and samples. We have found some containers (Erlenmeyer flasks, beakers, etc.) to be contaminated with the metal to be studied even though the container was brand new.

The samples should be digested, centrifuged, filtered, etc. to ensure that the total dissolved solids (tds) are below 0.5%. This will minimize plugging of the capillary tubing of the nebulizer ($1200) which feeds the ICP source. When trying to determine concentration of the metal ion(s) of interest either an external standard curve or standard addition (see below) can be used. When using an external standard curve it is important that the matrix for the standards is the same as that of the samples. Otherwise ion suppression phenomena acting on the samples but not on the standards will give erroneous results. This is the reason that standard addition is often employed. ICP standards can be purchased from Exaxol or VWR and diluted to the required concentrations. You can go to each of their websites and purchase the individual sodium and magnesium standard. These standards come in 2% nitric acid. I would recommend buying the 100ppm concentration standards as they are the cheapest, approximately $30-40 dollars. You have to be concerned with the stability of the standard solutions (and sample solutions as well).

The method of choice is standard addition, where you will take an aliquot of your sample solution and add known amounts of the metals (sodium and magnesium) of interest. A calibration curve is then generated from the ICP-MS data on all samples. The intercept corresponds to the negative of the concentration of metals in your sample solution (x-intercept = - [metal])

Your Procedure:

Standard Addition Procedure

- Create a large volume of 2% nitric acid.

  It is best to create a large volume of 2% nitric acid for your sample preparation so that the solvent of all samples is the same.

- Create a 10 ppb solution of the metals of interest (sodium and magnesium) in 2% nitric acid in a volume sufficient for all your samples. The metal solution should be the same throughout the run.

  You can purchase metal solutions for ICP-MS from Exaxol or VWR (see above) that are already in 2% nitric acid, then dilute these down to 10 ppb using the 2% nitric acid solution you've made.

- Digest your tablet to release metal and minimize clogging. Objective - create a stock solution of 10 ppb metal equivalents in 2% nitric acid. (A ppb translates to one ng of metal per ml of solution.)

Make up your sample by dissolving the sample in concentrated nitric acid, in order to digest the organic constituents. This can be done at room temperature. I would imagine that you probably don't need to leave the sample in the concentrated nitric acid for too long but for good measure I would leave it in for four (4) hours. This seems to be an average length of time for proteins, for which we have more experience. Then dilute the sample until you have what you believe is approximately 10ppb of the metal of interest. This solution will now be your stock sample solution

The following table is a guide for setting up your samples:

	Blank	Stock Sample Only	+ 10 ppb	+ 20 ppb	+ 30 ppb	+ 40 ppb
10 ppb metal equiv in sample	--	1 mL	1 mL	1 mL	1 mL	1 mL
10 ppb sodium and magnesium standard	--	--	1 mL	2 mL	3 mL	4 mL
2% nitric acid	5 mL	4 mL	3 mL	2 mL	1 mL	--

You may want to extend the range of metals, depending on the expected mole ratio of compound to metal in your sample.

This procedure as written would produce six samples for ICP mass analysis. You could get by with only the first two or three standard additions samples which would reduce the number of samples for analysis of each tablet to 4 or 5.

NOTE: Please label your samples completely.