EFFECTS OF METAL CONTAMINATION ON DEVELOPING RED-EARED SLIDER TURTLES (TRACHEMYS SCRIPTA) AND IMPLICATIONS FOR THE SPECIES AS A BIOMONITOR

By

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INTRODUCTION

The Red-eared Slider as a Biomonitor of Environmental Contaminants

Reptiles are experiencing declines in population numbers and in some cases local extirpation. A recent review of their conservation status (Gibbons et al. 2000) cites six factors as major contributors to their population declines: habitat loss, invasive species, disease, unsustainable use, global climate change, and environmental pollution. Despite grave risk from several anthropogenic factors, reptiles are the least-studied group with regard to environmental contaminants. Ironically, they typify many characteristics of good biomonitors of environmental contamination (Hopkins 2000).

Biomonitoring of environmental contamination involves the use of living organisms to assess the bioavailability and risk associated with exposure to pollution (Kettrup and Marth 1998). Walker et al. (2001) listed several key characteristics of candidate organisms for use in biomonitoring studies, including a wide distribution and high likelihood for exposure to the contaminant of concern, a quantifiable response to the contaminant, ability to survive at least moderate exposure, reproducibility of the response, and availability of existing toxicity data to assist in interpretation of the response.

Red-eared slider turtles (*Trachemys scripta*) exhibit many characteristics that would make them suitable biomonitors of environmental contamination in both aquatic and terrestrial systems. They are long-lived with a survival rate that is among the highest

of all animals once adulthood is attained (Gibbons 1990), thus facilitating study of chronic contaminant exposure (Hopkins 2000). They are largely sedentary with narrow home ranges and high site fidelity (Gibbons 1990) and are widely distributed across the United States (Ernst 1990), allowing for site-specific studies without the confounding effects of the study organism traveling from site to site. Their wide distribution is in part due to their excellent capabilities as habitat generalists. Red-eared sliders are amphibious, venturing on land primarily for reproductive purposes or basking (Gibbons 1990). They are opportunistic omnivores and experience incidental ingestion of water and sediment (Parmenter and Avery 1990), which are potential sources of contaminant uptake (Linder and Grillitsch 2000).

Red-eared sliders use terrestrial nests and are capable of successfully nesting in a variety of substrates and locations ranging from sandy and loamy soils with sparse vegetative cover to disturbed areas such as road banks, railroad grades, dikes, levees, and dams (Congdon and Gibbons 1990). The eggs have flexible shells that take up water from the surrounding substrate, which also might allow for transport of water-soluble contaminants across the eggshell (Parkard et al. 1987; Linder and Grillitsch 2000). As such, red-eared slider eggs may be used as biomonitors in several different soil types contaminated with a wide range of toxicants.

Trace metals, the focus contaminants of this study, are prominent environmental toxins. They occur in air, water, and soil via natural geological processes and anthropogenic activities such as mining and industrial operations (Burger 1992). Both essential and nonessential metals can pose toxic risks to organisms.

Risks associated with metals in soil are dependent on their availability (Basta and Gradwhol 2000). Many factors control the availability, or bioavailability, of metals in the environment, including the soil characteristics of pH, composition (particularly clay content), and amount of organic and inorganic matter present. As soil pH decreases and the surrounding environment becomes more acidic, the solubility and availability of metals increases (Walker et al. 2001 and Yong 2001). Generally, at any acidic pH, Pb is subjected to higher retention than Cd and Zn, which experience equal retention. The clay component of soil is generally poor at binding metals in the soil and rendering them unavailable. Conversely, soil organic matter effectively binds heavy metals, making them insoluble and unavailable. Thus, as the ratio of organic to inorganic matter decreases, so does the availability of the metals (Yong 2001). Ideally, the response of an organism used as a biomonitor of terrestrial metal contamination would reflect trends in metal bioavailability over time.

Reptiles and Heavy Metal Contamination

Several studies have shown that reptiles are capable of accumulating metals within various tissues. Alligators exposed to copper (Cu), zinc (Zn), mercury (Hg), lead (Pb), cadmium (Cd), chromium (Cr), iron (Fe), and arsenic (As) had detectable levels of those metals in muscle tissue. Accumulation of metals within alligator tissues can pose a risk to humans who eat alligator meat (Delaney et al. 1988). Burger (1992) demonstrated that pine snake (*Pituophis melanoleucas*) hatchlings accumulate Pb, Cd, Hg, Cr, selenium (Se), and manganese (Mn) in their skin and whole body tissues. Lead, Hg, and Cr were found in higher concentrations in the skin, and Burger (1992) speculated that ecdysis may be a method of elimination of these metals.

Sea turtles also accumulate metals within their tissues. Caurant et al. (1999) compared Cd, Cu, and Zn concentrations in the pancreas, muscle, kidney, and liver of leatherback (*Dermochelys coriacea*), loggerhead (*Caretta caretta*), and Kemp's ridley (*Lepidochelys kempii*) sea turtles. Pancreatic tissue, analyzed only in leatherbacks, had the highest concentrations of all metals studied. Cd and Cu were consistently high in liver and kidney of leatherbacks and loggerheads, whereas Zn was nearly homogeneous in all tissues with the heart showing the highest concentrations. Kemp's ridley turtles had lower concentrations of metals than leatherbacks and loggerheads.

Storelli et al. (1998) analyzed liver, lung, kidney, and muscle from beached loggerhead sea turtles for Hg, Pb, Cd, Cr, As, and Se. Liver contained the highest concentrations of Pb, Hg, and Se. The highest Cd levels were in kidney with decreasing levels found in the liver and muscle. Chromium was present in similar levels in all tissues with lung tissue having slightly higher concentrations. Arsenic was observed primarily in muscle tissue. When metal levels were examined as a function of size, smaller and presumably younger turtles had higher concentrations of all metals except As.

Snapping turtles (*Chelydra serpentina*) and red-eared sliders also accumulate metals. Overmann and Krajicek (1995) analyzed several tissues in snapping turtles caught in the Big River in Missouri's old lead belt and found relatively low levels of Pb in soft tissues, liver, kidney, and blood, and relatively high levels in calcified tissues, bone and carapace. Liver, blood, bone, and carapace Pb levels reflected concentrations at the capture locations of the turtles, with carapace levels being the highest. Positive relationships between liver Pb levels and Pb levels in blood, bone, and carapace also were noted. Snapping turtles accumulate several other heavy metals in their tissues as well.

Copper, Hg, Ni. Cd, and Zn have been detected in snapping turtle liver and kidney (Albers et al. 1986).

Red-eared sliders accumulate metals in a variety of tissues. After intraperitoneal injection of Cd, Zn, and Cu, Thomas et al. (1994) found Cd in liver, kidney, spleen, gonads, heart, and shell. The highest amounts of Cd were found in liver with far lower amounts observed in lung, muscle, brain and blood. Copper accumulated in liver, kidney, and ovarian tissues, and Zn was found primarily in the shell and ovary (Thomas et al. 1994).

Several studies also have examined the presence of metals within reptilian eggs. Stoneburner and Kushlan (1984) evaluated metal levels in freshly-laid eggs of the American crocodile (*Crocodylus acutus*). They analyzed eggs for aluminum (Al), Cd, cobalt (Co), Cr, Cu, Hg, molybdenum (Mo), nickel (Ni), Pb, and strontium (Sr) and found higher levels of Al, Cd, Cr, Cu, Mo, Ni, Pb, and Sr in eggshells than egg contents. Cobalt concentrations did not differ between the two egg compartments, and Hg concentrations were higher in egg contents than eggshell.

Sahoo et al. (1996) and Vazquez et al. (1997) examined distributions of heavy metals within eggs and hatchlings of olive ridley sea turtles (*Lepidochelys olivacea*) and eggshells of leatherback sea turtle (*Dermochelys coriacea*) eggs. Recently-laid eggshells of the olive ridley had higher concentrations of metals than the albumen-yolk portion of egg, and newly-hatched neonates had higher metal concentrations than freshly laid eggs. Presence of all metals investigated within eggs and hatchlings correlated with their presence in incubation sand, suggesting they were absorbed from the substrate, with Fe, Zn, and Pb being the most easily absorbed (Sahoo et al.1996). Leatherback eggshells

analyzed by Vazquez et al. (1997) also contained concentrations of Cd, Cu, Zn, Pb and Ni, all of which were present within the sand in which eggs were oviposited.

Similar to eggs of olive ridleys, eggs of slider turtles can accumulate metals from the substrate (Burger and Gibbons 1998). Burger and Gibbons (1998) analyzed egg contents and eggshells separately for metals derived from exposure to coal combustion wastes. Nearly all metals examined were found in higher amounts in egg contents than in eggshells. Pb, Hg, and Se were higher in egg contents, whereas Cr levels were significantly higher in eggshells. Hg and Cd accumulated nearly equally in both components.

Maternal transfer also may play a role in metal accumulation in eggs laid in the environment (Sakai et al. 1995; Burger and Gibbons 1998; Nagle et al. 2001). Some female birds burdened with Pb, Se, and Cr have the capacity to eliminate these contaminants from their own bodies by sequestering them into their eggs (Burger 1994). Similarly, loggerhead sea turtle females are capable of transferring essential metals to their eggs. They also can transfer Hg and Cd to their eggs, but in a limited capacity (Sakai et al. 1995). Of the metals studied in red-eared sliders (Se, As, Cd, Cr, and Cu), only Se was transferred from mother to egg and ultimately to hatchling (Nagle et al. 2001).

Physiological Energetics and Behavior as Indicators of Contaminant Exposure

Physiological energetics is the study of energy metabolism, or an organism's production of chemical, mechanical, and heat energy from the food and oxygen it consumes. Energetics depend on the First Law of Thermodynamics, which states that energy cannot be created or destroyed, only transformed from one form to another.

Animals consume food, assimilate the chemical potential energy, and transform it to other forms of potential chemical energy or into mechanical energy. All conversions occur at the cellular level to provide the energy necessary for cellular processes which, in turn, fuel other processes such as activity, growth and reproduction (Gordon et al. 1982, Withers 1992, and Schmidt-Neilsen 1997).

Energy metabolism per unit time is called the metabolic rate (MR). An organism's MR is variable over time and depends on factors such as temperature, activity, whether or not it has just eaten, and what it has eaten. Metabolic rate can be measured in several ways. The easiest method is measurement of the amount of oxygen the animal consumes over a period of time. Oxygen is the final electron acceptor for the electron transport chain, which is an integral part of ATP production. Increased oxygen consumption is the result of an increased need for energy and the resulting increased MR (Gordon et al. 1982, Withers 1992, and Schmidt-Neilsen, K. 1997).

The most commonly used measurement of metabolism in scientific studies is the basal or standard metabolic rate (BMR or SMR). It is a measure of the minimal amount of energy an animal must expend to stay alive. Standard metabolic rate (SMR) is the designation used for ectotherms and should be measured when an animal is at rest during its inactive period, post-absorptive, and not experiencing any stress. The temperature at which measurement is made must be specified because ectotherms do not retain any metabolic heat and their MRs are dependent on the ambient temperature (Withers 1992). During development, embryos within eggs absorb yolk as a source of fuel, and after hatching, hatchlings still contain yolk reserves. Thus, MRs of eggs and resultant hatchlings are considered to be resting metabolic rates (RMR) rather than standard

metabolic rates because neither the embryos nor hatchlings can be considered post-absorptive (O'Steen and Janzen 1999). Otherwise, RMRs are measured under the same conditions as SMRs.

There are at least five ways that organisms may respond to contaminants, with each imparting some energetic cost. The first is avoiding contaminants and expending energy to relocate and find new resources. Second is enduring the pollution while generating defenses against it, e.g., producing mucus to protect exposed areas. Third is detoxification of contaminants, which requires energy to process and transport contaminants to sites of excretion. Fourth is production of compounds that neutralize or sequester toxicants and prevent damage. Fifth is repairing damage caused by contaminants after they have been effectively neutralized or removed (Calow 1991). Physiological energetics has thus been suggested as one avenue for exploring impacts of environmental contamination on individuals and populations (Calow 1991; Widdows and Donkin 1991; and Calow and Sibly 1990). Because energy represents a common currency across animal groups, the evaluation of contaminant effects on energetic profiles of organisms might allow for clear intra-specific comparisons of response to pollutants. At the individual level, analysis of energetics can provide information on how contaminants influence energy acquisition and allocation to processes such as growth and reproduction (Widdows and Donkin 1991). At higher levels of organization, the study of energy transfer between trophic levels might indicate how contaminants influence food webs and entire communities (Newman 1998). Results also can be obtained in both the laboratory and the field, thus allowing contaminant effects to be determined outside the often-confounding laboratory environment (Calow 1991).

Integration of energetics and toxicology has occurred in studies involving several types of organisms, including mollusks, fish, anurans, and, in limited capacity, reptiles (Sobral and Widows 1997; Beters et al. 1999; Suresh et al. 1993; Berntssen and Lundebye 1993; Rowe et al. 1998; Hopkins et al. 1999; Nagle et al. 2001). However, no such studies have been conducted on turtle or even reptilian eggs.

Clams (*Ruditapes decussates*) exposed to sublethal concentrations of Cu exhibited respiration rates that increased to 145% that of control clams. Their scopes for growth were reduced to 23% of that in control clams, suggesting that energy normally allotted to growth was redistributed to detoxification efforts (Sobral and Widows 1997). Atlantic salmon (*Salmo salar* L.) parr reared on diets supplemented with Cd showed no differences in growth compared to a control group at the end of a 4-month experimental period. However, when fish were sacrificed and carcasses analyzed, those in the experimental group had significantly decreased amounts of protein, lipid, and glycogen, as well as reduced whole body energy content. Reductions in energy stores were attributed to reduced digestibility of Cd-contaminated feed and increased metabolic costs of dealing with Cd exposure (Berntssen and Lundebye 2001). The SMRs of bullfrog tadpoles (*Rana catesbeiana*) from a site contaminated with coal ash were 40-90% higher than those of tadpoles collected from a reference site. Reciprocal transplant experiments showed that tadpoles from eggs maintained in the polluted site had SMRs that were 39-175% higher than those in the reference site, whereas the site where eggs were originally oviposited had no effect on larval SMRs. Higher SMRs were presumably the result of exposure to coal ash pollution (Rowe et al. 1998). Banded water snakes (*Nerodia fasciata*) from the same site also were studied to determine if SMRs increased due to

contaminant exposure. Analysis of tissues indicated that snakes accumulated some contaminants present within the polluted site and their body burdens were higher than those of snakes from reference sites. Standard metabolic rates of snakes from the polluted site were, on average, 32% higher than SMRs of snakes from reference sites. Elevated SMRs of exposed snakes indicated an increased cost of maintenance and a potentially decreased scope for growth and reproduction (Hopkins et al. 1999).

Behavioral responses also can be used as nonlethal indicators of stress in response to environmental contamination (Newman 1998, Little 1990, and Walker et al. 2001). Behavior is often the end result of several different biochemical and physiological processes, and its alteration can be the result of changes in those processes due to contaminant exposure (Little 1990; Walker et al. 2001). Modification of the behavior of an animal can change its ability to obtain resources and mates and avoid risks, such as predators. These changes can affect the animal's ability to interact with its environment, possibly lending to subsequent effects at population and community levels (Little 1990). Common behaviors assessed in behavioral toxicology studies include: activity level, feeding, performance, predation, learning, and reproductive behavior (Newman 1998).

Several studies have evaluated effects of metal exposure on behavior. For example, Selvi et al. (2003) examined the effects of Cd exposure on adult water frogs (*Rana ridibunda*) and found that swimming behavior was altered compared to that of control frogs as Cd exposure levels increased. At lower concentrations, frogs swam almost exclusively near the water's surface, and, at higher concentrations, swimming speed was severely decreased. Strickler-Shaw and Taylor (1990) evaluated the effects of sublethal exposure to Pb on the acquisition and retention of avoidance learning in green

frog tadpoles (*Rana clamitans*). Tadpoles were maintained in water contaminated with 750 μg/L Pb for six days. The experiment was designed to condition tadpoles to discriminately avoid electric shock and test for the learned association between the paired stimuli of illumination change and electric shock. Lead-exposed tadpoles had increased response times and fewer avoidances than control tadpoles in learning acquisition tests. They also had higher response times and no avoidances in retention tests. Learning the appearance or scent of a predator is important in avoiding the predator. Lead exposure may hinder this process and increase the chance of predation for anuran tadpoles.

Lefcort et al. (1998) assessed the effects of exposure to Cd, Zn, and Pb singly and Cd and Zn mixtures on the anitpredatory behaviors of the Columbia spotted frog (*Rana luteiventris*). Cadmium exposure levels ranged from 10 to 50 mg/L, Zn ranged from 25 to 100 mg/L, and Pb ranged from 1 to 100 mg/L. The Cd-Zn mixtures contained equal concentrations of both metals, which ranged from 1 to 10 mg/L. Unexposed tadpoles used refugia and reduced their activity levels when presented with the odor of a trout, a fish that normally preys on Columbia spotted frog tadpoles. Exposed tadpoles did not seek refuge in the presence of fish odor and or reduce their already low activity levels.

A single study has examined the effects of Pb exposure on the behavior of red-eared slider turtle hatchlings. Burger et al. (1998) injected 3-week-old hatchlings with doses of lead acetate ranging from 0.25 to 2.5 mg/g. They examined the ability of hatchlings to right themselves once flipped on their backs. Larger hatchlings tended to flip over faster, and flipping time was positively correlated with the dose of Pb received.

The Tar Creek Superfund Site

The Tar Creek Superfund Site is located in Ottawa Co., Oklahoma and is inhabited by red-eared slider turtles, among other organisms (Conant and Collins 1998). In 1984, the area was granted Superfund status due to heavy metal contamination, a result of Zn and Pb mining from 1891 to 1970. Today there are 75 million tons of chat, a gravel-like substance left over from mining operations which contains Cd, Zn, and Pb, on the ground surface, (US EPA 2002). Area waters also are contaminated with metals (USGS 2000). When mining operations ceased, the mines filled with water and sulfide minerals dissolved, creating an acidic environment (US EPA 2002). The decrease in pH caused metals to dissolve into solution (Yong 2001 and Siegel 2002), and contaminated water then enters Tar Creek and other bodies of water via natural springs, boreholes, and open mine shafts (EPA 2002).

Effects of Metal Exposure

Organisms living near and in sites such as the Tar Creek Superfund Site potentially are exposed to Cd, Zn, and Pb contamination via groundwater, contact with chat, and airborne particles (US EPA 2002). Different animal species have varying sensitivities and different routes of metal uptake (Goyer 1991). Organisms accumulate Cd, a nonessential metal, via ingestion of contaminated water or food, and Cd may potentially cross the semipermeable eggshells of many reptilian eggs, thus exposing the developing embryo inside (Linder and Grillitsch 2000). Cadmium accumulates primarily in kidney and liver (Wren et al. 1995) and can replace Zn biochemically (Sparks 2003). It causes kidney (Sparks 2003) and liver damage (Goyer 1991). Accumulation of Cd

increases with age (Goyer 1991; Wren et al. 1995). Its biological half-time in organisms can be as long as ten years, and it is excreted via feces and urine (Friberg et al. 1979).

Zinc is an essential metal that is toxic in high concentrations. As an essential metal, it is an important component in many metalloenzymes and aids in wound healing (Goyer 1991, Sparks 2003). In excessive amounts, it can cause gastrointestinal stress in humans (Goyer 1991) and be lethal to plants and litter invertebrates (Beyer and Storm 1995). It can decrease liver catalase and cytochrome oxidase activity and inhibit respiration of isolated liver mitochondria (Subcommittee on Zinc 1979). Exposure to high levels can negatively effect growth rate and food intake (Elinder and Piscator 1979). Young animals seem more susceptible to Zn than older ones. However, its toxic effects are rarely seen, or rather rarely properly diagnosed, in larger wildlife (Beyer and Storm 1995). The biological half-time of Zn ranges from 150 to 500 days, and the metal is excreted primarily via the gastrointestinal tract (Elinder and Piscator 1979).

Lead is a highly toxic nonessential metal (Pain 1995; Goyer 1991) with adverse neurological effects (Goyer 1991, EPA 2002, and Sparks 2003) and the ability to inhibit activity of delta-aminolevulinic acid dehydratase (ALAD), an enzyme necessary for the second step of heme synthesis, in mammals (Goyer 1991). Exposure is detrimental to unborn children in that it shortens gestation and decreases birth weight. Exposed children also demonstrate learning difficulties and decreased cognitive abilities (Goyer 1991 and EPA 2002). Lead exposure also can cause anemia and kidney disease (Spraks 2003).

Many of the same effects also have been seen in wildlife. As previously mentioned, lead-exposed green frog tadpoles exhibited reduced performance as compared to reference tadpoles in discriminate avoidance learning tests (Strickler-Shaw

and Taylor 1990). Exposed hatchling red-eared sliders exhibited decreased survival compared to reference hatchlings. They also took longer righting themselves after being flipped over, which is an important predator avoidance skill (Burger et al. 1998). Overmann and Krajicek (1995) analyzed blood from snapping turtles for levels of ALAD and found that activity was negatively correlated with levels of Pb in tissues.

Objectives

The overall objective of this study was to better understand how reptiles such as the red-eared slider might be used to monitor metals at localities such as the Tar Creek Superfund site, and how these contaminants might impact resident populations of turtles. I examined effects of exposure of *T. scripta* eggs to Cd, Zn, and Pb throughout incubation on metal-contaminated substrates or via applications of metal solutions. The use of red-eared slider eggs to biomonitor environmental metal contamination is especially intriguing because there are few studies evaluating controlled exposure of reptile eggs to toxic elements (Linder and Grillitsch 2000). Because eggs are laid in terrestrial nests where they are in contact with substrate (Congdon and Gibbons 1990), the exchange of water with the surrounding substrate (Packard et al. 1987) provides the potential for uptake of contaminants dissolved in that water (Linder and Grillitsch 2000).

I evaluated energetic responses of red-eared sliders to Cd, Zn, and Pb exposure during and after embryonic development to gain insight into effects on energy use. Eggs are especially good subjects for metabolic rate measurements because they are relatively closed systems with respect to energy flow. Thus, effects of activity and uncontrolled feeding do not confound interpretation of egg metabolic rates (Vleck and Hoyt 1991). Changes in the energy allotment by hatchlings can alter the dynamics of the entire

population by changing the hatchlings' growth, survival, and reproduction (Newman 1998).

I also examined two hatchling behaviors, swimming speed and ability to right themselves once flipped on their backs, to gain additional information on physiological and biochemical processes that may be altered by metal exposure and accumulation and the potential survival of the organisms in the wild. The abilities to swim quickly and right themselves once placed on their backs are important skills for the survival of red-eared slider hatchlings. Swim speed is important in the acquisition of food, predator avoidance, and ability to seek refuge; the faster an individual is, the better it will perform these activities. The ability of a hatchling to right itself after being flipped on its back is important for avoiding death due to desiccation or predation.

The toxicity of complex mixtures of contaminants is also understudied (McCarthy and Shugart 1990). This study may produce useful information on the effects of Cd, Zn, and Pb mixtures, such as those found within the soil in the Tar Creek drainage. If red-eared slider turtles are effective biomonitors of environmental contamination, then their use may provide a relevant measure of the bioavailability of metals, which can be used to assess ecological and human health risks emanating from the contaminated site (McCarthy and Shugart 1990).

Specific objectives were to:

- Evaluate the utility of *T. scripta* eggs and hatchlings as biomonitors of terrestrial metal contamination.

- Determine if *T. scripta* embryos can accumulate Cd, Zn, and Pb through the eggshells from incubation substrates or application of metal solutions and whether eggshells or egg contents accumulate higher concentrations of the metals.
- Evaluate the effects of metal contamination in incubation substrate on the RMRs of developing embryos.
- Determine how much caloric energy contained within the yolk sac is consumed during embryonic development and whether Cd, Zn, and Pb contamination has an effect on that energy consumption.
- Evaluate effects of varying levels of metal contamination in incubation substrate on hatching success and time to hatching of eggs.
- Evaluate effects of embryonic Cd, Zn, and Pb exposure on birth mass of hatchlings.
- Evaluate effects of incubation substrate on whole body metal levels of hatchlings and determine if preferential accumulation occurred in the eggshell, turtle shell (carpace and plastron), or soft tissue (soft tissue and bone).
- Determine if metal-exposed hatchlings have different RMRs than hatchlings from eggs incubated in clean substrate.
- Determine if embryonic metal exposure has effects on the behavior and potential survival of red-eared slider hatchlings.

MATERIALS AND METHODS

Substrate Collection

Contaminated natural substrates were collected from randomly selected locations at two sites located within the Tar Creek Superfund Site in Ottawa Co., Oklahoma. Samples were collected with an acid-washed polypropylene plastic spade in April 2002. Sufficient sample was collected to fill an opaque 20-L polypropylene bucket, which was then covered with a lid and transported back to the laboratory. Substrates were held at room temperature before being used in experiments.

The selected sites were two mining impacted areas within the Tri-State Mining area. The first site, Catholic 40 (CF), is located on land owned by the Quapaw Tribe of Oklahoma just east of Beaver Creek and north of E0050 Road between State Route 137 and Lincolnville, OK (Figure 1). The 40-acre (16.2 ha) site consists of a riparian zone along Beaver Creek interspersed with chat piles and is inhabited by a population of red-eared slider turtles.

The second site, known as the Douthat Settling Pond (DSP), is located south of E0040 Road about 1 km west of the bridge crossing Tar Creek (Figure 1). It is presumably named for the now defunct mining town of Douthat, Oklahoma, which was located in the vicinity of the pond (Schehrer 2000). The land was owned by Central Mill and operated by Eagle-Picher, which processed most of the ore from the Picher Mining Field (Schehrer 2000; M. Garvin, Tribal Environmental Management Services, College

of Law, University of Tulsa, pers. comm. 2002). Waste waters from the milling processes were released into the pond and subsequently evaporated.

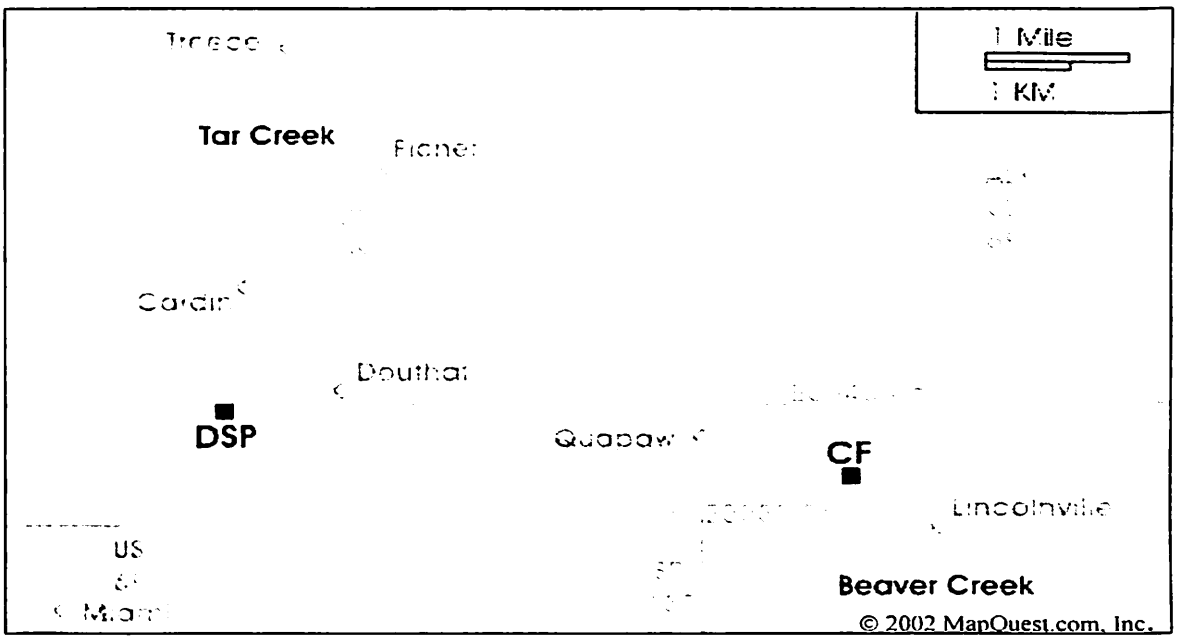

Figure 1. Locations of the Catholic 40 (CF) and Douthat Settling Pond (DSP) sites in Ottawa County, OK.

Egg Study 2002

In addition to the CF- and DSP-contaminated substrates, blasting sand was purchased from a local retailer for use as reference substrate. Sand was selected as a reference because of the similarity of its texture to the natural substrates. All substrates were sieved through a No. 5 (4 mm) USA standard testing sieve to obtain homogenous particle size and then dried at approximately 100°C to constant dry weight.

Water potential analysis

The water potential of each substrate was determined using a modified version of the filter paper method described by Campbell and Gee (1986). Water potential is the free energy of water within a system relative to the free energy of a pool of pure free water and is essentially a measure of the tendency of water to flow from a system of high potential to low potential (Papendick and Campbell 1981). It is dependent upon the pore

volume of the substrate, i.e., how much water it can hold, the sand, silt and clay content of the substrate, and dissolved solids within the substrate (Sparks 2003). *T. scripta* eggshells are semipermeable membranes through which water osmoses (Packard et al. 1987) and dissolved solids diffuse (Linder and Grillitsch 2000). Water potential determines the rates of osmosis and diffusion and, thus, was kept equal to prevent any confounding effects of some eggs absorbing or losing more water than others.

Sets of 5 100-g (± 0.1 g) samples of substrate were mixed with known amounts of distilled water and allowed to equilibrate for at least 18 hours at 29°C in airtight plastic containers. After equilibration, the 100-g samples were placed in 1 liter metal paint cans. Bottoms of the paint cans were lined with polyethylene disks cut to the diameter of the can, disks of Schleicher and Schuell no. 589 (VWR, West Chester, PA, USA) white ribbon filter paper with diameters slightly smaller than the can, and disks of paper towel cut to the same diameter as the can, respectively. The filter paper was treated with a 3% pentachlorophenol solution (Fisher Scientific, Pittsburgh, PA, USA) in ethanol and allowed to air dry to prevent its degradation by microbes living in the soil (Campbell and Gee 1986). Cans were then held at 29°C for 48 to 168 hours to allow water in the substrate to equilibrate with the filter paper. Immediately upon removing the cans from the incubator, the filter paper was weighed to the nearest 0.0001 g and the water potential of each sample was calculated according to the moisture curves devised by Campbell and Gee (1986). Water potential curves were constructed for each substrate and the amount of distilled water per gram of each substrate producing a water potential of −1.5 bars was interpolated.

Substrate characterization

Substrates were analyzed for texture, texture class (% percent sand, silt, and clay), pH, total soluble salts (TSS), and percent organic matter (OM). Analyses were performed by the Soil, Water & Forage Analytical Laboratory at Oklahoma State University, Stillwater, Oklahoma.

Substrate metal analyses

Total Cd, Zn, and Pb concentrations of sand, CF, and DSP samples were determined using US EPA Method 3051 (1994) for substrate digestion and US EPA Method 6010 (1996) for determination of metal concentrations within the filtrate. Analyses were performed by Accurate Environmental Laboratories, Stillwater, Oklahoma.

Additionally, samples were analyzed for exchangeable and readily soluble concentrations of Cd, Zn, and Pb using a modified version of the first step of the Potentially BioAvailable Sequential Extraction Procedure described by Basta and Gradwhol (2000). Bioavailability analyses also were performed by Accurate Environmental Laboratories, Stillwater, Oklahoma. Substrate samples weighing approximately 1 g (weighed to the nearest 0.001 g) were mixed with 20 mL 0.1 molar $Ca(NO_3)_2$ (Spectrum, Gardena, CA, USA) and shaken on a reciprocal mixer for 16 hours. Samples were centrifuged at 1152 x g and the supernatants were filtered through a 0.45-μm filter. To acidify the filtrate, 1.05 mL concentrated trace-metals grade nitric acid (Fisher Scientific, Pittsburgh, PA, USA) was added. Bioavailable concentrations of Cd, Zn, and Pb within the samples were measured using inductively coupled plasma-atomic emission spectrometry according to US EPA Method 6010 (1996).

Egg collection

Eighty-one eggs were collected from 14 female *T. scripta* captured from Sequoyah National Wildlife Refuge (SNWR), OK. Turtles were trapped using baited hoop nets, and gravid females were maintained in water until transported to the laboratory where they were injected with oxytocin to induce egg laying (Ewert 1979). Fifteen units of oxytocin per kilogram of turtle were injected with a 23-gauge needle on a 1-cc syringe. Each egg was weighed to the nearest 0.01 g on an OHAUS Scout balance and marked with a unique label using a No. 2 pencil indicating the female from which it came. Turtles were returned to SNWR.

Egg exposures

Eggs were subjected to 2 treatment regimes that resulted in metal exposure through either contaminated substrates or topical application of metals in solution (Table 1). Substrate treatments included the blasting sand reference and CF and DSP substrates.

Table 1. Number of eggs in each treatment group for the 2002 study.

	Substrate			Exposure Solution			
Treatment	**Sand**	**CF**	**DSP**	**CPaint**	**LPaint**	**MPaint**	**HPaint**
Number of Eggs	16	17	16	8	8	8	8

The treatments with metal solutions were conducted to ensure exposure of eggs to Cd, Zn, and Pb. They involved direct topical application of metal and blank solutions to the eggshells, followed by incubation in blasting sand. A stock metal solution, which also served as the most highly concentrated solution, was prepared by dissolving cadmium nitrate ($Cd(NO_3)_2 \bullet 4H_2O$, Mallinckrodt, Phillipsburg, NJ, USA), zinc nitrate ($Zn(NO_3)_2 \bullet 6H_2O$, Spectrum, Gardena, CA, USA), and lead nitrate ($Pb(NO_3)_2$, Fisher Scientific, Pittsburgh, PA, USA), in distilled water. This stock solution was diluted to

create the metal solutions for the other 2 experimental treatments (Table 2). Distilled water served as a control. The stock solution was made prior to oviposition of eggs and the other 2 metal solutions were made fresh each week. Each week during the incubation period, the eggs in the 4 treatments were completely submerged in their respective solution for two seconds and returned to the incubation substrate before drying completely.

Table 2. Cd, Zn, and Pb concentrations in metal solutions in 2002 study.

Treatment	Abbreviation	Cd (mg/L)	Zn (mg/L)	Pb (mg/L)
Control Paint	CPaint	0	0	0
Low Paint	LPaint	0.66	85	2
Medium Paint	MPaint	6.6	850	20
High Paint	HPaint	66	8500	200

All eggs were incubated in Sterlite plastic shoeboxes that were 30.8 cm long, 16.5 cm wide, and 7.94 cm deep and filled with 2500 g of incubation substrate. Eggs were incubated at 29.0°C, which is 0.2 C° less than the pivotal temperature that produces approximately a 50:50 ratio of male to female slider turtles (Willingham and Crews 1999). The shoeboxes with lids were not airtight, and aluminum foil was placed between the shoebox and the lid to reduce the amount of evaporative water loss during incubation. Shoeboxes were rotated daily to avoid potential effects of temperature gradients within the incubator.

Each clutch of eggs was distributed across all 7 treatments to account for clutch effects in the development of the eggs. Eggs were evenly spaced throughout the box and placed such that the unique marking was always facing up. They were nestled in the substrate such that only their lower halves were in contact with the substrate.

Enough water was added to each shoebox to attain a water potential of −1.5 bars and allowed to equilibrate for 24 hours. Eggs were placed in their respective boxes and then boxes were weighed to determine the initial mass of the substrate, water and eggs combined. Twice weekly, boxes were placed on a scale and distilled water was added to the box until the weight of the box matched the initial mass, thereby maintaining hydric conditions at a water potential of −1.5 bars throughout the incubation period.

Initial egg characterization

Ten eggs were randomly selected before the incubation period for determination of caloric content of yolk sacs and initial metal levels. Eggs were stored at -70°C prior to analysis, and then. Eggshells were cut away and saved for later metal analyses. As egg contents thawed, the white yolk, albumen, and chalaza portions were carefully separated from the yellow yolk and discarded. Eggshells and yolk sacs were placed in pre-labeled, pre-weighed 100 mL glass beakers and dried at 80°C until constant dry weight. Dried egg yolks were homogenized by grinding and compressed into 0.2 g (± 0.05 g), 6.35 mm diameter pellets with a Parr pellet press (Moline, IL, USA) and then exploded with a Parr 1425 Semi-micro bomb calorimeter (Moline, IL, USA). After exploding each sample, caloric content was calculated for the entire yolk sac using equations provided in the instruction manual. The caloric content of the 10 yolk sacs was then averaged.

Ten additional eggs were similarly stored at -70°C for determination of initial Cd, Zn, and Pb concentrations within eggshells and eggs contents separately. Eggs were removed from the freezer and the eggshells cut away. Eggshells and egg contents were placed in pre-labeled, pre-weighed 100 mL glass beakers and dried at 80°C until constant dry weight. Entire samples were digested via a modification of the method described by

Sahoo et al. (1996) Samples were kept in the 100-mL glass beakers in which they were dried for digestion and 15 mL of concentrated metals-grade HNO_3 (Fisher Scientific, Pittsburgh, PA, USA) was added to each sample. Samples were heated to 80 ± 5°C until dry and then reconstituted with 30% H_2O_2 (VWR, West Chester, PA, USA) and heated to 70 ± 5°C for an additional 30 to 90 minutes. Five mL of concentrated metals-grade HNO_3 was then added to the mixture, which was subsequently filtered through a 0.45-μm syringe filter and diluted to 100 mL with reagent grade water (RGW). These digests were analyzed using Flame Atomic Absorption spectrometry (FLAA) and Graphite Furnace Atomic Absorption spectrometry (GFAA) on a Perkin Elmer AAnalyst 700 (Appendix). Metal concentrations within the eggshells and contents served as reference points for metal accumulation during development.

Resting metabolic rates of developing embryos

Resting metabolic rates (RMRs) of embryos in each treatment were measured weekly, beginning three weeks post-oviposition, using a modified version of the method described by Peterson and Stone (2000). Sample sizes varied from week to week due to mortality and complications with equipment (Table 3). Eggs were placed in metabolic chambers and incubated at 29°C for 2 to 6 hours. Metabolic chambers were 125-mL plastic containers with screw-on lids. The threads for the lids were greased with vacuum grease to promote an airtight seal. A one-way male stopcock with luer connection was inserted into each lid. Before the containers were sealed, 15 mL air samples were taken from the ambient air with 20-cc syringes equipped with stopcocks. These samples were used for the determination of initial ambient oxygen concentrations. At the end of the 2 to 6 hour period, 15-mL air samples were taken through the stopcock with a 20-cc syringe

also equipped with a stopcock. Oxygen concentrations of all air samples were analyzed using a Sable Systems FC-1 oxygen analyzer (Las Vegas, NV, USA). Ten mL of each air sample was injected into the airflow of the system, which drew air from outside the building at a rate of 100 mL/min. All air was passed through Drierite and Ascarite to remove CO_2 and water, respectively. The amount of oxygen consumed during the 2-6-hour period was then calculated as the difference between the initial and final volumes of oxygen after correcting for chamber volume (Peterson 1990). RMRs were then expressed as milliliters of oxygen consumed per hour (mL O_2/h).

Table 3. The sample size (n) for metabolic rate measurements for each treatment group each week in 2002.

Week	Substrate			Exposure Solution			
	Sand	CF	DSP	CPaint	LPaint	MPaint	HPaint
3	16	17	16	8	8	8	8
4	16	17	15	8	8	8	8
5	15	16	16	8	8	8	8
6	15	16	16	8	8	8	8
7	14	16	16	8	8	8	8
8	14	16	15	8	8	8	8
9	13	16	15	8	8	8	7

The metabolic rate of an organism is a function of an organism's mass. Unfortunately, metabolism and mass are not directly proportional. It is for this reason that we need to know the mass of an organism to correctly interpret its metabolic rate relative to the metabolic rates of other organisms of differing mass (Gordon et al. 1982). In turtle eggs, it is impossible to determine the mass of a developing embryo without dissecting the egg and rendering further measurements impossible. It was assumed that the masses of the developing red-eared slider embryos were essentially the same at the beginning of the study. However, embryo masses increased during development, but not at the same pace because the growth of an embryo within an egg is dependent upon the size of the

egg as well as water intake (Packard et al. 1987). Therefore, the RMR results of slider turtle embryos must be considered cautiously toward the end of the study, as it is impossible to discern if observed effects were due to differences in size, metal exposure, or other sources.

Yolk sac analysis

Four hatchlings per treatment were sacrificed for bomb calorimetry analysis of their internalized yolk sacs to determine embryonic yolk consumption. Hatchlings were decapitated, double pithed, and stored in a –70° C freezer until their internalized yolk sacs were dissected. Yolk sacs were placed in pre-labeled, pre-weighed 50 mL glass beakers and dried at 80° C until constant dry weight was attained. They were subsequently analyzed for caloric content with bomb calorimetry as described for the yolk sacs from the newly laid eggs. The caloric content of the remaining yolk sac was subtracted from the average of the newly laid eggs to determine the approximate amount of yolk-derived caloric energy used by the hatchling during development.

Hatchling Study 2002

Hatching success and time to hatching for each group was recorded. As soon after hatching as possible, neonates were weighed to the nearest 0.01 g to determine birth mass.

Turtle metal analysis

Four or five hatchlings per treatment were sacrificed to determine whole body concentrations of Cd, Zn, and Pb (Table 4). Hatchlings were decapitated, double pithed, and stored in a –70° C freezer until their tissues were separated into turtle shell (specifically the carapace and plastron) and soft tissue (all other body tissue). Eggshells,

turtle shells, and soft tissues from each hatchling were placed into pre-labeled, pre-weighed 100 mL glass beakers and dried at 80°C until constant dry weight was attained. Eggshells from all hatchlings and hard and soft tissues from randomly chosen hatchlings only were digested and analyzed for metals as per the same modified version of the method described by Sahoo et al. (1996) used for newly laid egg contents and shells.

The sample sizes of each component were the same for all metal analyses (Table 4). All hatchlings and untreated eggs were analyzed for Cd, Zn, and Pb. Eggshells from all hatchlings were digested and analyzed for metal concentrations. When the different components were compared within the same treatment groups (i.e., whole turtle versus eggshell and eggshell vs. turtle shell vs. soft tissue), only hatchlings from which all components were obtained were used.

Table 4. Sample sizes (n) for each metal analysis of each component of hatchlings in all treatment groups in 2002.

		Substrate			Exposure Solution			
Treatment	**Untreated**	**Sand**	**CF**	**DSP**	**CPaint**	**LPaint**	**MPaint**	**HPaint**
Eggshell	20	13	14	14	7	8	8	8
Hard Tissue	-	4	5	4	4	4	4	4
Soft Tissue	-	4	5	4	4	4	4	4
Whole Hatchling	10	4	5	4	4	4	4	4

Egg Study 2003

The 2002 study was repeated in 2003 with several modifications.

Egg collection

First, rather than collecting eggs from induced females, 230 *T. scripta* eggs were purchased from Kliebert's Gator and Turtle Farm in Hammond, Louisiana and transported to Oklahoma State University. All eggs were laid on 14 May 2003 and arrived at OSU the next day. Upon arrival, each egg was weighed, randomly assigned to a substrate treatment (200 eggs) and distinctly marked with a No. 2 pencil according to its

substrate treatment and number, or sacrificed (30 eggs). Clutch association of each egg was unknown. Each treatment consisted of a sample size that was ≤ 40 eggs. Within each treatment, 12 eggs were randomly selected for weekly metabolic rate measurements of embryos and subsequent hatchlings, 14 eggs were randomly selected for whole body metal analysis of hatchlings, and 14 were randomly selected for hatchling yolk sac analysis.

Incubation substrates

The eggs were incubated on 5 different substrates; clean blasting sand, blasting sand spiked with metal levels (Table 5) and CF soil. The artificially contaminated substrates were produced by dissolving cadmium sulfate ($3CdSO_4 \bullet 8H_2O$, Fisher Scientific, Pittsburgh, PA, USA), zinc sulfate ($ZnSO_4 \bullet 7H_2O$, Fisher Scientific, Pittsburgh, PA, USA), and lead sulfate ($PbSO_4$, Aldrich, St. Louis, MO, USA), in RGW and then adding the metal solution to 8 kg of dry blasting sand to produce the concentrations of each metal within the substrate for each treatment. For the initial spiking, the 8 kg for each treatment was divided into three equal portions of 2.67 kg. Cadmium sulfate, Zn sulfate, and Pb sulfate of appropriate amounts for the treatment were dissolved in three separate liters of water and added to the 3 portions of sand. Upon addition of the metal solutions to the sand, the mixture was spun at 27 RPM on a rotary mixer for 2 to 6 hours. After mixing, the spiked sand was dried at 95°C until constant mass was attained. The substrate was then subjected to two additional cycles of rewetting. A rewetting cycle consisted of combining the three portions and then dividing the whole into two 4-kg portions. One liter of RGW was added to each 4-kg portion, and the mixture was spun on the rotary mixer for 2 to 6 hours. The 4-kg portions were then

dried at 95°C until constant dry mass. After the second rewetting cycle, the 4-kg portions for each treatment were combined and placed in a 5-gallon plastic bucket. The bucket was rolled down the hallway for 20 minutes to homogenize the substrate.

Table 5. The target concentrations of Cd, Zn, and Pb as a result of the sand spiking process.

Substrate	Cd (mg/kg)	Zn (mg/kg)	Pb (mg/kg)
CS	0	0	0
LS	10	1000	500
MS	25	2500	1250
HS	50	5000	2500

Similar to the 2002 study, substrates were analyzed for total and bioavailable metal concentrations by Accurate Laboratories, Stillwater, OK.

Egg exposures

In contrast to the approach used for incubating the eggs in 2002, individual eggs in 2003 were incubated in 250-mL plastic screw-lid containers drilled with a 1.6 mm diameter hole in the lid to allow air exchange. A similar hole was not required for the shoebox incubation chambers previously used because the shoebox lids were not airtight. Each incubation container was filled with 190 g of substrate, and reagent grade water was added to create a water potential of −1.5 bars and allowed to equilibrate with the substrate. Water potential of each was determined using the filter paper method (Campbell and Gee 1986) described previously. Forty containers were allotted to each substrate treatment (CS, LS, MS, HS, and CF). Eggs were incubated at the pivotal temperature of 29.2°C (Willingham and Crews 1999) and rotated daily in the incubator to avoid the effects of temperature gradients within the incubator. After 1 week, all infertile eggs were removed from the study, resulting in 38, 39, 38, 39, and 40 eggs in the control, CF, LS, MS, and HS treatment groups, respectively. Infertile eggs were identified by the

lack of an opaque white spot of the "top" of the egg, i.e., the side of the egg with the distinct identification marker.

Water lost due to evaporation from each container was replaced weekly similar to the 2002 study. The initial mass of the container, water, and egg combined was known and the weekly mass of the combination was subtracted from that to determine evaporative water loss. During container watering, eggs were removed from their respective containers and weighed to the nearest 0.01 g. Concurrently, water was replaced in the containers and the substrate stirred with a metal spatula to prevent the dissolved metal from diffusing to the bottom of the container.

Initial egg characterization

Fifteen randomly selected, newly laid eggs were sacrificed to determine the average initial caloric content of the yolk, whereas an additional 15 were sacrificed for determination of initial Cd, Zn, and Pb concentrations within eggshells and eggs contents. These eggs were initially stored and then analyzed as described in the 2002 study. Metal concentrations within the eggshells and contents served as reference points for metal accumulation during development.

Resting metabolic rates of developing embryos

Resting metabolic rates of the embryos within the eggs designated for metabolic rate measurements in each treatment were measured weekly as described for the 2002 study. Measurements commenced 20 days post oviposition. Sample sizes for most treatments changed during the course of incubation due to mortality (Table 6).

Table 6. The sample size (n) of each treatment group for weekly embryonic resting metabolic rate measurements in 2003.

Day	Substrate				
	CS	LS	MS	HS	CF
20	11	11	11	12	12
28	11	11	10	12	12
34	10	11	10	12	12
41	10	11	10	12	12
48	10	11	10	11	11
55	10	11	9	9	11

Yolk sac analysis

The hatchlings from the 14 eggs per treatment designated for yolk sac analysis were sacrificed the day they hatched as they were in the 2002 study for the determination of embryonic yolk consumption. Internalized yolk sacs were analyzed as described in the 2002 study.

Hatchling Study 2003

Hatching success and time to hatching for each group was recorded. As soon after hatching as possible, neonates were weighed to the nearest 0.01 g to determine birth mass.

Hatchlings designated for metabolic measurements and behavioral experiments were weighed on day of hatch to assess birth weight, and then placed back into their respective incubation chambers and in the incubator for another 6 to 8 days. This time allowed for complete absorption of their yolk sacs, which are slightly externalized at the time of hatch.

After 6 to 8 days post hatch, hatchlings were moved to one of three Rubbermaid plastic tubs filled with approximately 12 L of water. Water was changed every 10 days. Hatchlings were maintained at equal densities and removed only for metabolic rate

measurements and behavioral experiments. They were kept on a 16:8 hour light/dark cycle. Light was provided by two 75-watt light bulbs. They were not fed until all experiments were completed.

The hatchlings were divided into three groups based on their days of hatch. The 9 hatchlings that hatched on days 1 through 3 of hatching were placed in Group 1. Twenty-two hatchlings emerged from their eggs on days 4 through 6 of hatching and were placed in Group 2. Twenty hatchlings hatched on days 7 through 9 of hatching and were placed in Group 3. Hatchlings were maintained in respective groups for the conduction of metabolic rates and behavioral experiments.

Turtle metal analysis

The hatchlings from the 14 eggs per treatment designated for tissue metal analyses were sacrificed the day they hatched for the determination of whole body concentrations of Cd, Zn, and Pb as occurred in the 2002 study. Hatchlings were sacrificed and dissected as they were in the 2002 study. Metal analyses of eggshells, hard tissue, and soft tissue were conducted as they were in the 2002 study. In contrast to the 2002 study, only hatchlings from which eggshell, hard tissue, and soft tissue could be obtained were used for metal analyses and comparisons. Similarly, only untreated eggs from which eggshells and contents could be obtained were used for metal analyses and comparisons. Sample sizes are the same for all components within each treatment group unless otherwise specified. Actual sample sizes for each treatment group differed from 14 due to mortality during development and mishandling of digested samples (Table 7).

Table 7. Sample sizes (n) for metal analyses of each component of hatchlings from all treatment groups in 2003.

		Substrate				
Treatment	**Untreated**	**CS**	**LS**	**MS**	**HS**	**CF**
Eggshell	15	9	13	12	13	12
Hard Tissue	-	9	13	13	13	12
Soft Tissue	-	9	12	13	13	12
Whole Hatchling	15	9	12	13	13	12

Resting metabolic rates of hatchlings

Resting metabolic rates of the hatchlings from the surviving eggs designated for metabolic rate measurements in each treatment were measured weekly. The control, CF, and LS groups were composed of 11 hatchlings each and the MS and HS groups were composed of 9 hatchlings each. RMR measurements of hatchlings were performed similarly to the RMR measurements of eggs in both the 2002 and 2003 studies; only slight modifications were made. The same metabolic chambers, syringes, and oxygen analyzer were used.

Metabolic measurements commenced 1 week after the mean day of hatch for each group. Hatchlings were removed from their respective tubs, dried, and weighed to the nearest 0.01 g before placement in the metabolic chamber. While inside the metabolic chambers, hatchlings were placed into dark incubator and allowed to acclimate to the 29.2°C temperature for 60 to 90 minutes. After acclimation, the lights in the room were dimmed and the metabolic chambers were removed from the incubator. Ambient air was blown into each chamber with a small hand-held fan and the chambers closed but not sealed. Using a separate 20-cc syringe for each chamber, 20 mL of ambient air was pumped into the chamber and mixed by pumping three times. A 20-mL sample was then

drawn from each chamber for background oxygen concentrations and the chamber was sealed. Chambers and turtles were returned to the incubator for 1 to 2 hours.

After 1 to 2 hours, with the room still dark, the chambers and turtles were removed from the incubator. Fifteen-mL air samples were drawn from each chamber. All air samples, including the background samples, were analyzed as described in the 2002 egg study. Turtles were then returned to their respective tubs.

Swimming speeds

Hatchlings used in RMR measurements also were subjected to 2 behavioral experiments. The first examined swimming speed, whereas the second evaluated ability of the hatchlings to right themselves when flipped over on their backs.

Swimming speed was examined 3 times for each group of turtles. The mean post hatch ages for each trial of each group were 11, 22, and 33 days. The experimental design consisted of a 247-liter tank (122 cm x 47 cm x 43 cm). The tank was filled with approximately 220 L of water (42 cm in depth), and a platform was suspended at the water's surface at one end of the tank. The vertical and horizontal planes of the tank were marked in two-centimeter increments to allow for distance determination.

Turtles were placed at the edge of the platform facing the water and gently prodded with a dissecting probe to force them to dive into the tank if they did not do so of their own volition. Each turtle performed 5 consecutive dives for each trial. The diving trials were recorded using a Canon NTSC ZR45 MC digital video camcorder. The view of the camera ranged from the very edge of the diving platform to the other end of the tank. The first three dives that were acceptable were analyzed for swimming speed. The acceptability of a dive depended on whether turtle swam out away from the diving

platform or swam back underneath the platform. Because the area of the tank beneath the platform was not in the camera's view, dives during which turtles swam underneath the platform were excluded from analyses.

Swimming speed was determined by dividing distance swam by the time taken to swim that distance. The distance swam consisted of a linear, two dimensional vector (vertical and horizontal only) starting at the point of entry into the tank (assumed to be the same for all turtles) and ending when the turtle either hit the bottom or the end of the tank. The vertical and horizontal distances swam by the turtle were determined to the nearest centimeter from the mark reached by the turtle. The Pythagorean theorem was used to calculate the linear distance swam. Time was kept using the counter on the camera, allowing for accuracy to the nearest 0.03 sec while timing. Swim speed was then expressed as centimeters per second (cm/s).

Righting trials

Righting trials were performed 2 times for each group of turtles. The mean post hatch ages for each trial for each group were 16 and 32 days. The experimental design consisted of a wooden grid measuring 40.6 by 40.6 cm and divided into 16 equal sections of 10.2 cm^2 placed on Astroturf. One turtle was placed in each section on its back. The trials were recorded using a Canon NTSC ZR45 MC digital video camcorder. Turtles performed 1 flip per trial.

Trials were analyzed for time it took hatchlings to right themselves after being placed on their backs. Time, again, was kept using the counter on the camera and was expressed in seconds. A time limit of fifteen minutes was imposed on all trials. If the

turtle did not flip within 15 minutes of being placed on its back, it was considered a "did not flip".

Statistical Analyses

Statistical analyses were conducted using SAS (SAS Institute, Inc., Cary, NC, USA). The same statistical tests were performed for corresponding components of the 2002 and 2003 studies. Alpha was equal to 0.05.

Homogeneity of variances was determined for treatment groups involved in each statistical comparison using Levene's Test for Equal Variances (Ott and Longnecker 2001). Depending on the outcome, either actual data values or ranks of those values were used for comparisons. Where applicable, Tukey's *W* Procedure was implemented for means separation (Ott and Longnecker 2001).

Resting metabolic rates of embryos and amount of yolk-derived caloric energy used by hatchlings during development were compared using a parametric one-way ANOVA or a Kruskal-Wallis Nonparametric ANOVA (Ott and Longnecker 2001).

Mean concentrations of Cd, Zn, and Pb found within the eggshells, turtle shells, soft tissues, and whole bodies of hatchlings were compared among treatment groups using Kruskal-Wallis Nonparametric ANOVA (Ott and Longnecker 2001). Eggshell and whole hatchling comparisons included the mean metal concentrations of those components from the untreated eggs.

Metal concentrations found within the whole turtle were compared to the metal concentrations of corresponding eggshells using Wilcoxon's Signed Rank Test (Ott and Longnecker 2001). This comparison included the contents and eggshells of untreated eggs. Concentrations of Cd, Zn, and Pb found within the eggshells, turtle shells and soft

tissues of hatchlings were compared within treatment groups using Friedman's Test (Zar 1999).

An ANCOVA (analysis of covariance) was performed on the log-transformed variables of body mass and amount of oxygen consumed, swim speed and flipping time to determine if relationships between mass and RMR, swimming speed, or flipping time existed and if treatment had an effect on any of the physiological and behavioral variables measured (Ott and Longnecker 2001).

RESULTS

Substrate Characterization

For the 2002 study, each incubation substrate was classified as having an overall sandy texture. Percent sand in the substrates ranged from 87.5 to 100, whereas the silt and clay compositions ranged from 0 to 7.5% (Table 8). Substrate pH ranged from 7.1 to 7.5, and total dissolved salts (TSS) and organic matter (OM) ranged from 288 to 3074 mg/L, and 0.04 to 4.38%, respectively. In 2003, the control sand (CS) and the CF soil were the same substrates as used for 2002. All other substrates were 100 percent sand dosed with varying levels of metals. The substrates ranged in pH from 5.9 to 6.2, total dissolved salts (TSS) ranged from 3570 to 11532 mg/L, while the percent organic matter ranged from 0.07 to 0.08%.

Table 8. Composition, pH, and total salts (TSS), in incubation substrates from 2002 and 2003. OM = Organic matter. DSP = Douthat Settling Pond, CF = Catholic 40, CS = Control sand, LS, MS, HS = low, medium and high spiked sand, respectively.

Substrate	% Sand	% Silt	% Clay	pH	TSS (mg/L)	% OM
2002 Study						
DSP	90.0	5.0	5.0	7.5	3074	4.38
CF	87.5	5.0	7.5	7.1	721	2.40
Sand (CS)	100.0	0.0	0.0	7.4	288	0.04
2003 Study- also included CF and CS as listed for 2002						
LS	100.0	0.0	0.0	6.2	3570	0.08
MS	100.0	0.0	0.0	5.9	5664	0.07
HS	100.0	0.0	0.0	6.0	11532	0.07

Substrate Metal Content

In the 2002 study, control sand had the lowest total or bioavailable metal levels of any of the substrates, with concentrations ranging from below detection for Pb to a total Zn concentration of 6.2 mg/kg (Table 9). Of the 3 metals measured in the natural contaminated substrates (CF and DSP), Zn concentrations were the highest, followed by Pb and then Cd. The bioavailable fraction of metals in each substrate was always substantially less than the total fraction. Interestingly, although the total Zn concentration in the DSP substrate was higher than that for the CF substrate, bioavailable Zn was higher in the latter.

For the spiked substrates from the 2003 study, the actual measured metal concentrations (Table 9) were much lower than the target concentrations (Table 5). Total Cd levels ranged from 5.6 to 19 mg/kg, Zn ranged from 350 to 1100 mg/kg, and Pb ranged from 280 to 1600 mg/kg. As with the natural substrates, the bioavailable fraction of the metals in the spiked sand was usually less than the totals, particularly in the case of Pb. However, for Cd and Zn, some of the treatments had bioavailable levels that were equivalent to (e.g. Cd in MS) or exceeded total values (e.g. Cd and Zn in HS). When differences between total and bioavailable metals in the spiked sand did exist, they were usually less than those observed for the natural substrates (Table 9).

Table 9. Measured total and bioavailable metal concentrations in incubation substrates used to treat the eggs in 2002 and 2003. DSP=Douthat settling pond, CF=Catholic 40, CS=control sand, LS, MS, HS=low, medium and high-spiked sand, respectively. Cells marked with "-" indicate that metal concentrations were below detectable limits. Minimum detection limits were 0.02, 0.098, and 0.4 mg/kg for Cd, Zn, and Pb, respectively.

	Cd (mg/kg)		Zn (mg/kg)		Pb (mg/kg)	
Substrate	**Total**	**Bioavailable**	**Total**	**Bioavailable**	**Total**	**Bioavailable**
2002 Study						
DSP	56	1	9300	10	540	5.2
CF	20	4	3000	120	440	2.8
Sand (CS)	-	0.06	6.2	0.17	-	-
2003 Study - also included CF and CS as described for 2002.						
LS	5.6	5.2	350	240	280	170
MS	12	12	810	680	760	400
HS	19	23	1100	1300	1600	980

Metal Levels in Turtle Eggs and Hatchlings

Cadmium

2002: Accumulation from substrates

In the 2002 study, eggshells from undeveloped eggs had higher mean concentrations of Cd (7.8 μg/g) than did eggshells from eggs incubated on metal-contaminated substrate ($p<0.0001$,Figure 2a). Although not different from each other, eggshell Cd concentrations in the CF and DSP groups (both 2.2 μg/g) were higher than that found in eggshells from the group incubated on sand ($p<0.0001$). The eggshells of the untreated eggs also had higher concentrations of Cd than did their corresponding contents ($p<0.0001$), whereas whole hatchlings incubated on the control, CF, and DSP substrates had higher concentrations of Cd than did their corresponding eggshells (Figure 2a; $p\leq0.0305$).

In contrast to the results for eggshell metal levels, Cd levels in all hatchlings were greater than those in the contents of the undeveloped eggs ($p<0.0001$). There was no difference in Cd levels between whole hatchlings incubated on either CF or DSP substrates, although the average metal levels in both of these groups were higher than those in hatchlings from eggs incubated on the control substrate (sand) (Figure 2a). Hatchlings from the CF and DSP substrates had mean Cd concentrations of 8.7 and 9.0 μg /g dry tissue, respectively, whereas the mean concentration in hatchlings incubated on control substrate was 6.5 μg/g.

Both the whole hatchlings and associated eggshells from the CF and DSP groups contained lower total Cd concentrations than did the incubation substrates. Eggshells from the CF hatchlings also contained lower Cd concentrations than the bioavailable fraction in the CF substrate, although this relationship was reversed for eggshells from DSP eggs (Table 9, Figure 2a).

No significant difference was found among Cd concentrations in the turtle shell (carapace and plastron) of hatchlings incubated on natural contaminated and control substrates (Figure 2b). Concentrations ranged from 14.5 μg/g in hatchlings from the control substrate to 16.9 μg/g in hatchlings from the DSP substrate. However, levels of Cd in the soft tissues (whole hatchling minus the plastron and carapace) of hatchlings incubated on CF and DSP substrates (7.0 and 6.3 μg/g, respectively) were higher than the Cd concentrations found within the soft tissue of hatchlings incubated on control substrate (4.2 μg/g, $p=0.0042$, Figure 2b).

The Cd levels in the turtle shell, soft tissue and eggshell components were different from each other for all substrate treatment groups. In all groups, turtle shell had

the highest average Cd concentrations, soft tissue values were intermediate, and levels in the eggshells were lowest (Figure 2b; $p<0.0001$).

Whole hatchlings and hatchling components (eggshell, turtle shell, and soft tissue) from the control group contained average Cd concentrations that were higher than the total and bioavailable Cd concentrations in the control substrate. For the natural substrates, there were differences in the accumulation patterns between total and bioavailable metals. Specifically, average Cd levels in whole hatchlings and hatchling components from the CF and DSP groups were lower than total values in their respective incubation substrates, but usually higher than the bioavailable fractions. The one exception to this was for the eggshells from the CF group, in which Cd concentrations were lower than the substrate bioavailable fraction (Figure 2a&b).

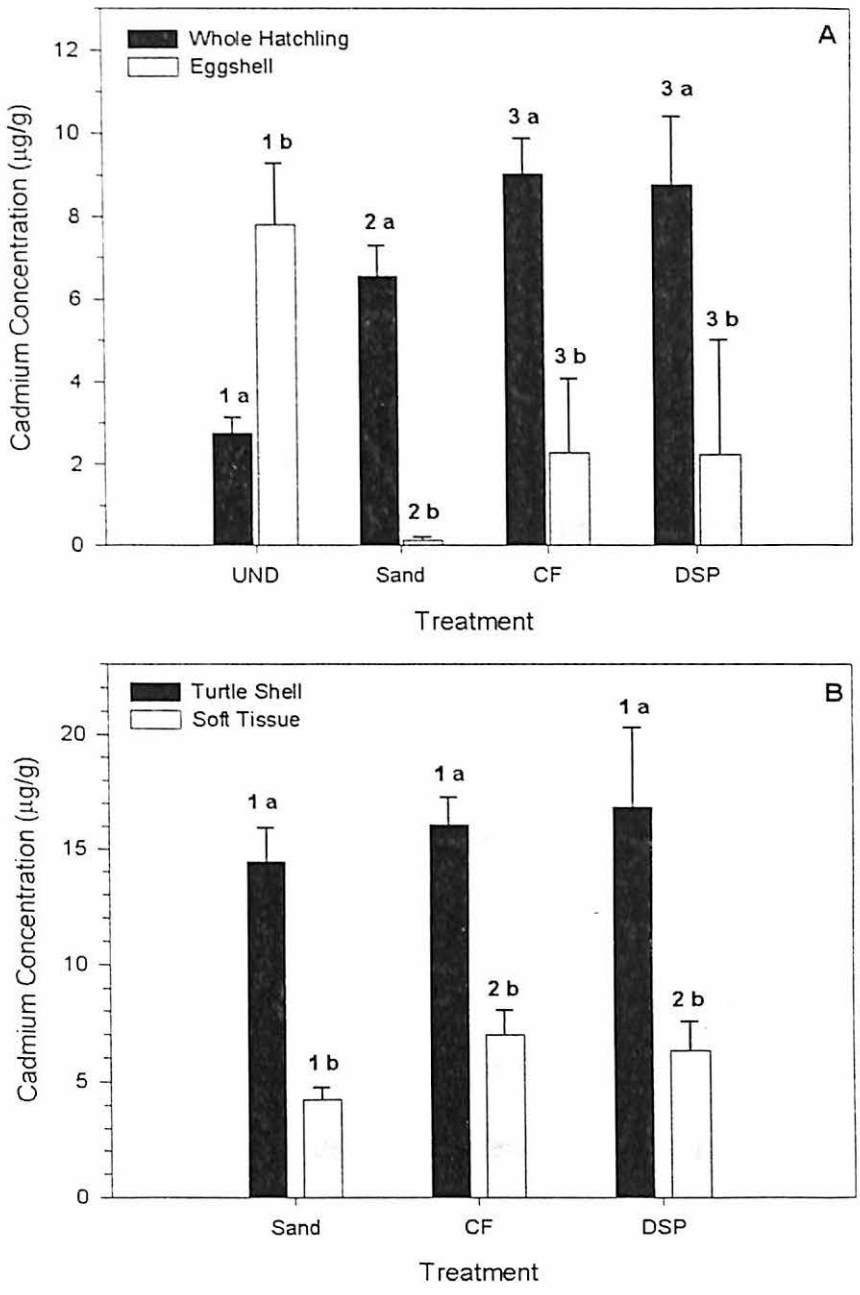

Figure 2. Average (± 1 SD) Cd concentrations found in the undeveloped eggs, eggshells, whole hatchlings (A), and hatchling components (turtle shell and soft tissue) (B) from eggs incubated on reference and natural contaminated substrates in 2002. UND=undeveloped eggs, Sand=reference substrate, CF=Catholic Forty, and DSP=Douthat Settling Pond. Bars with different numbers indicate different ($p<0.05$) Cd concentrations among treatments within a tissue type (whole hatchling or eggshell and turtle shell). Bars with different letters indicate significant differences between Cd concentrations in the different tissues within a treatment. Turtle shell and soft tissue accumulated higher Cd concentrations than eggshells within each treatment.

2002: Accumulation from aqueous applications

As seen with the eggs incubated on contaminated substrates, Cd levels in eggshells from undeveloped eggs (7.8 μg/g) were higher than Cd levels in eggshells from the CPaint, LPaint, and MPaint groups. However, the Cd concentration in the eggshells from hatchlings in the HPaint group were higher than all other groups, including the undeveloped eggs, with an average metal level of 60.9 μg/g. A positive dose-response was apparent for Cd levels in the eggshells, with shells from eggs in the MPaint group higher than those in eggshells from hatchlings in the LPaint and CPaint groups ($p<0.0001$, Figure 3a).

No dose response was apparent for Cd in whole hatchlings from the metal solution treatments as no significant differences were observed in the average Cd concentrations in hatchlings from any of the treatment groups, including the controls. Metal levels in all treated groups were ($p<0.0001$) greater than those measured in the contents of the undeveloped eggs. The average Cd concentrations in the whole hatchlings from the CPaint, LPaint, MPaint, and HPaint groups were 7.5, 7.5, 6.4, and 8.1 μg/g, respectively, vs. 2.7 μg/g in the contents of the undeveloped eggs (Figure 3a).

The eggshells of both the undeveloped eggs and the eggs from the HPaint group contained higher concentrations of Cd than either the contents of the undeveloped eggs or the HPaint hatchlings ($p=0.0003$ and 0.0305, respectively). Conversely, whole hatchlings in the CPaint, LPaint, and MPaint groups had higher concentrations of Cd than did their corresponding eggshells (Figure 3a; $p\leq0.0305$).

No significant difference was found among the Cd concentrations in the turtle shells of hatchlings exposed to metal solutions, with mean concentrations ranging from

14.3 μg/g in the MPaint group to 18.0 μg/g in the HPaint group (Figure 3b). Similarly, no significant difference was observed between Cd concentrations in the soft tissues of hatchlings exposed to metal and control solutions (Figure 3b). Mean Cd concentrations in soft tissue ranged from 4.1 μg/g in the MPaint group to 5.7 μg/g in the HPaint group.

In comparing the levels of Cd between eggshells, turtle shell, and soft tissue, metal levels were found to be higher in the turtle shell as compared to the soft tissue in hatchlings from all treatment groups ($p<0.0001$). In addition, metal levels in the turtle shell were also greater than that in the eggshells of all groups, except the HPaint group, which had highest average metal levels in the eggshells.

Whole hatchlings and components from the CPaint and LPaint groups accumulated Cd to concentrations that were higher than the Cd concentrations of their respective exposure solutions (7.2 and 7.5 μg/g, respectively, compared to 0 and 0.66 mg/L, respectively). Cadmium concentrations in the MPaint and HPaint samples (eggshells, whole hatchlings, and soft tissue) were either similar to metal levels in the respective exposure solutions (e.g. 64.4 μg/g in HPaint eggshells compared to 65.7 mg/L) or less than exposure solutions (all other samples, e.g. 4.06 μg/g in MPaint soft tissue versus 6.6 mg/L; Figure 3a&b). Soft tissue in all hatchlings, except HPaint hatchlings, contained higher Cd concentrations than their respective exposure solutions.

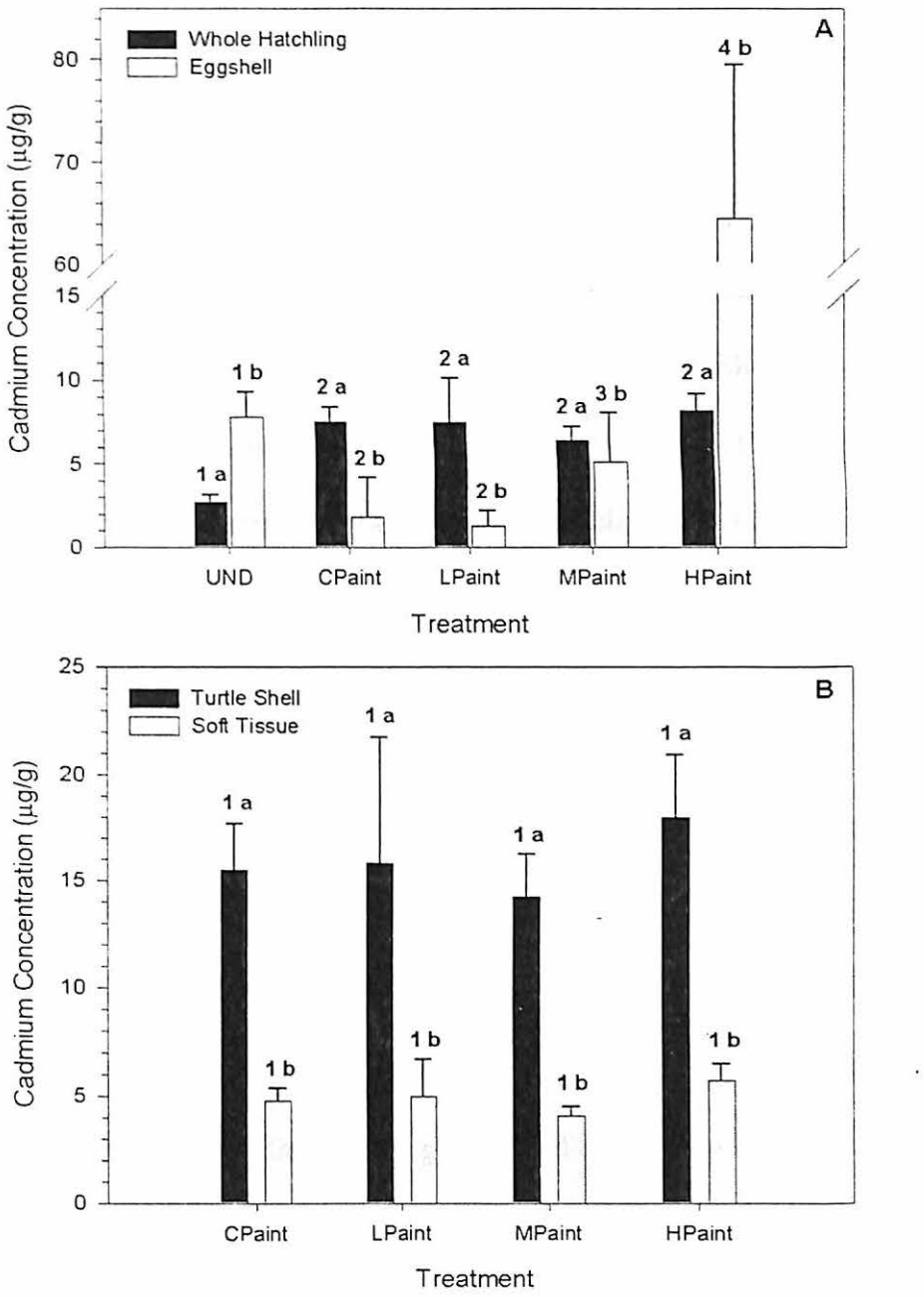

Figure 3. Average (± 1 SD) Cd concentrations found in the undeveloped eggs, eggshells, whole hatchlings (A), and hatchling components (turtle shell and soft tissue) (B) from eggs exposed to metal solutions in 2002. UND=undeveloped eggs, CPaint=control solution, LPaint=low concentration, MPaint=medium concentration, and HPaint=high concentration. Bars with different numbers indicate different ($p<0.05$) Cd concentrations among treatments within a tissue type (whole hatchling or eggshell and turtle shell). Bars with different letters indicate significant differences between Cd concentrations in the different tissues within a treatment. Turtle shell and soft tissue accumulated higher Cd concentrations than eggshells the control and LPaint treatments. Hard tissue accumulated higher Cd concentrations than eggshells in the MPaint treatment. Eggshells accumulated higher Cd concentrations in the HPaint treatment.

2003: Accumulation from substrates

In the 2003 study, the turtle eggs were incubated on both a naturally contaminated substrate (2002 CF) and on sand that had been dosed with metals. Cadmium levels in the eggshells ranged from 6.2 μg/g in the undeveloped eggs to 91.6 μg/g in the HS group, with levels in the eggshells from the dosed sand treatments higher than levels in undeveloped eggs or in eggs that were incubated on control sand or the CF substrate ($p<0.0001$, Figure 4a). In all cases, the average Cd concentrations in the eggshells were greater than metal levels in the egg contents or whole hatchlings ($P<0.0001$, Figure 4a).

Hatchlings from all 2003 treatment groups, including the CF group, accumulated Cd to concentrations that were higher than that found in the undeveloped egg contents ($p<0.0001$). As was observed for the eggshells, metal levels in the hatchlings from the sand-spiked treatments were greater than those from the control group (2.9, 2.7, and 4.1 μg/g, for LS, MS and HS, respectively, vs. 1.03 μg/g in the control group). There was no difference among the Cd concentrations in hatchlings from the LS, MS and HS groups. Cadmium concentrations in hatchlings from the CF group (1.92 μg/g) were similar to Cd concentrations in hatchlings from the control groups and lower than those in the LS, MS, and HS groups.

Cadmium concentrations in the turtle shells ranged from 2.2 μg/g in the CF group to 5.7 μg/g in the HS group (Figure 4b). There was no significant difference in the Cd concentrations of the turtle shells from the control or CF groups, although the levels in the turtle shell from hatchlings in the LS, MS, and HS groups were higher than the concentrations in both groups ($p<0.0001$). Cadmium concentrations in the soft tissues ranged from 0.51 μg/g in the control to 4.1 μg/g in the HS group (Figure 4b). Hatchlings

from the spiked and CF groups accumulated higher levels of Cd in their soft tissues compared to hatchlings from the control group ($p<0.0001$), although there was no difference between these treated groups. In contrast to the comparisons between the 2002 and 2003 data for the eggshells, the Cd concentrations in the hatchling tissues from the control sand and CF groups were lower in 2003 than in 2002.

In comparing metal levels among the eggshells and hatchling tissues, turtle shell contained higher concentrations of Cd than soft tissue for all treatment groups, including the control. Eggshells contained the highest Cd concentrations of all components analyzed ($p<0.0001$).

Whole hatchlings and components from the CS group accumulated Cd to concentrations greater than the total and bioavailable concentrations of Cd in the control substrate. This was also true for the eggshells from all hatchlings exposed to the metal-contaminated substrates. In contrast, Cd levels in the hatchlings and associated tissues from the LS, MS, and HS groups were lower than the total and bioavailable Cd concentrations in their respective substrates. Lastly, whole hatchlings and components from the CF group accumulated Cd to concentrations lower than both the total and bioavailable fractions in the CF substrate. Only the eggshells from the CF group accumulated Cd to a concentration higher than the bioavailable Cd concentration of the incubation substrate (Figure 4a&b, Table 9). These results are the opposite of that observed in 2002, in which the hatchlings from the CF substrate had higher Cd levels than the substrate bioavailable fraction and the eggshells had lower.

In 2002 and 2003, undeveloped eggs contained higher Cd concentrations in eggshells compared to egg contents. In the control and CF hatchlings from 2003, this

relationship was maintained, whereas in the control and CF hatchlings from 2002 the relationship was reversed. The partitioning of Cd in the eggshell, turtle shell, and soft tissue of hatchlings from the control and CF groups also changed from 2002 to 2003. In 2002, turtle shell Cd concentrations exceeded soft tissue Cd concentrations, which exceeded eggshell Cd concentrations for both groups. In 2003, turtle shell concentrations were still higher than soft tissue concentrations, but the eggshell concentrations were the highest of all three in both groups.

There were also several differences between the metal concentrations observed in the eggs and hatchlings between the two years. Eggshells from undeveloped eggs had similar Cd concentrations in 2002 and 2003 (7.8 and 6.2 μg/g, respectively), but the Cd concentrations in the egg contents from 2003 were less than those from 2002 (0.5 μg/g versus 2.8 μg/g). Similarly, the Cd concentrations in the whole hatchlings, turtle shell, and soft tissue from the control and CF groups also were less in 2003 compared to 2002. Control hatchlings from 2003 contained 1.0, 2.4, and 0.5 μg/g Cd in their whole bodies, turtle shell, and soft tissue compared to 6.5, 14.5, and 4.2 μg/g, respectively, in 2002. Hatchlings from the 2003 CF group contained 1.9, 2.2, and 1.8 μg/g Cd in their whole bodies, turtle shell, and soft tissue compared to 9.0, 16.1, and 7.0 μg/g, respectively, in 2002 hatchlings. On the other hand, eggshell Cd concentrations did increase in the control and CF groups from 2002 to 2003 (0.13 and 2.3 μg/g, respectively, compared to 6.6 and 7.8 μg/g, respectively).

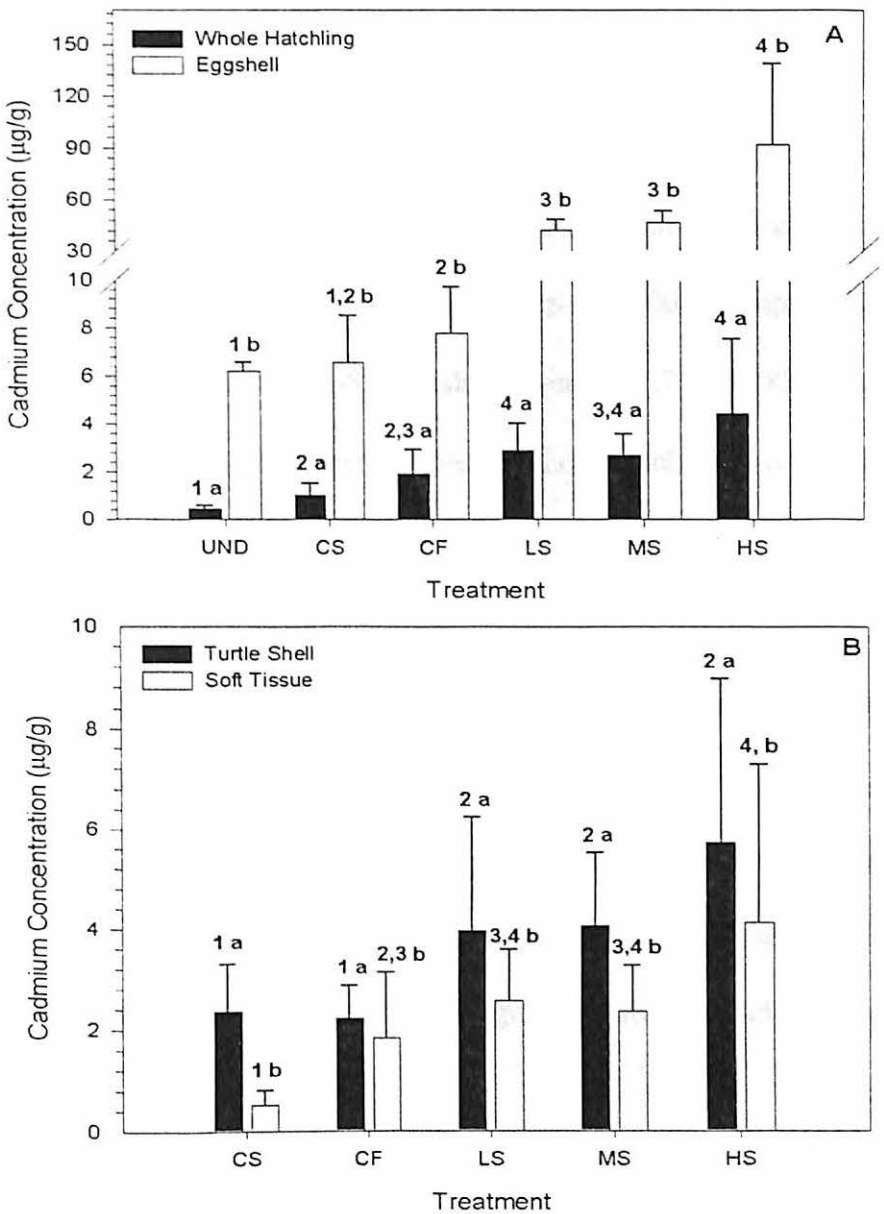

Figure 4. Average (± 1 SD) Cd concentrations found in the undeveloped eggs, eggshells, whole hatchlings (A), and hatchling components (turtle shell and soft tissue) (B) from eggs incubated on artificial and natural contaminated substrates in 2003. UND=undeveloped eggs, CS=control substrate, CF=Catholic Forty, and LS=low substrate, MS=medium substrate, and HS=high substrate. Bars with different numbers indicate different ($p<0.05$) Cd concentrations among treatments within a tissue type (whole hatchling or eggshell and turtle shell). Bars with different letters indicate significant differences between Cd concentrations in the different tissues within a treatment. Eggshells accumulated higher Cd concentrations than turtle shell and soft tissue within each treatment.

Zinc

2002: Accumulation from substrates

Zinc levels in both eggshells and hatchlings from eggs incubated on the CF and DSP substrates were greater than levels in eggshells and contents of undeveloped eggs as well as eggshells and hatchlings from the control group ($p<0.0001$, Figure 5a). The average Zn concentrations in CF and DSP eggshells were 232.7 and 283.6 μg/g, respectively, whereas the average concentrations in whole hatchlings were 423.4 and 403.6 μg/g, respectively. Average Zn concentrations in the undeveloped eggs were 8.4 μg/g in eggshells and 58.4 μg/g in egg contents. Values for the control group were 21.1 and 62.6 μg/g for eggshells and hatchlings, respectively. There were no significant differences between the Zn concentrations of undeveloped eggs and control eggs and hatchlings. Similarly, Zn levels in both of the contaminated groups were not different. Zinc levels were higher in the contents of undeveloped eggs and hatchlings compared to the eggshells, although this difference was only significant for the undeveloped eggs ($p=0.0003$, Figure 5a).

No significant differences were found among Zn concentrations in the turtle shells of hatchlings incubated on natural contaminated and control substrates (Figure 5b). Zinc concentrations ranged from 104.7 μg/g in the control group to 230.2 μg/g in the DSP group. Although not different from each other, the concentrations of Zn found in the soft tissues of hatchlings incubated on CF and DSP substrates (503.6 and 455.8 μg/g, respectively) were higher than the concentrations found within the soft tissue of hatchlings incubated on the control substrate (50.3 μg/g, Figure 5b; $p=0.0056$).

Hatchlings incubated on the control substrate had higher Zn concentrations in turtle shells as compared to soft tissues and eggshells and higher Zn concentrations in soft tissues compared to eggshells ($p<0.0001$). The Zn concentrations in the turtle shell, soft tissue and eggshells of hatchlings from the contaminated substrates were not different, although the average metal levels in the soft tissues of both groups were higher than levels in the other two components (Figure 5b).

Whole hatchlings and components from the control group accumulated Zn to concentrations that were higher than the total and bioavailable concentrations of Zn in the sand substrate, whereas levels in hatchlings and components from the CF and DSP groups were lower than the total and higher than the bioavailable Zn concentrations of their respective incubation substrates (Figure 5a&b and Table 9).

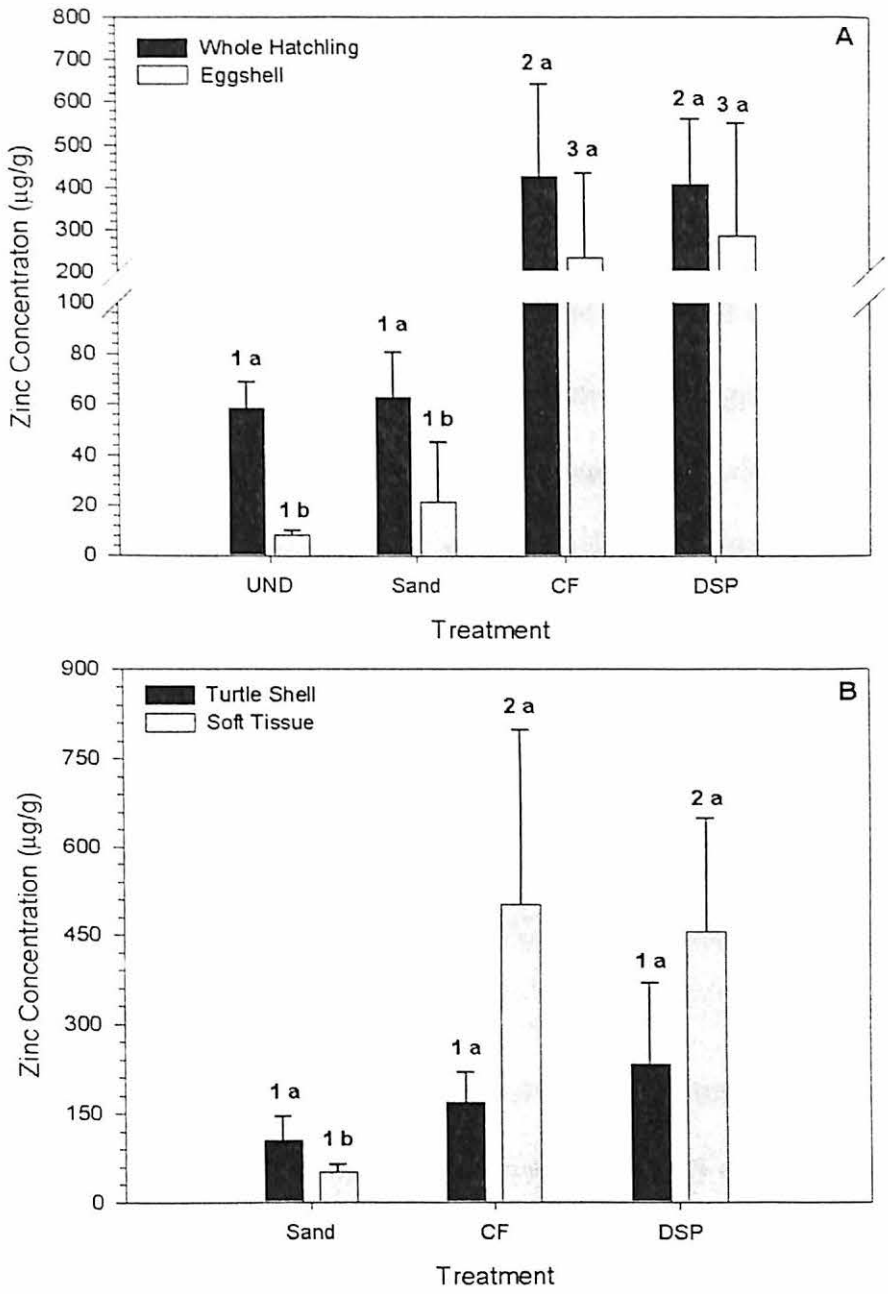

Figure 5. Average (± 1 SD) Zn concentrations found in the undeveloped eggs, eggshells, whole hatchlings (A), and hatchling components (turtle shell and soft tissue) (B) from eggs incubated on reference and natural contaminated substrates in 2002. UND=undeveloped eggs, Sand=reference substrate, CF=Catholic Forty, and DSP=Douthat Settling Pond. Bars with different numbers indicate different ($p<0.05$) Zn concentrations among treatments within a tissue type (whole hatchling or eggshell and turtle shell). Bars with different letters indicate significant differences between Zn concentrations in the different tissues within a treatment. Turtle shell and soft tissue accumulated higher Zn concentrations than eggshells within the control treatment.

2002: Accumulation from aqueous applications

Of the three groups of eggs exposed to Zn through the metal solutions, only hatchlings from the group treated with the highest concentrations (the HPaint group) contained higher whole body Zn levels (233.9 μg/g) than the control hatchlings (61.1 μg/g, p=0.0016, Figure 6a). In contrast, the eggshells from the metal-treated groups all had higher Zn levels than eggshells from controls or undeveloped eggs ($p<0.0001$). A dose-dependent accumulation of Zn was apparent in the eggshells, with controls having an average concentration of 21.2 μg/g, LPaint with 102.1μg/g, MPaint with 696.1 μg/g and HPaint with the highest at 6775.3 μg/g.

The contents of undeveloped eggs had higher concentrations of Zn than did their eggshells (p=0.0003). Similarly, the hatchlings from the control, MPaint and HPaint groups had higher concentrations of Zn as compared to their corresponding eggshells (p=0.0305). There was no significant difference in Zn levels between hatchlings and eggshells from the LPaint group (Figure 6a).

As seen for the whole body Zn levels, the average metal concentrations in the turtle shell and soft tissue from the HPaint treatment were the only ones different from the controls (p=0.0084 and 0.0278, respectively) Concentrations of Zn in the turtle shell and soft tissue of HPaint hatchlings were 244.3 and 196.7μg/g, respectively, whereas the levels in control hatchlings were 56.6 and 74.4 μg/g, respectively (Figure 6b). Zinc levels in the turtle shell from the HPaint groups were also greater than levels in the turtle shell from the LPaint group (p=0.0084).

Hatchlings from the control group accumulated higher concentrations of Zn in their turtle shells as compared to their soft tissues and eggshells. Zinc concentrations in

their soft tissues were higher than Zn concentrations in their eggshells ($p<0.0001$). Hatchlings from the LPaint group accumulated similar concentrations of Zn in their turtle shells, soft tissues, and eggshells. Hatchlings from the MPaint and HPaint groups accumulated the highest concentrations of Zn in their eggshells compared to their turtle shells and soft tissues ($p=0.0345$ and 0.0156, respectively), No significant difference was observed between the turtle shell and soft tissue Zn concentrations in hatchlings from the MPaint group as well as the HPaint.

Whole hatchlings and components from the control group contained Zn concentrations that were higher than the Zn concentration in the control solution. The whole hatchlings, turtle shells, and soft tissues from the LPaint group contained Zn concentrations similar to those found in the LPaint solution. Eggshells from the LPaint group contained higher Zn concentrations compared to the LPaint solution. Whole hatchlings and components from the MPaint and HPaint groups accumulated Zn to concentrations lower than the Zn concentrations in their respective exposure solutions (Figure 6a&b and Table 2).

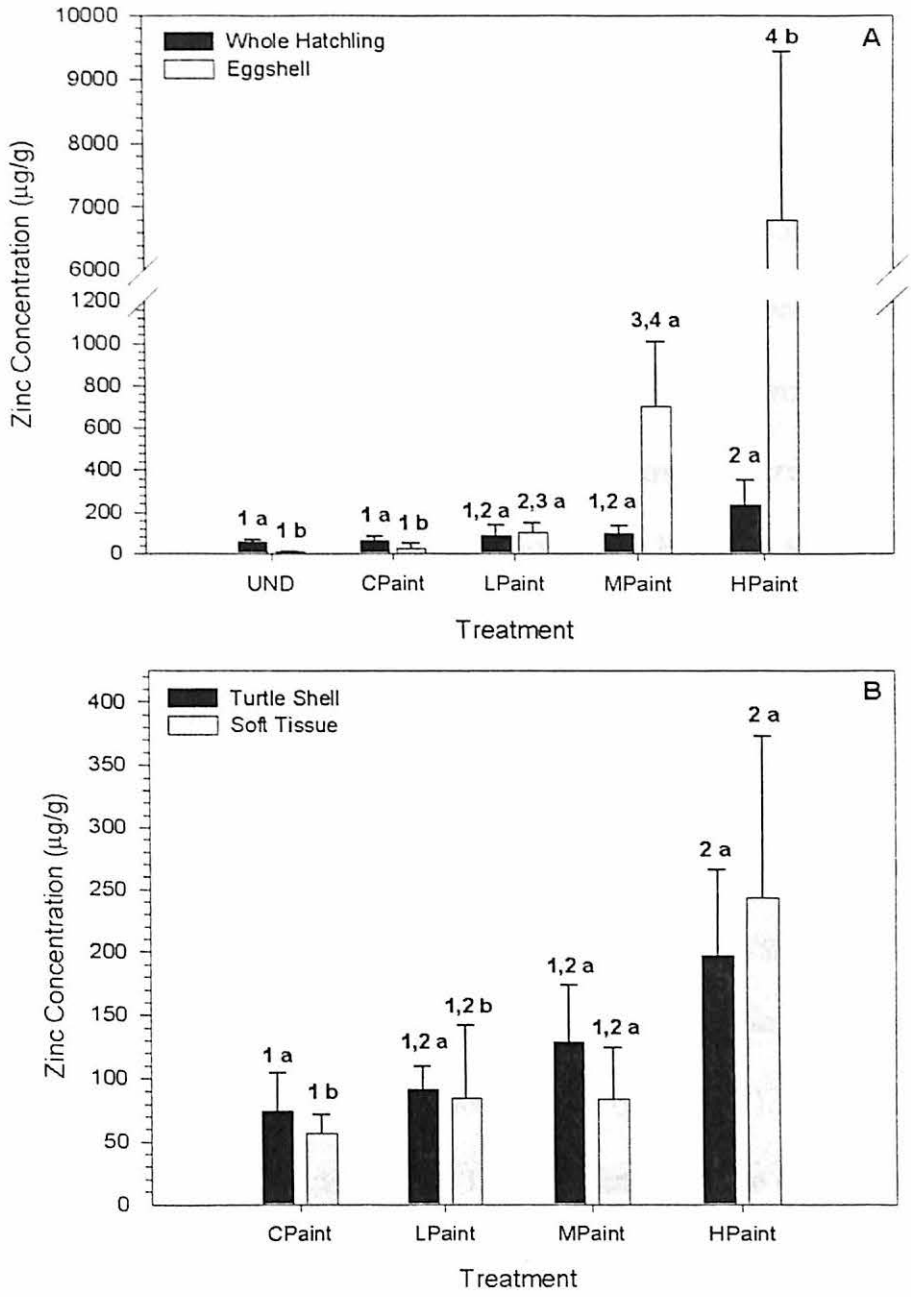

Figure 6. Average (± 1 SD) Zn concentrations found in the undeveloped eggs, eggshells, whole hatchlings (A), and hatchling components (turtle shell and soft tissue) (B) from eggs exposed to metal solutions in 2002. UND=undeveloped eggs, CPaint=control solution, LPaint=low concentration, MPaint=medium concentration, and HPaint=high concentration. Bars with different numbers indicate different ($p<0.05$) Zn concentrations among treatments within a tissue type (whole hatchling or eggshell and turtle shell). Bars with different letters indicate significant differences between Zn concentrations in the different tissues within a treatment. Turtle shell and soft tissue accumulated higher Zn concentrations than eggshells the control treatment. Eggshells accumulated higher Zn concentrations than turtle shell and soft tissue in the LPaint, MPaint, and HPaint treatments.

2003: Accumulation from substrates

In the 2003 study, eggshells from undeveloped eggs were found to contain an average Zn concentration of 9.5 μg/g. Zinc concentrations in eggshells from hatchlings in all treatment groups, including the control, were higher than this initial concentration ($p<0.0001$). Zinc accumulation ranged from 63.1 μg/g in eggshells from hatchlings in the control group to 9,433 μg/g in eggshells from hatchlings in the HS group (Figure 7a). Zinc concentrations in eggshells from the three spiked treatments were higher than those from the CF eggs ($p<0.0001$). Within the spiked groups, Zn levels in the eggshells from the LS and MS groups were not different, although values for the MS eggshells were higher (3,483 vs. 4,104 μg/g). Zinc concentrations in the HS eggshells were at least twice as high as these values and were different from both the LS and MS shells ($p<0.0001$).

The average Zn concentration in the contents of undeveloped eggs was 85.4 μg/g. Although, Zn levels in hatchlings from the control groups (77.0 μg/g) did not differ from this initial level, in ovo exposure to increasing Zn concentrations during development resulted in significant accumulation in all spiked groups ($p<0.0001$). Hatchling Zn concentrations ranged from 165.2 μg/g in the LS treatment group to 422.1 μg/g in the HS group (Figure 7a), with levels in hatchlings from both the CF and HS groups greater than those in the LS group. Although Zn levels in the HS hatchlings also were higher than those in the CF and MS groups, these values were not different due to the relatively high variability in the HS values.

Undeveloped eggs contained higher concentrations of Zn in their contents than in their eggshells ($p<0.0001$). As for the control and CF groups, no difference existed between the Zn concentrations within the hatchlings and the eggshells. In the spiked

groups, eggshells contained higher concentrations of Zn compared to the whole hatchlings (Figure 7a; $p<0.0001$).

Hatchlings from only the spiked treatment groups accumulated higher concentrations of Zn in their turtle shell compared to hatchlings from the control group ($p<0.0001$). Zn concentrations in the spiked treatment groups ranged from 160.0 μg/g in the LS group to 1,185 μg/g in the HS group, whereas turtle shell from hatchlings in the control group had a mean Zn concentration of 96.0 μg/g (Figure 7b). Turtle shell from hatchlings in the HS group also had higher concentrations of Zn than turtle shell from hatchlings in the MS, LS, and CF groups ($p<0.0001$). Mean Zn concentrations in the soft tissues of all hatchlings ranged from 66.9 μg/g in the control group to 258.0 μg/g in the CF group (Figure 7b). All experimental groups had higher Zn concentrations in their soft tissue as compared to the control group ($p<0.0001$), but were not different from each other.

In the control group, the turtle shell contained the highest concentration of Zn ($p=0.0008$), whereas the metal levels in the eggshell and soft tissue were not different from each other (Figure 7b). In the HS, MS, and LS groups, eggshells contained the highest concentrations of Zn compared to the other two components ($p<0.0001$). In the HS group, the turtle shell contained the next high concentration of Zn ($p<0.0001$), whereas in the MS and LS groups, Zn concentrations in the hard and soft tissues did not differ . Within the CF group, soft tissue contained the highest Zn concentrations, followed by eggshell, and the turtle shell contained the lowest ($p<0.0001$).

Whole hatchlings and components from the control group contained Zn concentrations that were greater than the total and bioavailable metal fraction within the

incubation substrate, whereas those from the CF group accumulated Zn to concentrations that were lower than the total in the CF substrate but higher than the bioavailable fraction. In the LS and MS and HS groups, eggshells accumulated Zn to concentrations greater than the total and bioavailable Zn concentrations in their respective substrates. Whole hatchlings, turtle shells, and soft tissues contained Zn levels that were either less than the total and bioavailable fractions or, in the case of the turtle shell for the HS group, similar to the total and bioavailable fractions. Whole hatchlings and soft tissue from the HS group accumulated Zn to concentrations lower than the total and bioavailable Zn concentrations of the incubation substrate. Zinc concentrations of eggshells from the HS group exceeded the total and bioavailable concentrations of the incubation substrate (Figure 7a&b and Table 9).

In 2002 and 2003, the relationships between Zn concentrations in the undeveloped eggs and the whole hatchlings from the CF group and the corresponding eggshells were the same. However, the relationships between those two components in the control groups changed from one year to the next. In 2002, control hatchlings contained higher Zn concentrations than their eggshell, but, in 2003, no difference was observed. The partitioning of Zn in all turtle egg and hatchling components also changed between years in the control and CF groups. In 2002, turtle shell from the control hatchlings contained higher concentrations than soft tissue, which contained higher concentrations than the eggshell. In 2003, turtle shell still contained the highest Zn concentrations, but there was no difference between the soft tissue and eggshell concentrations. As for the CF group, there was no difference among components in 2002, but in 2003, the soft tissue contained the highest Zn concentrations, followed by the eggshell, then the turtle shell.

As observed for cadmium, many of the actual concentrations between years did not agree. Undeveloped eggs in 2003 contained higher Zn concentrations than undeveloped eggs in 2002 (85.4 μg/g versus 58.4 μg/g). Both hatchlings and eggshells from the CF group contained higher Zn concentrations in 2002 compared to 2003 (423.4 and 232.7 μg/g, respectively, versus 228.9 and 176.2 μg/g, respectively).

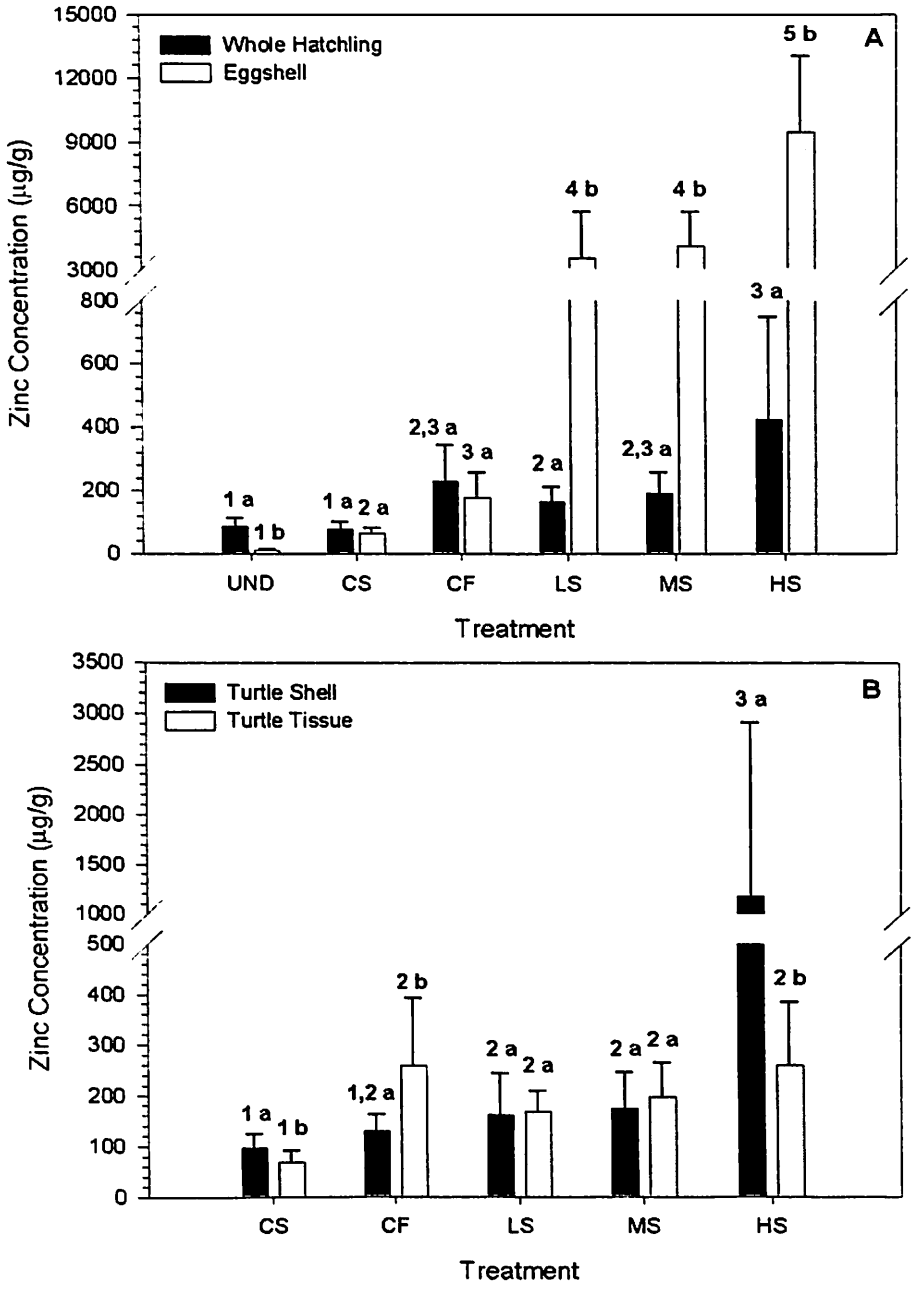

Figure 7. Average (± 1 SD) Zn concentrations found in the undeveloped eggs, eggshells, whole hatchlings (A), and hatchling components (turtle shell and soft tissue) (B) from eggs incubated on artificial and natural contaminated substrates in 2003. UND=undeveloped eggs, CS=control substrate, CF=Catholic Forty, and LS=low substrate, MS=medium substrate, and HS=high substrate. Bars with different numbers indicate different ($p<0.05$) Zn concentrations among treatments within a tissue type (whole hatchling or eggshell and turtle shell). Bars with different letters indicate significant differences between Zn concentrations in the different tissues within a treatment. Turtle shell accumulated Zn to higher concentrations than eggshells in the control treatment. Soft tissue accumulated Zn to higher concentrations in the CF treatment. Eggshells accumulated higher Zn concentrations than turtle shell and soft tissue within LS, MS, and HS treatments.

Lead

2002: Accumulation from substrates

Lead concentrations in the eggshells of eggs incubated on the CF and DSP substrates (17.8 and 13.1 μg/g, respectively) were ($p<0.0001$) higher than levels in the eggshells of undeveloped eggs or control eggs (0.34 and 1.0 μg/g, respectively, Figure 8a). Similarly, hatchlings in the CF and DSP substrate groups accumulated ($p<0.0001$) higher concentrations of Pb (27.8 and 18.9 μg/g, respectively) than undeveloped egg contents (0.38 μg/g) and hatchlings from the control group (1.3 μg/g). No significant differences were observed between the Pb concentrations of undeveloped eggshells and contents, as well as between the Pb concentrations in the eggshells and whole hatchlings from any of the substrate groups (Figure 8a).

No significant differences were observed among the Pb concentrations in the turtle shells of hatchlings incubated on the metal-contaminated and control substrates (Figure 8b). Mean Pb concentrations ranged from 2.2 μg/g in the control group to 7.7 μg/g in the DSP group. Although not different from each other, the concentrations of Pb found within the soft tissues of hatchlings incubated on CF and DSP substrates (50.0 and 30.0 μg/g, respectively) were higher than the Pb concentrations found within the soft tissue of hatchlings incubated on control substrate (0.40 μg/g, $p=0.0042$, Figure 8b).

Lead levels in both the control and DSP hatchlings were similar among eggshells, turtle shells, and soft tissues. The eggshells and soft tissues of hatchlings from the CF group had similar Pb levels, although concentrations in both components were higher than those in the turtle shells ($p=0.0075$).

Lead concentrations in whole hatchlings and components from the control group

were higher than the total and bioavailable concentrations of the control substrate. Hatchlings and components from the CF and DSP groups had Pb concentrations that were lower than the total concentrations of their respective incubation substrates but higher than the bioavailable fractions (Figure 8a&b, Table 9).

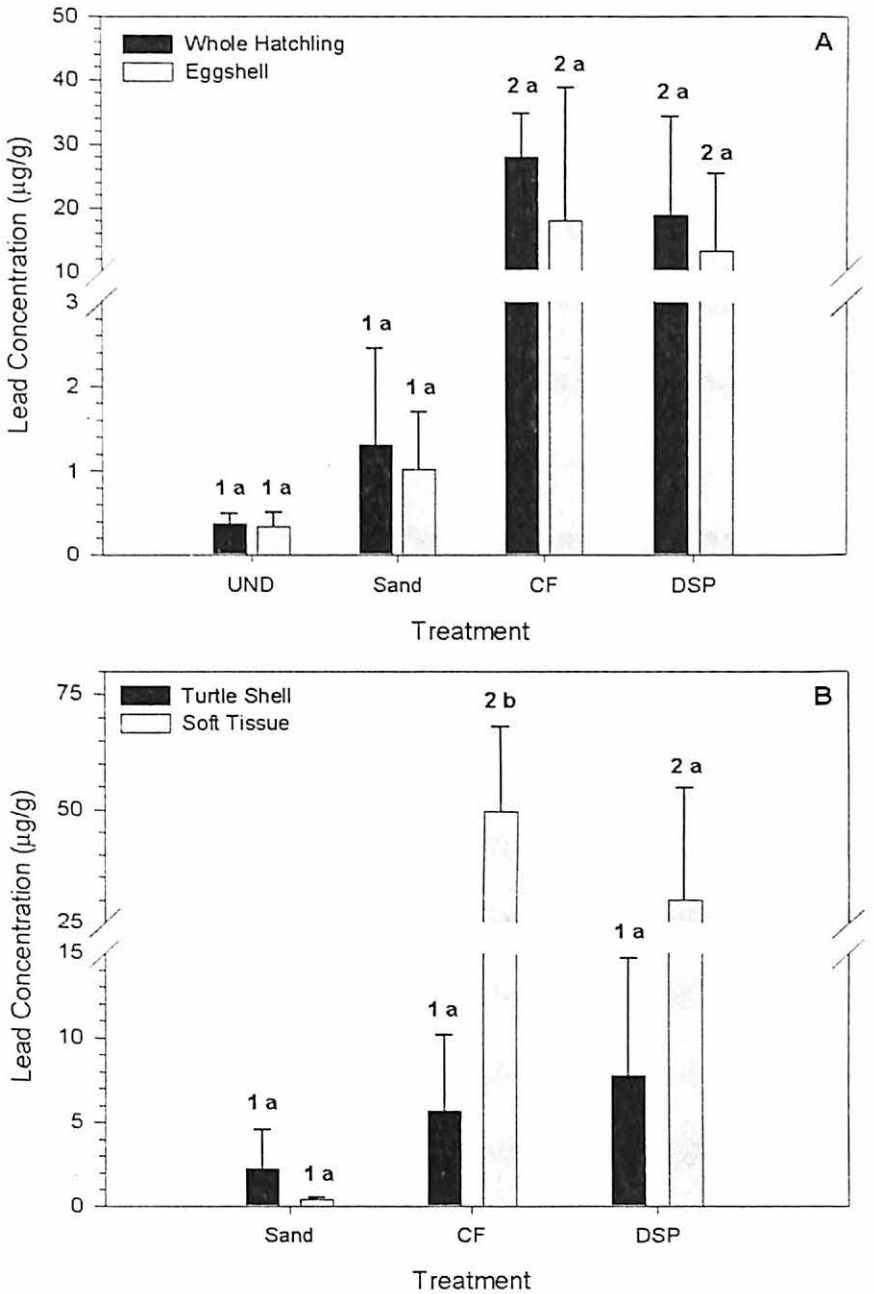

Figure 8. Average (± 1 SD) Pb concentrations found in the undeveloped eggs, eggshells, whole hatchlings (A), and hatchling components (turtle shell and soft tissue) (B) from eggs incubated on reference and natural contaminated substrates in 2002. UND=undeveloped eggs, Sand=reference substrate, CF=Catholic Forty, and DSP=Douthat Settling Pond. Bars with different numbers indicate different ($p<0.05$) Pb concentrations among treatments within a tissue type (whole hatchling or eggshell and turtle shell). Bars with different letters indicate significant differences between Pb concentrations in the different tissues within a treatment. Turtle shell and soft tissue accumulated higher Pb concentrations than eggshells within the control treatment. Eggshells accumulated higher Pb concentrations than turtle shell in the CF treatment.

2002: Accumulation from aqueous applications

Lead concentrations in the eggshells of eggs treated with the metal solution exhibited a dose-dependent increase. Average Pb levels in shells from undeveloped eggs, controls, LPaint, MPaint and HPaint were 0.34, 1.1, 6.8, 42.2 and 390.3 μg/g, respectively (Figure 9a). The Pb concentrations in the HPaint shells were greater ($p<0.0001$) than all others, followed by the LPaint and MPaint groups which did not differ statistically, and then the control and undeveloped eggs.

Hatchlings from the metal solution-treated eggs grouped somewhat differently than the eggshells in terms of Pb concentrations. Levels in whole hatchlings from the MPaint and HPaint groups (4.9 and 12.4 μg/g, respectively) were greater ($p<0.0001$) than average levels in from the control hatchlings (2.0 μg/g), LPaint hatchlings (0.83 μg/g) and undeveloped eggs contents (0.38 μg/g, Figure 9a). However, levels in control hatchlings were also greater ($p<0.0001$) than those in LPaint hatchlings and undeveloped eggs. There was no significant difference between the MPaint and HPaint groups

No significant differences were observed between the Pb concentrations contained in the eggshells and contents of undeveloped eggs and the eggshells and whole bodies of control hatchlings. Hatchlings from the MPaint and HPaint groups contained higher Pb concentrations in their eggshells compared to their whole bodies ($p=0.0305$, Figure 9a).

Turtle shells and soft tissues from hatchlings in the HPaint group (13.0 and 11.9 μg/g, respectively) had higher Pb concentrations than turtle shells and soft tissues from the hatchlings in the LPaint (0.6 and 1.1 μg/g, respectively) and control groups (0.4 and 3.6 μg/g, respectively, $p<0.0001$, Figure 9b). Turtle shell Pb concentrations in the MPaint

group (7.3 μg/g) did not differ from those in the LPaint and control groups, however, soft tissue Pb concentrations in the MPaint group (2.5 μg/g) were higher than those in the LPaint and control groups (0.6 and 0.4 μg/g, respectively, Figure 9b, $p<0.0001$).

Similar to the eggs incubated on the control substrates, hatchlings from the control group treated with deionized water contained comparable concentrations of Pb in their eggshells, turtle shells, and soft tissues. For the treated groups, eggshell Pb levels were consistently greater than those in the body tissues. Hatchlings from the LPaint group contained higher Pb concentrations in their eggshells than their soft tissues, and higher Pb concentrations in their soft tissues than their turtle shells ($p<0.0001$). In both the MPaint and HPaint groups, turtle shells and soft tissues contained similar Pb concentrations that were less than those in the corresponding eggshells ($p=0.0345$ and 0.0156, respectively, Figure 9b).

Whole hatchlings and components from the control group contained Pb concentrations that were higher than the Pb concentration in the exposure solutions, whereas concentrations in the hatchlings, turtle shells, and soft tissues from the LPaint, MPaint, and HPaint groups were lower than the concentrations in their respective exposure solutions. Eggshells from the three experimental groups accumulated Pb to concentrations that exceeded those of their respective exposure solutions (Figure 9a&b and Table 2).

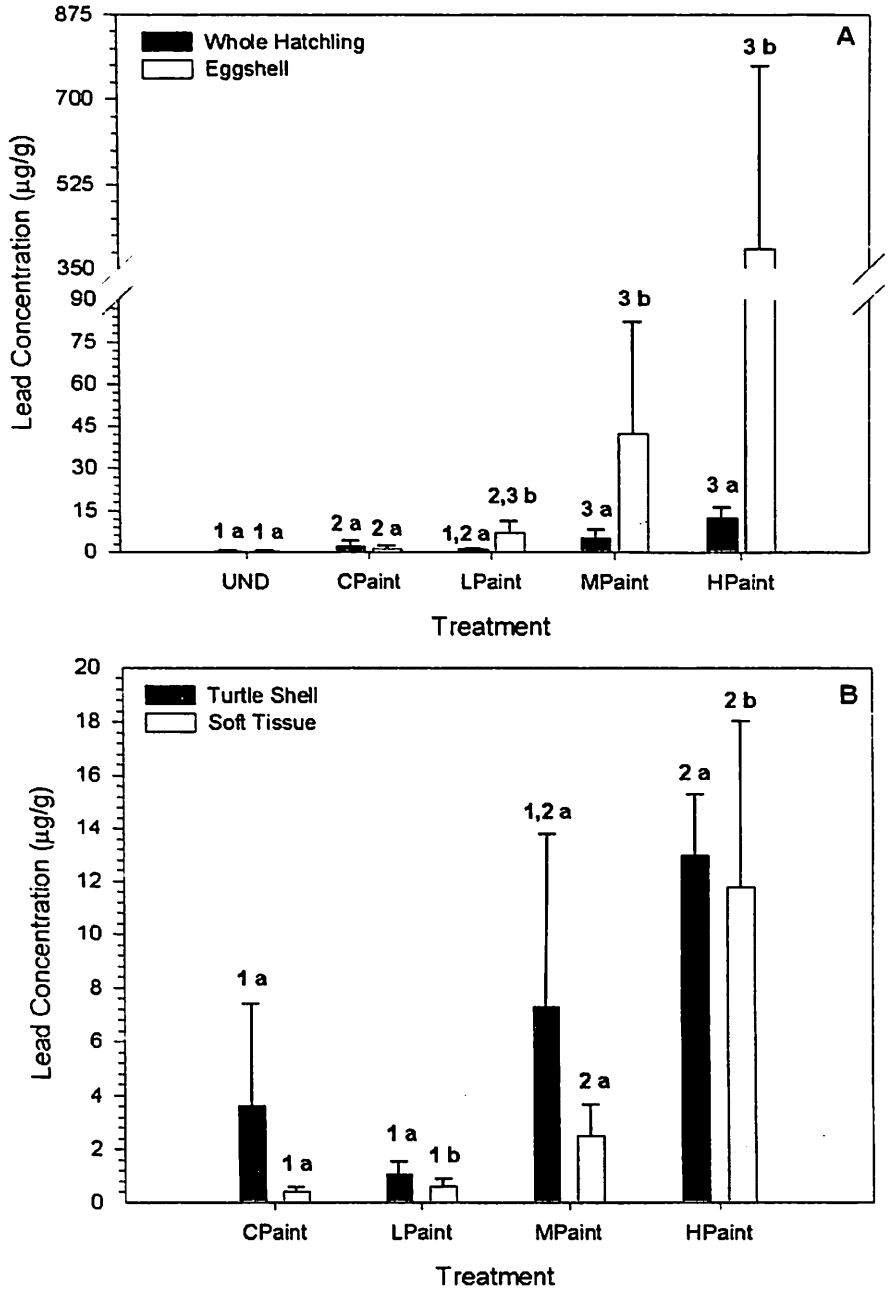

Figure 9. Average (± 1 SD) Pb concentrations found in the undeveloped eggs, eggshells, whole hatchlings (A), and hatchling components (turtle shell and soft tissue) (B) from eggs exposed to metal solutions in 2002. UND=undeveloped eggs, CPaint=control solution, LPaint=low concentration, MPaint=medium concentration, and HPaint=high concentration. Bars with different numbers indicate different ($p<0.05$) Pb concentrations among treatments within a tissue type (whole hatchling or eggshell and turtle shell). Bars with different letters indicate significant differences between Pb concentrations in the different tissues within a treatment. Eggshells accumulated higher Pb concentrations than turtle shell and soft tissue in the LPaint, MPaint, and HPaint treatments.

2003: Accumulation from substrates

In the 2003 study, a dose-dependant uptake of Pb was noted in both the eggshells and the whole hatchlings. Eggshells from undeveloped eggs contained 0.93 μg/g of Pb, whereas eggshells from eggs in all treatment groups had greater mean concentrations of Pb after incubation ($p<0.0001$, Figure 10a). Eggshell Pb concentrations ranged from 5.4 μg/g in the control group to 548.7 in the HS group. Eggshells from hatchlings in the spiked and CF treatment groups accumulated larger concentrations of Pb than eggshells from hatchlings in the control group, and all concentrations were different from each other ($p<0.0001$). The order of treatment groups from lowest to highest was CF, LS, MS, HS with 31.7, 152.9, 310.4 and 548.7 μ g/g, respectively.

Undeveloped egg contents contained an average Pb concentration of 0.4 μg/g. Similar to that observed in eggshells, incubation on the various substrates caused significant ($p<0.0001$) increases in the Pb concentrations of hatchlings from all treatment groups, including the control ($p<0.0001$, Figure 10a). Lead concentrations ranged from 1.5 μg/g in the control group to 233.1 μg/g in the HS group. All treatment groups accumulated greater concentrations of Pb as compared to the control group ($p<0.0001$) with hatchlings from the HS, MS, and LS groups containing higher Pb concentrations than hatchlings in the CF group ($p<0.0001$), and hatchlings from the HS and MS groups containing higher Pb concentrations than hatchlings from the LS group ($p<0.0001$).

Undeveloped eggs contained greater concentrations of Pb in their eggshells compared to their contents ($p=0.0069$). This relationship maintained itself throughout all treatment groups after incubation, with hatchlings from all groups having lower mean Pb concentrations than their respective eggshells (Figure 10a; $p<0.001$).

Hatchlings from the spiked treatment groups accumulated higher Pb concentrations in the turtle shell as compared to hatchlings from the control and CF groups ($p<0.0001$). Lead concentrations in the spiked treatment groups ranged from 34.8 μg/g in the LS group to 124.6 μg/g in the HS group, whereas turtle shell from hatchlings in the control and CF groups contained mean Pb concentrations of only 2.6 and 6.0 μg/g, respectively (Figure 10b). Turtle shell from hatchlings in the HS and MS groups also had higher concentrations of Pb than turtle shell from hatchlings in the LS group and CF groups ($p<0.0001$).

Lead concentrations in the soft tissues of hatchlings in the spiked and CF treatment groups were greater than concentrations in the control group ($p<0.0001$). Mean Pb concentrations ranged from 16.8 μg/g in the CF group to 262.5 μg/g in the HS group, whereas the mean levels in the soft tissue of the control hatchlings was 0.5 μg/g (Figure 10b). Soft tissue Pb concentrations in the CF group (1.8 μg/g) were higher than those in the controls ($p<0.0001$), but lower than those in the soft tissues from all the spiked treatment groups (LS=2.6 μg/g, MS=2.4 μg/g, and HS=4.1 μg/g, $p<0.0001$). Hatchlings in the HS and MS groups contained higher concentrations of Pb in their soft tissue compared to hatchlings in the LS group ($p<0.0001$).

Lead concentrations in the eggshells, turtle shells, and soft tissues of all hatchlings differed from each other (Figure 10b). In the control group, the eggshells contained the highest Pb concentrations followed by the turtle shell, with the soft tissue containing the lowest concentration ($p<0.0001$). Throughout the remaining treatment groups, eggshells still contained the largest highest concentration of Pb, however, the soft tissue contained the next highest, and the turtle shell contained the lowest concentration ($p<0.0001$).

Three different trends with regard to Pb uptake from the substrate were observed in this study. Whole hatchlings and components from the control group contained concentrations of Pb that were higher than the total and bioavailable fractions in the incubation substrate, whereas hatchlings and components from the CF group accumulated Pb to concentrations that were less than the total Pb concentration of the incubation substrate, but greater than the bioavailable Pb concentration. Finally, whole hatchlings from the LS, MS, and HS groups accumulated Pb to concentrations that were less than the total and bioavailable concentrations of their respective incubation substrates (Figure 10a&b and Table 9).

Several of the relationships between the egg and hatchling components changed from 2002 and 2003 within the undeveloped eggs and the control and CF groups. In 2002, the relationship between the Pb concentrations in the undeveloped egg contents and eggshells and whole hatchlings and eggshells was the same for all three groups: undeveloped egg contents and whole hatchlings contained similar Pb concentrations to their corresponding eggshells. However, in 2003, the eggshells from all groups contained higher Pb concentrations than the corresponding egg contents and whole hatchlings. In 2002, there was no difference among the Pb concentrations in the eggshell, turtle shell, and soft tissue of control hatchlings. Eggshell and soft tissue also contained similar concentrations in CF hatchlings from 2002, but they were higher than the Pb concentrations in the turtle shell. In 2003, hatchlings from both the control and CF groups contained the highest Pb concentrations in the eggshells, with the turtle shell containing higher concentrations than the soft tissue in control hatchlings and the soft tissue containing higher concentrations than the turtle shell in CF hatchlings.

Lead concentrations in eggshells from undeveloped egg were similar in 2002 and 2003 (0.3 μg/g and 0.4 μg/g, respectively), but undeveloped egg contents contained higher Pb concentrations in 2003 (0.9 μg/g) than in 2002 (0.4 μg/g). Several differences occurred among egg components from the control and CF groups between both years as well. Eggshells from the control and CF groups in 2002 had lower Pb concentrations than eggshells from the control group in 2003 (1.02 and 17.8 μg/g, respectively, compared to 5.4 and 31.7 μg/g, respectively). However, in 2003, the whole hatchlings and soft tissue from the CF group contained lower Pb concentrations than in 2002. In 2002, whole hatchling and soft tissue Pb concentrations were 27.8 and 50.0 μg/g, respectively, and in 2003 the concentrations were 14.39 and 16.8 μg/g, respectively.

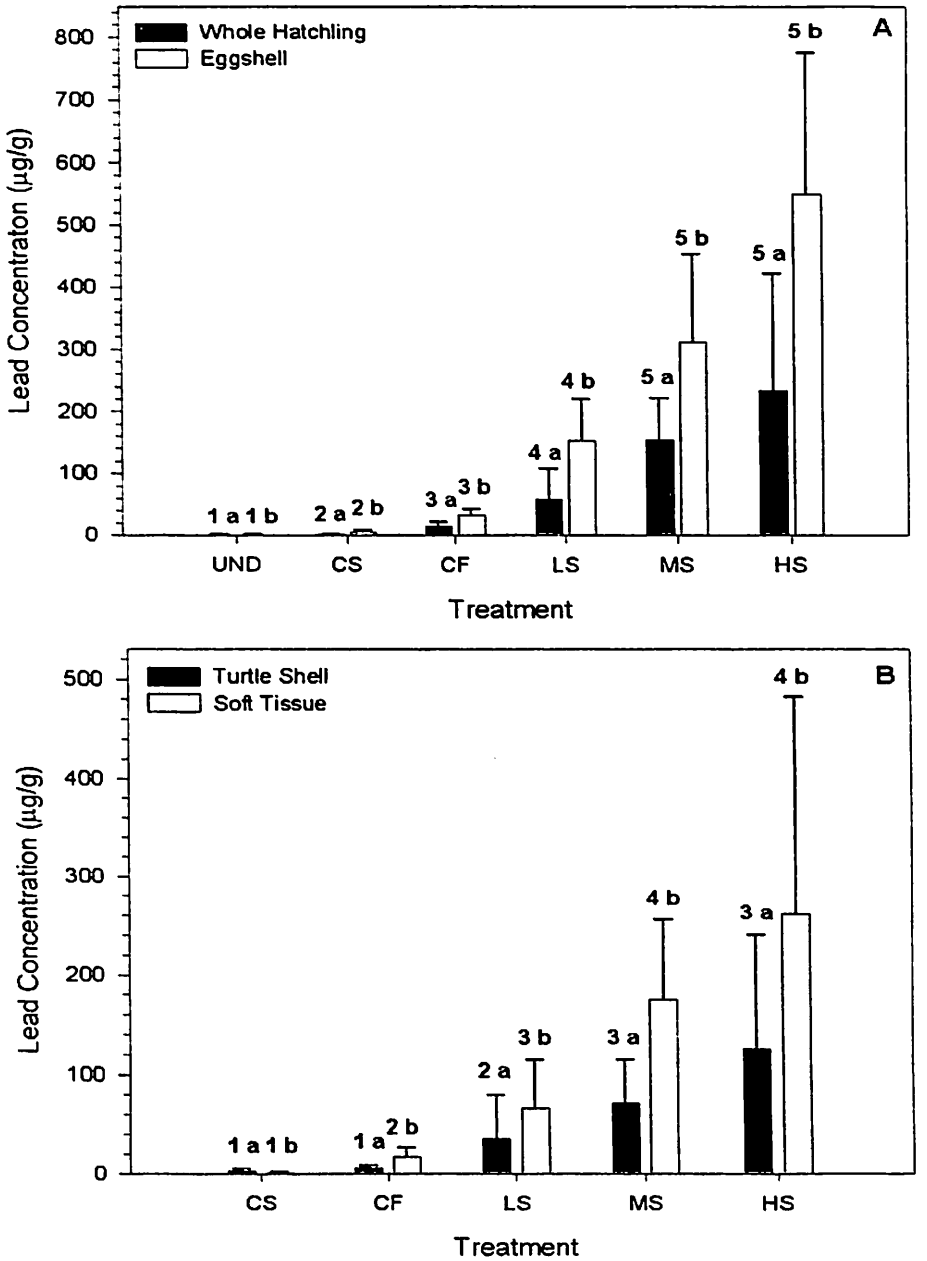

Figure 10. Average (± 1 SD) Pb concentrations found in the undeveloped eggs, eggshells, whole hatchlings (A), and hatchling components (turtle shell and soft tissue) (B) from eggs incubated on artificial and natural contaminated substrates in 2003. UND=undeveloped eggs, CS=control substrate, CF=Catholic Forty, and LS=low substrate, MS=medium substrate, and HS=high substrate. Bars with different numbers indicate different ($p<0.05$) Pb concentrations among treatments within a tissue type (whole hatchling or eggshell and turtle shell). Bars with different letters indicate significant differences between Pb concentrations in the different tissues within a treatment. Eggshells accumulated higher Pb concentrations than turtle shell and soft tissue within all treatments.

Effects of Metal Exposures on Embryos and Hatchlings

Resting metabolic rates of embryos

The mean RMRs (measured in mL O_2/h at 29.0°C in 2002 and 29.2°C in 2003) of embryos from the 2002 and 2003 studies increased throughout development during the weeks measured (Figure 11a-c). Measurements taken at the end of week 3 for the 2002 substrate study indicated that mean oxygen consumption ranged from 0.04 mL O_2/h in the CF group to 0.06 mL O_2/h in the DSP group, whereas measurements during week 9 ranged from 0.84 mL O_2/h in the control group to 0.93 mL O_2/h in the DSP group (Figure 11a). The RMRs among groups did not differ during weeks 3, and 5 through 9. During week 4, embryos incubated on the control substrate consumed oxygen at a higher rate (0.10 mL O_2/h) than did embryos incubated on the CF substrate (0.06 mL O_2/h, p=0.0013).

For the aqueous exposures conducted in 2002, the average RMRs taken at the end of week 3 ranged from 0.04 mL O_2/h in the HPaint group to 0.06 mL O_2/h in the LPaint group, whereas measurements during week 9 ranged from 0.84 mL O_2/h in the HPaint group to 0.92 mL O_2/h in the CPaint group (Figure 11b). The RMRs among groups did not differ during weeks 3, 4, and 7 through 9. During week 5, embryos in the CPaint group had a higher rate of oxygen consumption (0.22 mL O_2/h) than did embryos in the HPaint group (0.18 mL O_2/h, p=0.0275) and during week 6, embryos in the MPaint group consumed more oxygen (0.37 mL O_2/h) than did embryos in the LPaint substrate (0.31 mL O_2/h, p=0.0446).

For the 2003 study, the average RMRs ranged from 0.10 mL O_2/h in the LS group to 0.12 mL O_2/h in the HS group on day 20 post-oviposition, whereas on day 55 post-

oviposition, measurements ranged from 0.95 mL O_2/h in the HS group to 1.19 mL O_2/h in the LS group (Figure 11c). Variability among the groups began to increase toward the end of the incubation, although there were no significant differences among the RMRs of any groups during any period of measurement.

Yolk sac analysis

In 2002, there were no significant differences between the average number of calories used by hatchlings during development among the three substrate treatment groups or the aqueous exposure groups. Among the substrate groups, the average caloric difference between yolk in undeveloped eggs and hatchlings at emergence ranged from 9,921 calories in the DSP group to 11,280 calories in the control group (Table 10). In 2003, hatchlings incubated on the HS substrate consumed fewer calories during incubation than did hatchlings incubated on the control, LS and CF substrates ($p<0.0001$; Table 10).

Table 10. The average (± 1 SD) caloric difference between yolk content of eggs at the start of incubation and yolk sacs of hatchlings at emergence from the eggs in 2002 and 2003. Numbers that do not share at least 1 common letter are different at $\alpha=0.05$.

2002	Substrate			Exposure Solution			
	DSP	CF	Sand	CPaint	LPaint	MPaint	HPaint
Calories Consumed	9922 ± 745	11074 ± 1078	11280 ± 721	10380 ± 463	10412 ± 1393	11315 ± 718	10114 ± 1317
2003	**Incubation Substrate**						
	CS[a]	CF[a]	LS[a]	MS[a, b]	HS[b]		
Calories Consumed	12905 ± 1247	12990 ± 1043	13110 ± 894	12372 ± 1966	11811 ± 1110		

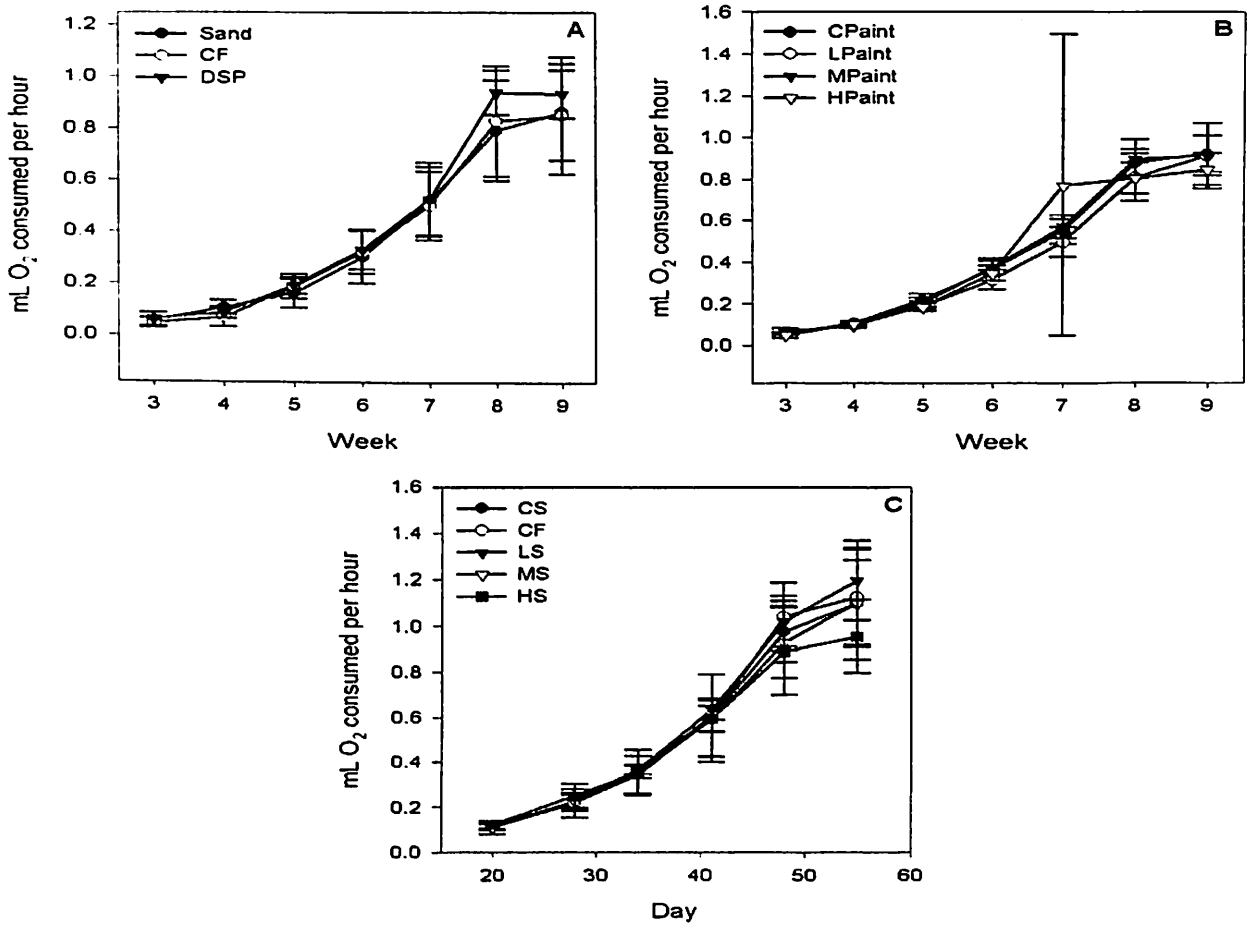

Figure 11. Average (± 1 SD) resting metabolic rate (RMR) of developing embryos from eggs incubated on natural contaminated substrates (A) or exposed to metal solutions (B) in the 2002 study, measured during weeks 3 through 9 after oviposition. C: Average (± 1 SD) RMR of developing embryos from eggs incubated on natural and artificial contaminated substrates in the 2003 study measured on days 20, 28, 34, 41, 48, and 55 after oviposition. UND=undeveloped eggs, Sand=reference substrate, CF=Catholic Forty, and DSP=Douthat Settling Pond (A). UND=undeveloped eggs, CPaint=control solution, LPaint=low concentration, MPaint=medium concentration, and HPaint=high concentration (B). UND=undeveloped eggs, CS=control substrate, CF=Catholic Forty, and LS=low substrate, MS=medium substrate, and HS=high substrate (C).

Hatch effects

No differences were observed in the number of days to hatch or the weight of hatchlings at hatch among any of the treatments in both the 2002 and 2003 studies. In 2002, the mean number of days to hatch ranged from 61 to 64 days and average hatch weight ranged from 7.5 to 8.9 g (Table 11). In 2003, the mean days to hatch ranged from 61 to 62 days and the average weight ranged from 8.2 to 8.7 g (Table 11).

Table 11. The average (± 1 SD) days to hatch and hatch mass for hatchlings in all treatment groups for both years of study.

2002	Substrate			Exposure Solution			
	Sand	**CF**	**DSP**	**CPaint**	**LPaint**	**MPaint**	**HPaint**
Days to Hatch	63.6 ± 2.4	63.0 ± 1.8	62.2 ± 2.3	63.0 ± 2.4	63.3 ± 2.1	63.1 ± 1.4	61.8 ± 1.5
Hatch Mass (g)	8.43 ± 0.97	8.34 ± 0.67	8.81 ± 0.68	8.93 ± 0.55	8.52 ± 1.17	8.80 ± 0.82	7.50 ± 0.45
2003	**Incubation Substrate**						
	CS	**CF**	**LS**	**MS**	**HS**		
Days to Hatch	62.3 ± 2.2	61.9 ± 2.5	61.5 ± 2.3	62.2 ± 2.0	61.4 ± 2.5		
Hatch Mass (g)	8.60 ± 1.00	8.68 ± 1.16	8.47 ± 1.16	8.46 ± 1.19	8.20 ± 1.26		

Resting metabolic rates of hatchlings

Resting metabolic rates of 2003 hatchlings were measured weekly for the first four weeks after hatching. Overall, mass was positively correlated with hatchling RMRs ($p \leq 0.0006$), and at ages 6-8 days, 13-15 days, and 27-29 days, treatment also had significant effects on hatchling RMRs ($p \leq 0.0014$).

At 6-8 days, hatchling RMRs appeared to be influenced in a dose-dependent fashion, with the exception of the CF group. Although not different from each other, hatchlings from the HS and MS groups had higher RMRs than hatchlings from the control and CF groups. RMRs of HS hatchlings also were higher than those of LS

hatchlings, which, in turn, were higher than those of CF hatchlings. The line depicting the relationship between log-transformed mass and metabolic rate for LS hatchlings had a smaller slope and a smaller correlation coefficient than the lines depicting the relationship for control, CF, MS, and HS hatchlings (Table 12, Figure 12). At 13-15 days, the dose relationship between RMRs and substrate metal concentration was not as apparent. RMRs of the hatchlings from the LS and HS groups were higher than those of the control and CF hatchlings but similar to those of MS hatchlings. The slopes comparing log mL O_2/h to log mass and the corresponding correlation coefficients were reduced in the CF and LS groups (Table 12, Figure 13). During the third week of RMR measurement (age 20-22 days), treatment did not have a significant effect on the metabolic rates of any of the hatchlings (Table 12, Figure 14). At age 27-29 days, hatchlings from the HS group again consumed oxygen at a higher rate than hatchlings from all other treatment groups, and hatchlings from the control, CF, LS, and HS groups demonstrated a correlation between mass and RMR (Table 12, Figure 15).

Table 12. Regression equations relating log mass to log oxygen consumption rate (mL O2/h) for all hatchlings combined and individual treatment groups during all periods of hatchling RMR measurement in 2003. y= log mL O_2/h and m=hatchling mass.

Treatment	Equation	R^2
Days 6-8		
All	y = 0.6913 [log(m)] - 1.1287	0.3249
CS	y = 0.6914 [log(m)] - 0.7010	0.4915
CF	y = 0.7117 [log(m)] - 0.7668	0.5552
LS	y = 0.1110 [log(m)] - 0.1479	0.0099
MS	y = 1.1663 [log(m)] - 1.1131	0.4775
HS	y = 1.3908 [log(m)] - 1.2260	0.7654
Days 13-15		
All	y = 0.7504 [log(m)] - 0.7470	0.2199
CS	y = 1.1458 [log(m)] - 1.4480	0.3667
CF	y = 0.6364 [log(m)] - 0.7020	0.1769
LS	y = 0.5165 [log(m)] - 0.4664	0.0599
MS	y = 1.3470 [log(m)] - 1.3085	0.3348
HS	y = 1.5120 [log(m)] - 1.0289	0.6732
Days 20-22		
All	y = 0.8813 [log(m)] - 0.9040	0.2099
CS	y = 2.1440 [log(m)] - 2.0464	0.3612
CF	y = 0.8735 [log(m)] - 0.9335	0.1331
LS	y = 1.5680 [log(m)] - 1.5473	0.4798
MS	y = 1.3061 [log(m)] - 1.3066	0.2319
HS	y = 0.7085 [log(m)] - 0.7214	0.3138
Days 27-29		
All	y = 0.7386 [log(m)] - 0.8851	0.1731
CS	y = 2.5166 [log(m)] - 2.5077	0.4177
CF	y = 0.9022 [log(m)] - 1.0969	0.3760
LS	y = 1.3985 [log(m)] - 1.5234	0.6440
MS	y = 1.0521 [log(m)] - 1.2067	0.2526
HS	y = 1.2002 [log(m)] - 1.1690	0.4782

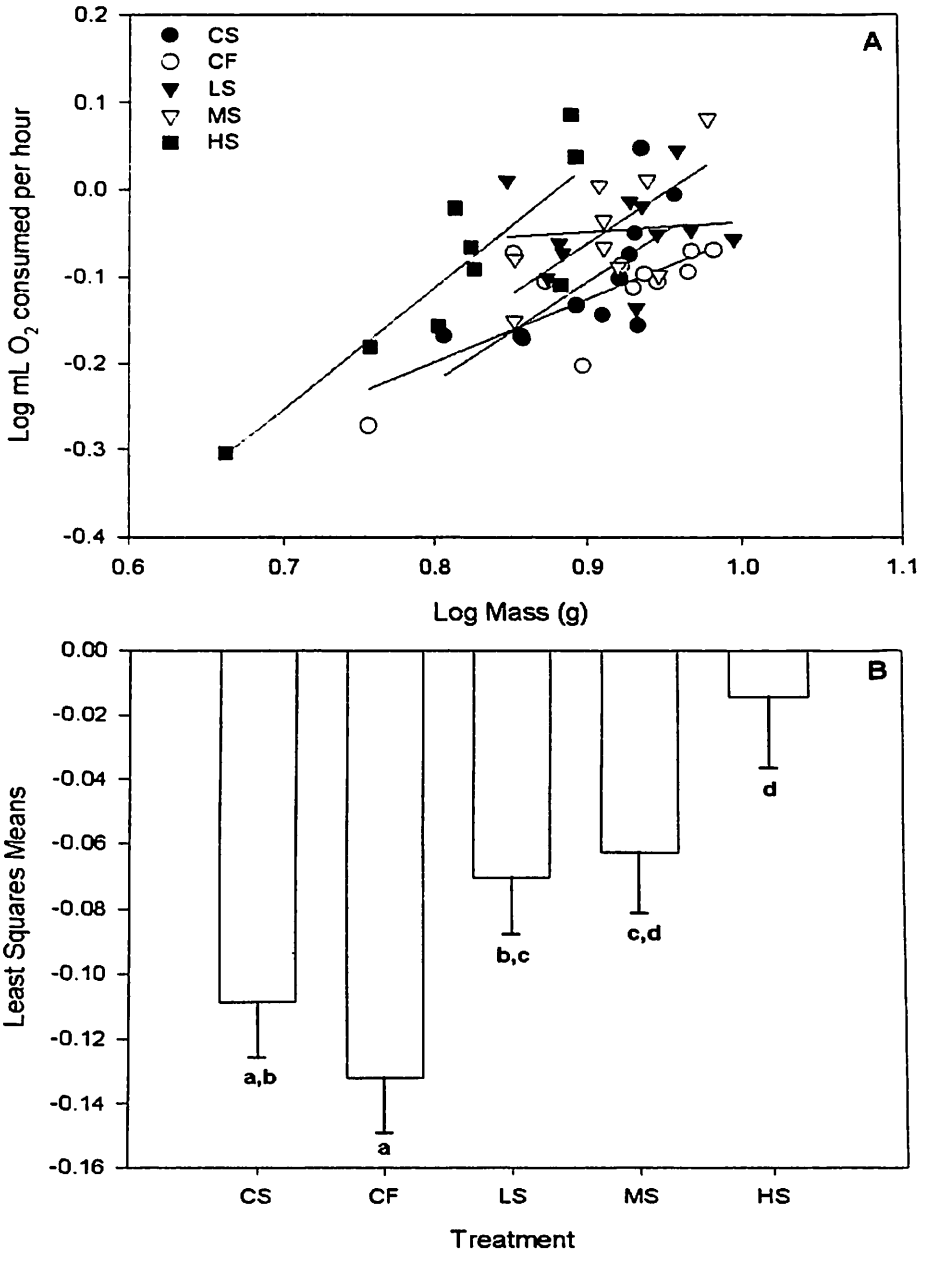

Figure 12. Log mL O_2 consumed/h vs. log mass of hatchlings ages 6 to 8 days from the 2003 study (A). The adjusted treatment means, or least squares means, of each treatment group (B). The least squares means are the mean mL O_2/h values adjusted for the effect of mass. Bars with different letters indicate significant ($p<0.05$) differences. UND=undeveloped eggs, CS=control substrate, CF=Catholic Forty, and LS=low substrate, MS=medium substrate, and HS=high substrate.

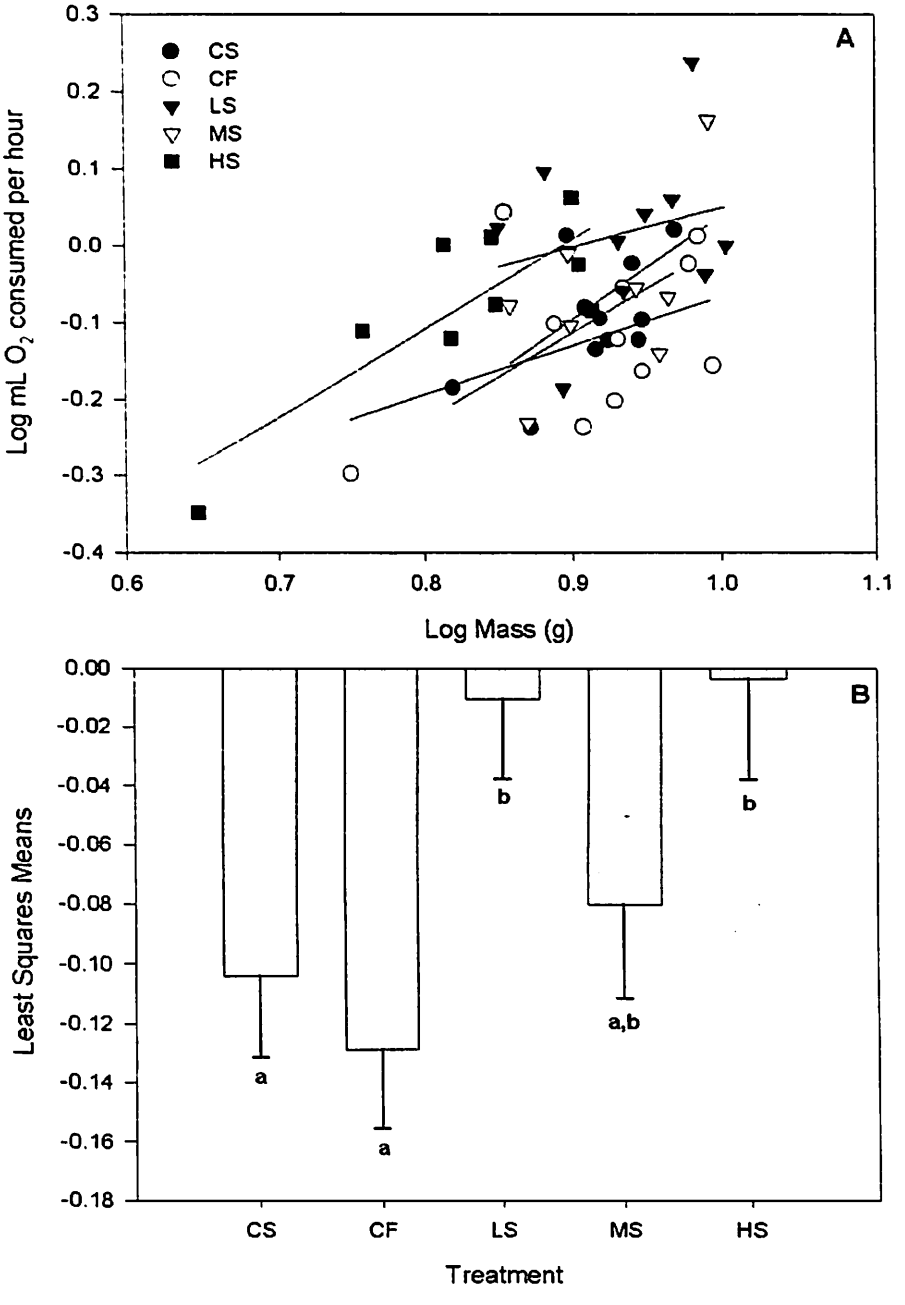

Figure 13. Log mL O_2 consumed/h vs. log mass of hatchlings ages 13 to 15 days from the 2003 study (A). The adjusted treatment means, or least squares means, of each treatment group (B). The least squares means are the mean mL O_2/h values adjusted for the effect of mass. Bars with different letters indicate significant ($p<0.05$) differences. UND=undeveloped eggs, CS=control substrate, CF=Catholic Forty, and LS=low substrate, MS=medium substrate, and HS=high substrate.

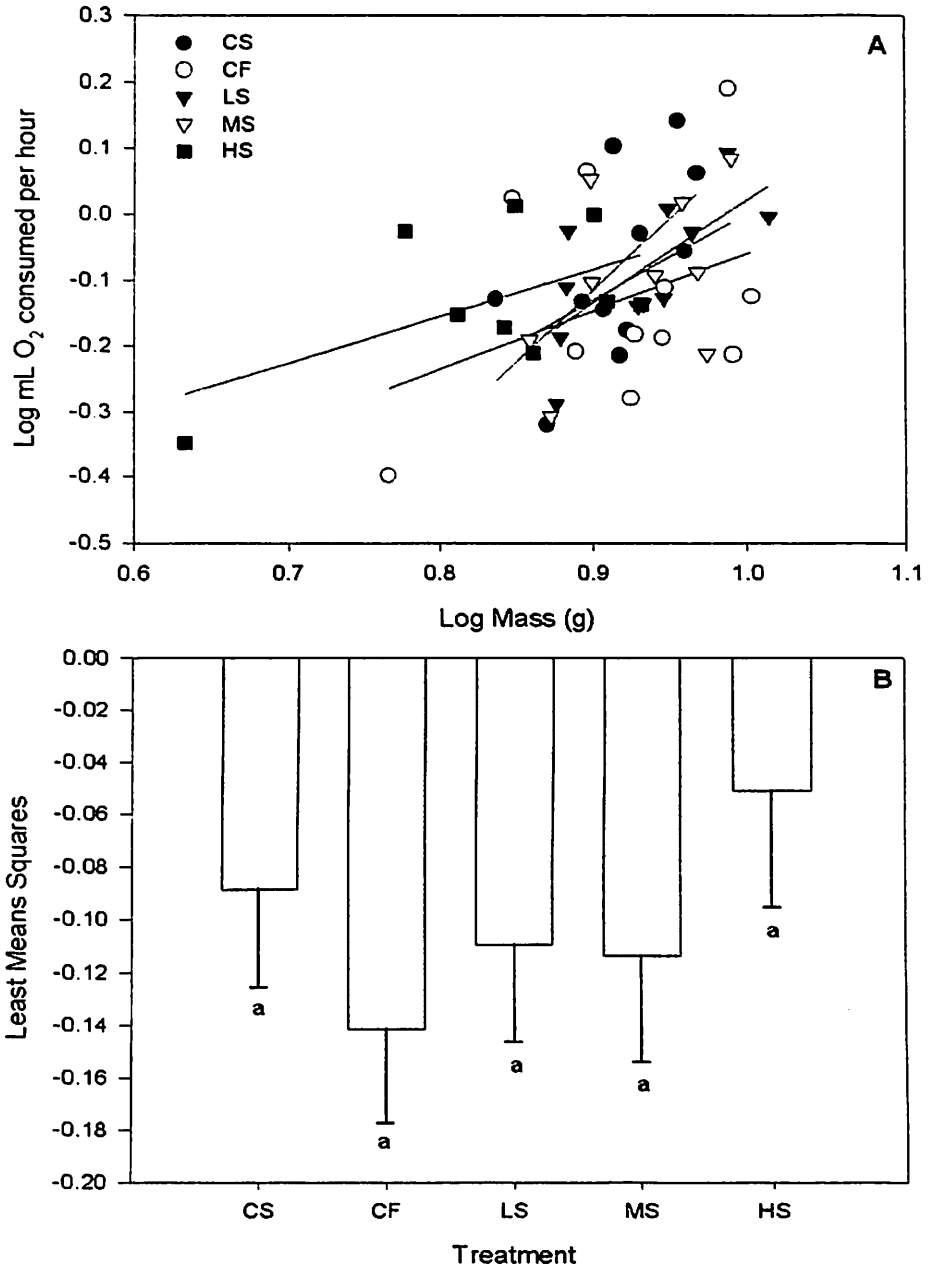

Figure 14. Log mL O_2 consumed/h vs. log mass of hatchlings ages 20 to 22 days from the 2003 study (A). The adjusted treatment means, or least squares means, of each treatment group (B). The least squares means are the mean mL O_2/h values adjusted for the effect of mass. Bars with different letters indicate significant ($p<0.05$) differences. UND=undeveloped eggs, CS=control substrate, CF=Catholic Forty, and LS=low substrate, MS=medium substrate, and HS=high substrate.

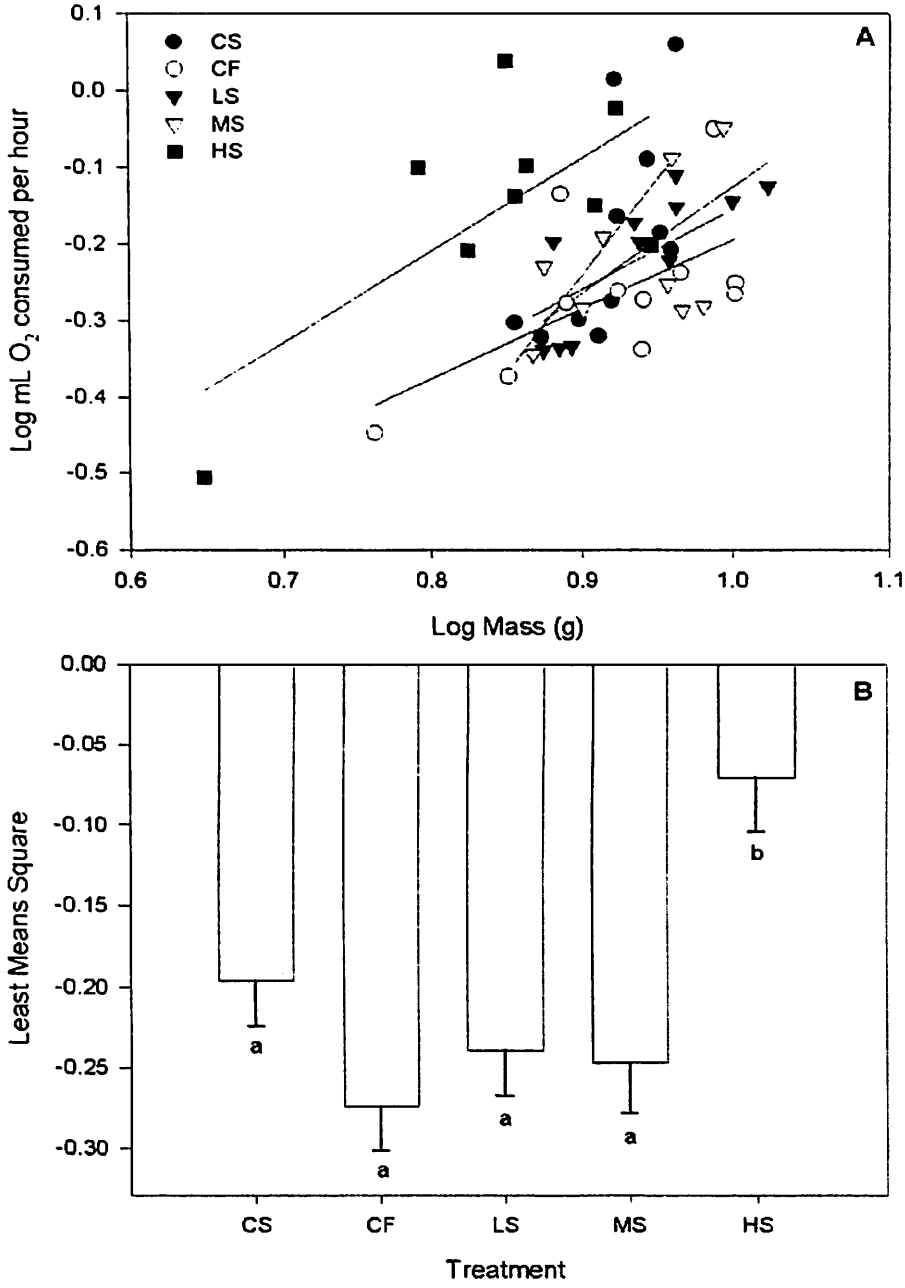

Figure 15. Log mL O_2 consumed/h vs. log mass of hatchlings ages 27 to 29 days from the 2003 study (A). The adjusted treatment means, or least squares means, of each treatment group (B). The least squares means are the mean mL O_2/h values adjusted for the effect of mass. Bars with different letters indicate significant ($p<0.05$) differences. UND=undeveloped eggs, CS=control substrate, CF=Catholic Forty, and LS=low substrate, MS=medium substrate, and HS=high substrate.

Swimming speeds

Swim speeds of hatchlings were evaluated 10-12, 20-22, and 32-34 days after hatching. Mass accounted for some of the variability in hatchling swim speeds at ages 10-12 and 32-34 days (Table 13, p=0.0142 and 0.0012, respectively), however, treatment had no effect on swim speed during any period of evaluation. Mass ranged from 4.59 to 11.55, 4.29 to 10.3, and 4.41 to 10.6 g from youngest to oldest treatment groups, respectively, and swim speed ranged from 11.5 to 35.1, 13.1 to 37.5, and 10.2 to 36.03 cm/s, respectively.

Table 13. The average (± 1 SD) mass and swim speed of hatchlings from all treatment groups for each period of swim speed evaluation in 2003. Age ranges marked by an asterisk indicate evaluation periods when mass influenced swim speed.

	10-12 Days*	**20-22 Days**	**32-34 Days***
Average Mass (g)	7.97 ± 1.11	8.20 ± 1.16	8.23 ± 1.13
Average Swim Speed (cm/s)	25.8 ± 5.7	24.3 ± 5.8	24.6 ± 5.2

Righting trials

The hatchling flipping trials indicated no apparent relationship between exposure to the metals and the time it took for the hatchlings to right themselves after being placed on their backs, regardless of hatchling age (15 to 17 days versus 31 to 33 days). Correlation coefficients between the time needed to instigate flipping and the mass of the hatchlings was 0.15 for the 15 to 17 day age group and 0.10 for the 31-33 day age group. These results indicate that mass accounted for a relatively minor portion of the variability in the time it took hatchlings to right themselves after they were placed on their back. The amount of time it took hatchlings, age 15 to 17 days, to right themselves ranged from

7.73 seconds to 12 minutes and 9 seconds. Five hatchlings, from the control, LS, and MS groups did not right themselves at all within the 15-minute observation period. At age 31-33 days, time to flip ranged from 3.1 seconds to 13 minutes and 57 seconds. Six hatchlings from the control, LS, MS, and HS groups did not right themselves at all within the 15-minute observation period.

DISCUSSION

General Metal Uptake By Eggs

Exposure of *T. scripta* eggs to Cd, Zn, and Pb throughout incubation on metal-contaminated substrates or via applications of metal solutions resulted in metal accumulation in tissues of hatchlings. Hatchling metal concentrations were higher than those found in undeveloped egg contents, with this accumulation often occurring in a dose-dependent fashion. Accumulation of metals from the exposure sources was not surprising because flexible-shelled reptilian eggs absorb water from the interstitial zone of surrounding substrate (Packard et al. 1987), and this uptake of water has been identified as a means for transport of contaminants into the egg (Linder and Grillitsch 2000).

Relatively few studies have examined the metals present within turtle hatchlings after incubation on contaminated substrate. Sahoo et al. (1996) examined metal levels in olive ridley sea turtle hatchlings after incubation on metal-contaminated substrate containing Cd, Co, Cr, Cu, Fe, Ni, Mn, Pb, and Zn. Substrate metal concentrations ranged from 1.5 mg/g for Cd to 55.0 mg/g for Pb to 88.4 mg/g for Zn. Although no reference substrate was used, they reported increased concentrations of all metals compared to the concentrations found within the contents of undeveloped eggs. Metal levels in whole hatchlings were reported to reach 2.0, 17.3, and 20.5 mg/g for Cd, Zn, and Pb, respectively, whereas undeveloped egg contents contained <1, 4.3, and 3.6 mg/g of Cd,

Zn, and Pb, respectively. However, their results should be considered cautiously due to the unrealistically high concentrations used and their reported minimum detection limit of 1 mg/g. The numeric data, if reported in concentrations three orders of magnitude lower (μg/g), would correspond well with the μg/g proportions of metals reported by Vazquez et al. (1997), Burger and Gibbons (1998), and Nagle et al. (2001), and may indicate an error in the units used.

Nagle et al. (2001) measured metal concentrations in red-eared slider hatchlings incubated on contaminated and reference substrates. The contaminated substrate contained elevated levels of As, Cd, Cr, Cu, and Se (25.1, 0.04, 6.9, 10.57, and 2.6 mg/kg, respectively) compared to the reference substrate (0.9, 0.03, 0.05, 2.2, 0.2 mg/kg, respectively). In contrast to the results of the current study, Nagle et al. found no difference between the metal concentrations of hatchlings incubated on the two substrates. The difference may be due to the lower metal levels in the contaminated substrate, although direct comparisons with the present work are only possible for Cd. In their study, Cd levels in both the contaminated and reference substrates were less than 1 mg/kg and differed by only 0.01 mg/kg, and hatchling Cd concentrations from the exposed and reference groups were both 0.03 μg/g. There was no indication of the bioavailable fraction of metals, which can significantly influence uptake of metals and associated effects (Boyd and Williams 2003 and Paquin et al. 2003).

Metal bioavailability

The importance of considering bioavailability in toxicity assessments of aquatic, sediment and soil systems has been clearly demonstrated by a number of studies (Gueguen et al. 2004, Ciutat and Boudou 2003, and Basta and Gradwhol 2000). For most

metals, the bioavailable or active fraction is the water-soluble or free ion component (Walker et al. 2001). Factors that influence metal bioavailability include pH (Yong 2001 and Siegel 2002), cation exchange capacity (CEC, Sparks 2003) and organic matter (Yong 2001). In water, pH has the largest impact on metal bioavailability (Siegel 2002), and, in soil, all three factors play a role (Siegel 2002, Yong 2001, and Sparks 2003).

The important influence of bioavailability on metal uptake was demonstrated in that accumulation of metals within the whole body, eggshell, soft tissues and carapace and plastron of red-eared slider hatchlings was usually dependent on the bioavailable rather than the total metal concentrations present in the exposure source. For instance, eggshells from the HPaint group in the exposure solution study accumulated Cd, Zn, and Pb to concentrations (64.4, 6775.3, and 390.3 μg/g, respectively) higher than those found in eggshells in the DSP group (2.2, 283.6, and 13.09 μg/g, respectively), despite having total concentrations of individual metals that were lower than or comparable to those found in the DSP group (65.8, 8507.0, and 280.5 mg/L, respectively, versus 56, 9300, and 540 mg/kg, respectively). The total concentrations of each metal were dissolved in the HPaint solution and were bioavailable, whereas only small portions of the total Cd, Zn, and Pb concentrations in the DSP were bioavailable (1, 10, and 52 mg/kg, respectively).

Additionally, in 2003, the CF substrate had a total Zn concentration of 3000 mg/kg and a bioavailable Zn concentration of 120 mg/kg, and total and bioavailable concentrations of the HS substrate were 1100 and 1300 mg/kg, respectively. Hatchlings from the CF group had lower whole body Zn burdens compared to hatchlings from the HS group (228.6 μg/g versus 422.1 μg/g) despite the HS substrate having a lower total

Zn concentration again reflecting relative bioavailable metal concentrations of the exposure sources rather than relative total metal concentrations.

These and other similar results from the current study support the findings of Boyd and Williams (2003), who reported that total concentrations of Cd, Zn, and Pb, among other metals, within a substrate were not indicative of actual exposure concentrations experienced by organisms exposed to that substrate. Factors such as pH, CEC, and amount of organic matter present within the substrate (also cited by Siegel 2002, Yong 2001, and Sparks 2003) affected the amount of metals actually bioavailable to nematodes (*Caenorhabditis elegans*). The amount of organic matter present was the best predictor of Cd and Zn availability, whereas pH and CEC were better predictors of Pb bioavailability. The LC50 values of nematodes exposed to substrates with varying characteristics and metal concentrations reflected the trends in metal bioavailability rather than trends in total metal concentration by decreasing as the bioavailable concentrations of metals increased, thus indicating increased exposure to and accumulation of those metals.

Metal partitioning in components of undeveloped and hatched eggs

Measurable levels of Cd, Zn, and Pb were present in undeveloped eggs in 2002 and 2003. For both years, eggshells contained higher Cd concentrations and lower Zn concentrations than egg contents. In 2002, no difference was observed between Pb concentrations in eggshells and contents of undeveloped eggs, whereas, in 2003, Pb concentrations were higher in eggshells.

Sahoo et al. (1996) reported higher Cd levels in eggshells of undeveloped olive ridley sea turtle eggs compared to egg contents, which was observed in 2002 and 2003,

and higher Pb levels in the eggshells compared to egg contents, which was observed only in 2003. However, contrary to my results, they reported higher Zn levels in the eggshells than egg contents.

Burger and Gibbons (1998) found no difference in undeveloped red-eared slider eggs between Cd concentrations in undeveloped egg contents and eggshells (0.067 and 0.013 μg/g, respectively), but did see higher Pb concentrations in egg contents compared to eggshells (0.687 μg/g compared to 0.219 μg/g). The results from the current study differ from those reported by Burger and Gibbons, as Cd concentrations were higher in eggshells than undeveloped egg contents, and Pb concentrations in eggshells were either similar to or higher than those in egg contents.

In addition to examining metal levels in undeveloped egg contents and hatchlings after incubation on metal contaminated substrate, Sahoo et al. (1996) also examined metal levels in eggshells from undeveloped and hatched olive ridley sea turtle eggs. Similar to the 2002 studies, eggshell Cd levels were lower after incubation on contaminated substrate than before (1.3 mg/g compared to <1 mg/g). Contrary to the 2002 and 2003 studies, they did not report increases in eggshell Zn and Pb concentrations during development. Zinc and Pb concentrations were similar in undeveloped and hatched eggshells (13.0 and 11.0 mg/g, respectively, compared to 16.6 and 15.6 mg/g, respectively).

Bioavailability of the metals influenced whether they accumulated most in the eggshells or in the hatchlings themselves. In the 2002 and 2003 substrate studies, the whole red-eared slider hatchlings exposed to lower bioavailable Cd concentrations (those present in the CF and DSP substrates in the 2002 study) contained higher Cd

concentrations than their eggshells, but as the bioavailable Cd concentrations increased (to levels present in the 2003 study), the relationship reversed and eggshells contained higher Cd concentrations. The same dependency on bioavailable metal concentrations was apparent in the relative Zn concentrations in whole hatchlings and eggshells. In the 2002 CF and DSP groups, whole hatchlings contained higher Zn concentrations than eggshells, and, in the 2003 substrate groups, eggshells contained higher Zn concentrations than whole hatchlings. The relative concentrations of Pb in whole hatchlings and eggshells also were dependent on metal bioavailability; at the lowest bioavailable Pb concentrations, no difference existed between the Pb concentrations in hatchlings and their eggshells. However, as Pb bioavailability increased, eggshells accumulated higher Pb concentrations than hatchlings.

Similar trends were observed in the 2002 exposure solution study. At lower bioavailable Cd and Zn concentrations, whole hatchlings contained higher Cd and Zn concentrations than eggshells, whereas the relationship reversed itself as the bioavailable concentrations increased. As for Pb, exposure to lower bioavailable concentrations resulted in the presence of similar Pb concentrations in the eggshell and whole hatchling, but as bioavailable Pb concentrations increased, eggshells contained higher Pb concentrations than whole hatchlings.

In some cases, bioavailability also appeared to play a role in the fate of metals between the hatchling components. At the lower bioavailable Cd concentrations present in the 2002 substrate and exposure solution studies, turtle shell accumulated the highest Cd concentrations, followed by soft tissue, and then eggshell. As the bioavailable Cd levels increased to those in the 2003 substrate study, turtle shell still accumulated higher

concentrations than soft tissue, but eggshell accumulated higher concentrations than both hatchling tissues. The order in which Zn accumulated in the various tissues when eggs were exposed to minimal bioavailable concentrations was turtle shell, soft tissue, and then eggshell. However, as Zn bioavailability increased, the only clear trend was that eggshells began accumulating the highest concentrations; there was no consistent pattern in Zn partitioning within the turtle shell and soft tissue. At lower bioavailable Pb concentrations, soft tissue generally accumulated the highest concentration compared to eggshell and turtle shell, which did not differ. As Pb bioavailability increased, eggshells began accumulating the highest concentrations, followed by soft tissue and then turtle shell.

Because no previous studies have attempted to relate metal accumulation in specific turtle tissues with metal bioavailability, there is nothing with which to compare these results. Perhaps when bioavailability was highest, there was greater mobility of metals in the substrates and a greater overall concentration became associated with the outside of the eggshell. The high Cd, Zn, and Pb concentrations within the eggshells of red-eared slider hatchlings exposed to increased bioavailable concentrations of those metals suggest that the eggshells play a protective role against metal exposure during embryonic development. Red-eared slider eggshells are composed of calcium carbonate, or aragonite. Their function is to protect embryos from biotic and abiotic factors that could adversely affect development (Congdon and Gibbons 1990). The aragonite matrix of the shell probably reacts with metal ions dissolved in the pore water present in the incubation substrate as it passes through the membrane. This reaction then may result in the immobilization of the metals, thus preventing them from entering the egg and

harming the embryo inside. If this is indeed a function of the eggshell, then it is not completely efficient, as evidenced by the accumulation of metals within the slider turtle hatchlings.

Sahoo et al. (1996) also compared the relative metal levels in whole olive ridley sea turtle hatchlings and hatched eggshells. Whereas eggshells from undeveloped eggs had higher concentrations of Cd, Zn, and Pb than egg contents, only the Cd concentrations of whole hatchlings and hatched eggshells differed. Instead of Cd concentrations being higher in the eggshells as they were in the undeveloped eggs, they were higher in the hatchlings (<1 mg/g compared to 2.0 mg/g). Exposure to the lower bioavailable Cd concentrations present in the CF and DSP substrates produced results similar to those of Sahoo et al., i.e., higher Cd concentrations in whole red-eared slider hatchlings than in eggshells. However, exposure to the higher bioavailable Cd concentrations present in the spike substrates resulted in higher Cd concentrations in the eggshells. The Zn and Pb results from the current study did not agree with those from the Sahoo et al. study except in the case of exposure to the lower bioavailable Pb levels found in the CF and DSP substrates in 2003, when the Pb levels did not differ between whole hatchlings and hatched eggshells.

Perhaps the differences among the relative metal concentrations in hatchlings and hatched eggshells observed in the Sahoo et al. study and the current study are attributable to differences in metal exposure levels between the two studies. Although the bioavailable metal fractions of the incubation substrate were not reported by Sahoo et al., the total concentrations of Cd, Zn, and Pb were 1.5, 55.0, and 88.4 mg/g, respectively. Assuming there was an error in the units used and the actual units were intended to be

μg/g, then the metal concentrations were lower than the total metal concentrations in the substrates used in the current study.

Although the partitioning of Cd, Zn, and Pb within the soft tissue (i.e., the relative concentrations of metals within different organs and tissues) was not examined in the *T. scripta* hatchlings used in the current study, it may be helpful to look at studies involving adult turtles to glean some insight on the matter. Several studies have examined the concentrations of heavy metals in the liver, kidney, and muscle tissues of sea turtles including loggerhead (Storelli et al. 1998 and Caurant et al. 1999), leatherback (Caurant et al. 1999), and green sea turtles (*Chelonia mydas*, Sakai et al. 2000). Cadmium and Zn were present in the highest concentrations in the liver and kidney in all species with preferential Cd accumulation in the kidney and no preferential Zn accumulation in either organ. In the Storelli et al. (1998) study, Pb was found primarily in the liver, whereas it was only detected in the kidney in the Sakai et al. study (2000).

Thomas et al. (1994) and Burger (2002) analyzed metal levels in two emydid turtles. Thomas et al. determined the relative locations of Cd and Zn, among other metals, in red-eared slider turtles after a series of intraperitoneal injections of Cd. Relative metal levels were determined in the liver, kidney, spleen, heart, lung, muscle, shell, brain, blood, and ovary of the turtles. The liver and kidney contained the largest percentages of the whole body Cd burden where it was bound by metallothionein, whereas the shell and ovary contained the largest percentages of the whole body Zn burden. The next highest proportions of Zn were found in the liver and kidney. Burger (2002) analyzed, among other metals, the Cd and Pb concentrations in wet liver and muscle tissue in diamond back terrapin turtles (*Malaclemys terrapin*). The liver contained higher Cd concentrations

compared to muscle, and no difference existed between the liver and muscle Pb concentrations.

Overmann and Krajicek (1995) evaluated concentrations within various tissues of snapping turtles inhabiting Missouri's old lead belt. They analyzed wet muscle, brain, liver, carapace, blood, carapace, and bone tissue. Blood, carapace, and bone contained the higher Pb concentrations than muscle, brain, and liver. Bone contained the highest Pb concentrations compared to all other tissues examined, supporting the claim that bone is a primary storage compartment (Tsuchiya 1979 and Linder and Grillitsch 2000). The turtle shell, similar to bone, is another hard tissue that may serve to sequester and immobilize metals within a turtle's system.

Although Cd accumulated preferentially in hard tissue in this study, turtle shell was not analyzed in the other studies. Thus, we can only speculate on the partitioning of Cd within the soft tissue alone. Cadmium may have been present primarily in the liver and kidney, as was observed in other studies (Storelli et al. 1998, Caurant et al. 1999, Sakai et al. 2000, Thomas et al. 1994, and Burger 2002) and presumably bound by metallothionein (Thomas et al. 1994). Trends in the partitioning of Zn in the current study supported the preferential accumulation of the metal in the turtle shell followed by the liver and kidney in the soft tissue asserted by Thomas et al. (1994), Zinc may be stored in soft tissues for the synthesis of Zn-dependent macromolecules (Thomas et al.1994). The preferential partitioning of Pb in the soft tissue of hatchlings (which included bone) supported the findings of Overmann and Krajicek (1995) in which the highest Pb concentrations were in the bone where it becomes immobile and unavailable within the organism's system.

Differences in accumulation among different metals

Bioaccumulation factors (BAF) help in the comparison of metal accumulation from sources containing different metal concentrations. They are the quotient of the metal concentration within the organism divided by the metal concentration in the environment (Walker et al. 2001). In this case, they are the hatchling concentrations of a particular metal divided by the concentration of that metal in the incubation substrate. They are not applicable to the exposure solution treatments because those were applied only once a week and it is impossible to determine the exact amount of exposure experienced by the eggs in these treatment groups.

In the 2002 substrate study the only BAF calculable for the control group was for Zn because it was the only detectable metal in the substrate. The mean BAF for Zn in the control group was 10.09. However, this is more a reflection of the amount of Zn present within the egg contents before incubation on the substrate than the accumulation of Zn from the substrate because there was no difference between the levels of Zn found in the undeveloped egg contents (58.4 μg/g) and control hatchlings (61.1 μg/g). Bioaccumulation factors for the CF and DSP groups were 0.45 and 0.16, respectively, for Cd, 0.14 and 0.4, respectively, for Zn, and 0.06 and 0.03, respectively, for Pb. Hatchlings accumulated Cd in the highest proportions, followed by Zn, followed by Pb.

In 2003, the BAF for the control group was similar to that of the 2002 control group for Zn, 12.41 and again was a reflection of the amount of Zn already present in the undeveloped egg contents. No difference was observed between the levels of Zn found in the undeveloped egg contents (85.4 μg/g) and control hatchlings (77.0 μg/g). The CF group in 2003 had lower BAF values for Cd, Zn, and Pb (0.1, 0.07, and 0.03,

respectively) than the 2002 CF group. However, similar to the 2002 CF hatchlings, the 2003 CF hatchlings accumulated the highest proportion of Cd, followed by Zn, followed by Pb. The BAFs for Cd in the LS, MS, and HS groups were 0.48, 0.22, and 0.23, respectively. With regards to Pb, the BAF values were 0.19, 0.20, and 0.12, respectively. In the LS group, hatchlings accumulated the highest proportion of Cd, followed by Zn, followed by Pb, the pattern that was observed in the 2002 study and the CF group from the 2003 study. However, as the metal concentrations increased in the incubation substrates as they did in the MS and HS groups, hatchlings accumulated the highest proportions of Zn, followed by Cd, followed by Pb.

The trends in the BAF values may be explained by the behavior of Cd, Zn, and Pb when reacting with substrates and turtle eggs. According to Alloway and Ayers (1997), in substrate, Pb is one of the most readily adsorbed divalent cations, followed by Zn and Cd. Thus, Pb is generally less available than Zn, which is less available than Cd. This pattern is supported by the trends in the BAFs in the 2002 substrate study and in the LS group of the 2003 substrate study. In these cases, red-eared slider embryos accumulated higher proportions of Cd from the substrate than Zn and higher proportions of Zn from the substrate than Pb. In the MS and HS substrates, Pb was still taken up in the smallest proportion with Cd in the next smallest proportion, and Zn in the highest proportion. The change in order between Cd and Zn may be a reflection of the relative bioavailable portions of those metals within the substrates. In the LS substrate the ratio of available Zn to Cd was 46:1, whereas in the MS and HS substrates, the ratios were 57:1 and 56:1, respectively. Thus, with an increased ratio of available Zn to available Cd, an increase in the proportion of Zn accumulated may have followed.

Sources of metal exposure

Although the primary sources of metal exposure in the 2002 and 2003 studies were the incubation substrates and exposure solutions, they were not the only sources. Undeveloped eggs from both 2002 and 2003 had surprisingly high Cd concentrations, with the concentrations in the eggshells (7.8 and 6.2 μg/g, respectively) exceeding those in the egg contents (2.7 and 0.5 μg/g, respectively) by 3 to 14 times. Whole egg Zn concentrations were also high, with the egg contents containing concentrations (58.4 and 85.4 μg/g, respectively) 7 to 9 times higher than those found in eggshells (8.4 and 9.5 μg/g, respectively). Even Pb was present within the egg contents (0.4 and 0.4 μg/g, respectively) and eggshells (0.3 and 0.9 μg/g, respectively), although in concentrations lower than those of Cd and Zn.

The suspected sources of the metals within the undeveloped eggs are the female turtles from which the eggs came. The transfer of essential and nonessential metals into eggs has been reported in loggerhead sea turtles (Sakai et al. 1995), diamondback terrapin turtles (*Malaclemys terrapin*, Burger 2002), and red-eared slider turtles (Burger and Gibbons 1998 and Nagle et al. 2001). Burger and Gibbons (1998) suggested that maternal transfer may be a mechanism by which female turtles can reduce their own metal burdens and reduce toxicity. Sahoo et al. (1996) also found metals in olive ridley sea turtle eggs.

The fact that Burger and Gibbons (1998) observed residues of Cd and Pb in undeveloped red-eared slider eggs is not surprising because they were derived from female turtles that inhabited a site contaminated with Cd, Cr, Hg, Mn, Pb, and Se. In the present study, the females from which eggs were harvested in 2002 were collected from the Sequoyah National Wildlife Refuge in eastern Oklahoma, whereas eggs were

purchased from a turtle and alligator farm in Hammond, LA, in 2003. Unfortunately, the background metal levels for these locations is unknown, but the residues in the undeveloped eggs illustrate the importance of these ancillary data and the utility of the turtle egg as an indicator of metals from multiple sources.

The presence of Zn in undeveloped eggs is not surprising. The higher Zn concentrations in egg contents compared to eggshells (unlike the case of Cd) is logical because Zn is an essential metal, and the female must provide the material necessary for embryonic development and maintenance as well as some of the material necessary for the maintenance and growth of resultant hatchlings (Congdon and Gibbons 1990). Because Zn is an essential element, it should follow that female turtles make it available to their offspring by depositing it into their eggs.

Discrepancies among studies

There were several instances in the current study in which the metal accumulation results from the three individual exposure experiments (2002 substrate and solution, 2003 substrate) did not agree, despite similarities such as incubation substrate and bioavailable levels of metals. For example, in 2002, hatchlings from the CF groups contained higher Cd concentrations than control hatchlings, but, in 2003, there was no difference between hatchling Cd burdens in the two groups. This difference may stem from the amount of Cd present in the undeveloped egg contents before each study. The contents of undeveloped eggs in the 2002 study had higher Cd concentrations than the contents of undeveloped eggs in the 2003 study (2.7 μg/g in 2002 vs. 0.5 μg/g in 2003). This relationship is reflected in the relative concentrations of Cd found within the hatchlings from the CF and

control groups from both years (9.0 and 6.5 μg/g, respectively, in 2002 and 1.9 and 1.0 μg/g, respectively in 2003).

Another case pertains to the Cd concentrations found in the eggshells of hatchlings in the CF groups from both years. Cadmium levels in eggshells from the 2002 CF group (2.3 μg/g) were less than Cd levels in the eggshells of undeveloped eggs (7.8 μg/g), whereas Cd levels in the 2003 CF group eggshells (7.8 μg/g) exceeded the Cd levels in the eggshells of undeveloped eggs (6.2 μg/g). Eggshells from the 2003 CF group accumulated higher concentrations of Cd compared to those of the 2002 CF group despite the eggshells of undeveloped eggs from both years having similar Cd concentrations. The discrepancy may be attributable to a difference in the procedures used to replace evaporative water lost from substrates during incubation. The 2003 substrates were stirred weekly with the addition of water, whereas the 2002 substrates remained untouched. This weekly turnover of incubation substrate could have resulted in increased overall Cd exposure by reducing concentration gradients that may have developed next to the egg as metals were taken up from the interstitium.

In aquatic systems, the presence of organisms in the sediment may similarly increase metal bioavailability through bioturbation. Ciutat and Boudou (2003) found that it causes significant metal release into the water column from contaminated sediments. In the presence of *Hexagenia rigida* nymphs (the bioturbation source), benthic bivalves (*Corbicula fluminea*) accumulated higher metal concentrations than when the nymphs were not present to disturb the bottom sediment and increase metal bioavailability.

Mixing of substrates in the 2003 study also may be the cause of difference in the relative Pb concentrations found in whole hatchlings and eggshells from the control and

CF groups in 2002 and 2003. In 2002, whole hatchlings from the control and CF groups contained Pb concentrations similar to those in their respective eggshells (1.3 and 27.8 μg/g in hatchlings, respectively, compared to 1.0 and 17.8 μg/g in eggshells, respectively), whereas whole hatchlings in those same groups in the 2003 study contained lower Pb concentrations than their respective eggshells (1.5 and 14.4 μg/g, respectively, versus 5.4 and 31.7 μg/g, respectively). The mixing of substrates in 2003 could have altered the Pb concentration gradient present within the pore water of the substrate that developed next to the egg, thereby increasing the rate and amount of Pb uptake.

Another reason for the variation among the 3 exposure experiments may be the difference in the Pb concentrations found in the undeveloped eggs from both years. Undeveloped eggs from the 2003 study already contained higher Pb concentrations in the eggshells than egg contents (0.9 μg/g versus 0.4 μg/g), whereas the Pb concentrations in the undeveloped egg contents and eggshells did not differ in 2002 (0.4 μg/g compared to 0.3 μg/g).

Discrepancies between the 2002 exposure solution study and both substrate studies can be explained by differences in exposure methods. In several cases, hatchlings and eggshells from the exposure solution study contained lower metal concentrations than whole hatchlings and eggshells in the substrate studies even though bioavailable metal concentrations were higher in exposure solutions. Eggshells from the HS group in 2003 contained higher Cd concentrations than eggshells in the HPaint group from 2002 (91.6 μg/g versus 64.4 μg/g) in spite of the fact that the HPaint substrate had bioavailable Cd concentrations almost three times as high as that in the HS substrate. In 2002, hatchlings from the CF and DSP groups appeared to accumulate Zn to higher

concentrations (423.4 and 423.6 μg/g, respectively) than those in the MPaint and HPaint exposure solution groups (93.2 and 233.9 μg/g, respectively) despite the higher Zn bioavailability in exposure solutions. Also in 2002, hatchlings from the CF and DSP groups accumulated Pb to higher concentrations (27.8 and 18.9 μg/g, respectively) than those in the HPaint exposure solution group (12.0 μg/g) although Pb bioavailability was higher in the exposure solution. Such differences may be attributable to the differences in frequency of exposure to metal solutions versus contaminated substrate. Application of metal solutions occurred only once a week, whereas exposure to contaminated substrates was constant.

Effects of Metal Exposures on Embryos and Hatchlings

Resting metabolic rates of developing embryos

According to Calow (1991), contaminant exposure can be energetically costly. Three main reasons exist for the expenditure of energy as a result of metal exposure during embryonic development of turtles: Production of compounds that neutralize or sequester metals and prevent damage; repair of damage caused by metals after they have been effectively neutralized or removed; detoxification of the contaminants, which requires to process and transport metals to points of excretion, i.e., urine and feces.

Because of the added energy expense that can be caused by contaminant exposure, I expected red-eared slider turtle embryos and hatchlings to exhibit elevated RMRs due to their efforts in metal regulation, damage repair, and detoxification. With this increased energy expenditure, I also expected metal-exposed hatchlings to have allotted more of the caloric energy contained within the yolk sacs of their eggs for regulation, repair, and detoxification. Thus, considering the increased caloric energy

diverted to processes dealing with metal exposure, we expected that resultant hatchlings would take longer to develop and be smaller upon emergence from the egg.

No other studies have examined the effects of metal exposure via incubation substrate on the metabolism of turtle or even reptilian embryos. However, effects of metals exposure via other routes, such as food and ambient environment, on metabolic rates of other species have been studied and are reported to cause increased SMRs in clams (Sobral and Widows 1997), bullfrog tadpoles (Rowe et al. 1998), and banded water snakes (Hopkins et al. 1999), decreased and increased RMRs in the carp, *Cyprinus carpio*, depending on whether the metal exposure was lethal or sublethal, respectively (Suresh et al. 1993), and decreased RMRs in red-eared slider turtle hatchlings (Nagle et al. 2001).

Very few differences occurred among RMRs of developing *T. scripta* embryos in the 2002 substrate and exposure studies. In the 2003 substrate study, no differences in embryonic RMRs were observed among the treatment groups during any week. The only difference among treatment groups in the 2002 substrate study occurred during week 4, in which exposed embryos from the CF group had lower RMRs than embryos from the control group. However, this pattern did not persist throughout the rest of the incubation period and did not occur again in 2003 in embryos in eggs incubated on the same substrates. Suresh et al. (1993) observed a decrease in RMRs of carp when exposed to lethal Cd levels. The metal levels examined in this study, however, were not lethal, as all eggs hatched and no mortality occurred subsequent to hatching. Nagle et al. (2001) observed decreased RMRs in red-eared slider hatchlings that had elevated Se levels compared to control hatchlings and reported a lack of data on the depression of metabolic

rate due to Se exposure. Thus, the occurrence of lower RMRs in CF embryos compared to control embryos during week 4 after oviposition may have been just an anomaly, particularly since the difference did not persist and did not occur within the same treatments the following year.

In the 2002 exposure solution study, significant differences occurred twice during development on separate occasions between two different sets of treatment groups. During week 5, the HPaint group embryos had lower mean RMR than embryos from the control group, again supporting the results of Suresch et al. (1993) and Nagle et al. (2001). However, just as seen in the 2002 substrate study, this pattern also did not persist through the rest of embryonic development, nor was it consistent between the different exposures that were conducted.

During week 6, embryos from the MPaint group had a higher mean RMR than embryos from the LPaint group but not from the controls. An increase in metabolic rate as a result of metal exposure supports the original hypotheses of the present study and the results reported by Sobral and Widows (1997), Rowe et al. (1998), and Hopkins et al. (1999). However, the observed differences were once again not persistent and so it is not possible to effectively interpret their proximate cause or relevance.

Embryonic yolk utilization

Yolk sac consumption, or calorie consumption, goes hand in hand with embryonic RMR measurements. In addition to expecting that embryos exposed to elevated metal levels would have higher RMRs, we also expected those embryos to utilize more of their yolk sacs during incubation. Because the former did not occur, it follows that the latter also did not occur. Despite the occurrence of some differences among embryonic RMRs

in the 2002 substrate and exposure solution studies, there were no significant differences among the treatment groups with respect to the number of calories consumed during development.

Conversely, in the 2003 substrate study, no differences were observed among weekly embryonic RMR measurements, whereas there were differences observed in the amount of calories consumed among treatment groups. Embryos in the HS group consumed fewer calories than embryos in the control, LS, and CF groups. Although no significant differences were observed, embryos from the HS group did have discernibly lower RMRs than embryos from all other groups on days 48 and 55 after oviposition. The decrease in RMRs of this group may have been enough to cause a significant decrease in calorie consumption during development.

Hatch effects

We hypothesized that embryonic metal exposure would increase the amount of time it took for red-eared slider turtles to emerge from the egg and would decrease the weight at hatch. Contrary to our expectations, no difference was observed in the time between oviposition and hatch among the treatment groups within any study.

As previously discussed, Nagle et al. (2001) studied the effects of incubation in metal contaminated soil on the incubation time and metal burdens of red-eared slider embryos and hatchlings. The contaminated incubation substrate they used contained elevated As, Cd, Cr, and Se levels. As with the current study, hatchlings incubated on the contaminated substrate did not have a significantly altered incubation time or hatch mass as compared to those from the reference substrate. However, one key difference between hatchlings from the Nagle et al. study and the current study must be noted: in the Nagle

study, there was no difference between the whole hatchling metal burdens in hatchlings from the contaminated and reference substrates, whereas, in the current study, there were.

Resting metabolic rates of hatchlings

Generally, as an organism gets larger, its oxygen requirements increase, but not at a one to one ratio (Gordon 1982). The red-eared slider hatchlings used in the 2003 substrate study conformed to that generality. If mass is considered as a covariate of RMR, then it is possible to compare the RMRs of hatchlings of different sizes and determine if there are any effects due to different experimental conditions. In the current study, hatchlings' RMRs were measured weekly after metal exposure during incubation had ceased.

A dose response to embryonic metal exposure in hatchling RMR was apparent during the first, second, and fourth weeks of metabolic measurement, which supports the results of Rowe et al. (1998), Hopkins et al. (1999), and Suresh et al. (1993). Rowe et al. and Hopkins et al. examined the effects of metal mixtures on energetics of bullfrog tadpoles reared in contaminated water and banded water snakes from a contaminated site, respectively, and Suresh et al. examined the effects of single metal exposure on carp via ambient water. All metal-exposed organisms had higher MRs than organisms from reference sites or control organisms, supporting Calow's (1991) assertion that contaminant exposure can increase an organism's energetic cost of living.

Surprisingly, during the third week, no differences among the RMRs treatment groups were observed. It is unclear why this happened only during the third week, however, it may have been the beginning of a trend toward the equalization of hatchling RMRs, as hatchlings had not been exposed to metals since emergence from the eggs and

they were in the process of clearing the metals from their systems. During the fourth week, RMRs were similar among all groups, except the HS group, which had the highest RMRs. Perhaps continued measurements would have decreasing differences among groups from week to week until no more were observed. No studies on the effects of metal depuration or detoxification on the metabolic rates of organisms are available for comparison.

There were some instances during the four weeks of MR measurements when the slopes of the equations relating mass and RMR were remarkably low (LS during week 1 and CF and LS during week 2) and coupled by low r^2 values. These instances may be attributable to hatchling activity during the measurement period. If hatchlings were active within the metabolic chamber while it was sealed, they would have consumed more oxygen, and the resultant metabolic rate measured would have been higher than the organism's actual resting metabolic rate.

Hatchling swim speed and righting ability

Studies of performance and/or behavior of organisms can indicate the ecological relevance of contaminant exposure. Although these types of evaluations on reptiles are generally lacking, several have examined the effects of metal exposure on the behavior and general performance of anuran adults and tadpoles. Raimondo et al. (1998) reported decreased swimming speed and predator avoidance in bullfrog tadpoles exposed to Al, As, Cd, Cr, Hg, and Se. Selvi et al. (2003) reported that Cd exposure altered the swimming behavior and decreased swimming speed of adult water frogs (*Rana ridibunda*). Lefcort et al. (1998) reported a decline in the proficiency of anitpredatory behaviors of Columbia spotted frog tadpoles (*R. luteiventris*) when exposed to Cd, Zn,

and Pb singly and mixtures of Cd and Zn. Among the behaviors they examined were the use of refugia when presented with a predator's odor and avoidance in the presence of an actual predator.

Similar to the tadpoles in Raimondo et al. (1998), swim speed in my work was correlated with mass in two of the three measurement periods. However, embryonic metal exposure did not have an effect on the swim speeds of hatchlings from the current study, as no difference existed among the groups during any measurement period. Due to the lack of differences among groups, it can be inferred that swimming speed is not an adequate assessment of the effects of embryonic metal exposure on red-eared slider hatchlings.

The flipping trials with the hatchlings were even more equivocal with regard to contaminant effects than the swimming trials were. The time hatchlings took to right themselves after being flipped on their backs was not correlated with either mass or embryonic exposure level. Some hatchlings righted themselves almost immediately after being place on their backs, whereas other hatchlings never attempted to right themselves for the duration of the 15 min observation period.

In their 1996 experiment, Burger et al. (1998) injected 3-week-old *T. scripta* hatchlings with lead acetate and examined the ability of the hatchlings to right themselves once flipped on their backs. The time it took a hatchling to right itself was dependent upon the hatchling's size and dosage of Pb received. Larger hatchlings tended to flip over faster and flipping time was positively correlated with the dose of Pb received. Hatchlings receiving a dose of 1 mg/g lead acetate took longer to right themselves than did control hatchlings and hatchlings dosed with 0.25 mg/g. Exact Pb concentrations of

the hatchlings were not determined, but the dose of 2.5 mg/g was higher than the Pb burdens of hatchlings exposed to the highest Pb levels in the current study (233.1 μg/g in HS hatchlings).

Comments on the lack of effects in hatchlings associated with embryonic metal exposure

The developing red slider embryos clearly exhibited uptake of Cd, Zn and Pb from their respective substrates or treatment solutions, with hatchlings having up to 9, 420 and 230 μg/g of each metal in their soft tissues, respectively. Even so, there were few significant effects observed on the energetic or behavioral parameters evaluated. The only other effect was a dose dependent increase in the metabolic rates of exposed hatchlings within the first two weeks after hatching. However, that response appeared to diminish after the first two weeks.

These results are intriguing because studies with other species have indicated effects when metal body burdens were similar to or less than those observed in the turtles. For example, Rowe et al. (1998) reported elevated RMRs in bullfrog tadpoles containing elevated concentrations of As, Cd, Cr, Cu, and Se compared to compared to control tadpoles. Exposed tadpole metal concentrations were 25.95, 4.32, 27.25, 55.12, 25.27 μg/g of each metal, respectively, and their RMRs were 175.7% higher than those of control tadpoles. Conversely, Nagle et al. (2001) reported decreased RMRs in red-eared slider hatchlings with increased Se burdens (7.36 μg/g) over control hatchlings (1.63 μg/g). Lefcort et al. (1998) reported decreased activity and predator avoidance in Columbia spotted frog tadpoles exposed to Zn only, Pb only, and Cd, Zn, and Pb in combination. In the Zn-only and Pb-only exposed tadpoles, metal concentrations ranged from 14.6 to 18.6 μg/g and 2.1 to 8.8 μg/g, respectively. In the mixture-exposed tadpoles,

Cd, Zn, and Pb concentrations ranged from 0.2 to 5.8 μg/g, 11.5 to 28.8 μg/g, and 8.2 to 23.1 μg/g, respectively.

Several reasons could exist for the lack of observed effects in the red-eared slider turtles from the present study. First, the exposure levels of Cd, Zn, and Pb utilized in this study may not have been high enough to induce any negative effects in the *Trachemys scripta* hatchlings. Second, the effects we expected to see, i.e., increased metabolic rates and decreased performance, were observed in anuran tadpoles. Although both turtles and frogs are ectothermic vertebrates, many differences exist in potential contaminant exposure routes and uptake mechanisms between the two taxa. Tadpoles live completely in the water where their potential exposure sources are the water and the food they eat. There is no buffer between them and the surrounding environment. Turtle embryos are encased in eggshells that are in contact with substrate. Their exposure source is the water that diffuses through the eggshell, carrying dissolved metal ions. The eggshell is a calcium carbonate buffer that protects the embryo from the external environment (Congdon and Gibbons 1990) and can potentially bind contaminants, preventing them from entering the egg (Linder and Grillitsch 2000). Once tadpoles and turtle hatchlings have absorbed the metals, physiological and morphological differences may alter the effects of accumulation. Size (Gordon 1982) and ability to produce metal regulating proteins may alter energetics responses to exposure (Calow 1991). Turtles have shells in which metals can be sequestered and immobilized (Overmann and Krajicek 1995 and Linder and Grillitsch 2000), whereas tadpoles do not.

The lack of toxic effects observed in the *T. scripta* hatchlings due to Cd and Zn accumulation may be due to regulation of the metals by the metal-binding protein

metallothionein. Metallothionein synthesis is induced by the presence of Cd and Zn, among other metals, within both invertebrates and vertebrates (Petering et al. 1990). It binds Cd and transports it primarily to the liver and kidneys where the metal is sequestered and unable to interact with a target site and induce toxicity (Thomas et al. 1994). In general, metallothionein binds to Zn in the intestine and transports it throughout the body, making it available for the synthesis of Zn-dependent macromolecules (Petering et al. 1990 and Roesijadi 2000). The ability of the Zn-metallothionein complex to unload Zn ions makes it an important tool in the detoxification of Cd. Cadmium often binds to non-thionein proteins, inhibiting their function. The Zn-metallothionein complex can release its Zn ion, which displaces the Cd ion from the non-thionein protein, thereby reversing the harmful effects of Cd. The released Zn ion induces the synthesis of additional metallothionein, which can regulate Zn or serve in the further detoxification of Cd (Roesijadi 2000).

Red-eared slider turtles produce metallothionein upon introduction of Cd into their systems (Thomas et al. 1994), and the metalloenzyme serves in the detoxification of Cd. The fact that *T. scripta* produces metallothionein may explain, at least in part, the lack of adverse toxic effects due to the accumulation of Cd and Zn. The increased RMRs observed in hatchlings from the artificial contaminated substrate groups could have been caused by the production of metallothionein for the sequestration of Cd. Thus, if Cd was sequestered, it was unable to inflict any other harmful effects.

The mechanisms regulating or sequestering Pb within an organism's system are different from those that regulate Cd and Zn. In humans, Pb is compartmentalized primarily within bone, blood and soft tissue (Tsuchiya 1979). Lead also

compartmentalizes in carapace and plastron in snapping turtles (Overmann and Krajicek 1995), which can also serve as Pb storage compartments (Linder and Grillitsch 2002), in addition to the aforementioned tissues (Overmann and Krajicek 1995). Redistribution of Pb to the bone occurs after its initial distribution to various organs and tissues. In the bone, Pb remains essentially unavailable to affect target sites, and so does not cause toxicity. Over time, 90% of the body's unabsorbed Pb burden in excreted via feces (Tsuchiya 1979). The absorbed portion of the body's Pb burden is eliminated via urine, gastrointestinal excretions, and hair, nails, and sweat (Tsuchiya 1979 and Linder and Grillitsch 2000).

Red-eared slider turtles may exhibit similar Pb regulation mechanisms. In the 2002 and 2003 substrate studies, Pb appeared to accumulate more in the soft tissue portion of the hatchlings compared to the hard tissue portion. This supports the previously described partitioning of Pb in the soft tissue and bone, especially since the term soft tissue was inclusive of bone. Unfortunately, the amount of Pb in the bone relative to other soft tissues was not determined.

Conversely, hatchlings in the 2002 exposure solution study appeared to accumulate Pb in the turtle shell over the soft tissue. If we assume that most of the Pb found within the soft tissues of the hatchlings from all three studies was actually sequestered in the bone, as reported by Overmann and Krajicek (1995) in snapping turtles, then the lack of adverse effects due to Pb accumulation would be reasonable. Similarly, Pb sequestered in the hard tissue (carapace and plastron of the hatchlings) also would be unable to induce any toxic effects (Overmann and Krajicek 1995 and Linder and Grillitsch 2000).

The difference in Pb partitioning in whole hatchlings from the substrate and exposure solution studies may be due to differences in the exposure methods. Eggs in the aqueous solution study were dipped in the metal solutions and returned to the incubation substrate before completely air-drying. Thus, any metal ions on the underside of the egg, where exposure to the soft tissues of the embryos would have been greatest, could have diffused into the underlying substrate, whereas the metal ions on the top of the egg had to remain on the eggshell or diffuse through the eggshell. Turtles develop in the egg such that their orientation is carapace up with their legs and tail curled underneath them when they begin to hatch (personal observation). Any metals that diffused through the top of the egg would have gone into the embryo's carapace. In the substrate studies, all contact with the metals was on the underside of the egg, closer to the soft tissues. Hence any metal ions that diffused through the eggshell from the substrates entered soft tissue first.

This pattern of Pb partitioning in hatchlings exposed to metals via incubation substrate in the wild may not hold. Red-eared slider females oviposit eggs in terrestrial nests composed of holes they dig themselves. After they finish laying eggs, they fill in the hole with the excavated substrate (Congdon and Gibbons 1990). Clutches are small enough that all eggs are in contact with the incubation substrate on all sides after burial (personal observation), thus, diffusion of contaminants through the eggshell can occur from all directions, not just the top and bottom.

Another aspect that may have influenced the toxicity of the metals is that they occurred in mixture rather than as single entities, and so may have influenced their relative toxicities (Stewart 1999, Hopkin and Spurgeon 2000, and Walker et al. 2001). In the environment, Zn is usually present in concentrations that are 50 to 100 (Walker et al.

2001; N. Basta, Associate Professor, Ohio State University, pers. comm. 2003) times greater than those of Cd. The interaction between Cd and Zn extends to an organism's system. In an organism exposed to Cd and Zn, the toxic effects of Cd are observed when the ratio of Zn exposure to Cd exposure is less than ten. As the ratio decreases, Cd toxicity increases (Hopkin and Spurgeon 2000 and Walker et al. 2001).

Cadmium is a highly toxic, nonessential metal capable of biochemically replacing Zn (Sparks 2003) and its availability (Stewart 1999) and toxicity (Stewart 1999, Aravind and Vara Prasad 2003 and Herkovitz and Perez-Coll 1990) to organisms are reduced in the presence of Zn. According to Stewart (1999), metal mixtures can influence the bioavailability of individual metals within that mixture. In an aqueous system containing Cd, Cu, Ni, Pb, and Zn, increasing concentrations of Cu, Ni, Pb, and Zn, but not the concentration of Cd resulted in increasing available concentrations of Cd in the water. It appeared that the different metals were competing with binding sites on the substrate present in the system. In the presence of higher concentrations of other metals, Cd had reduced capacity for binding to the substrate. However, increasing concentrations of Cu, Ni, Pb, and Zn also decreased the accumulation of Cd by a freshwater mussel (*Pyganodon grandis*), again indicating the competition among metals for binding sites. It appeared that the ratio of Zn to Cd (43:1) had the greatest impact on Cd binding and accumulation (Stewart 1999).

The antagonistic relationship between Cd and Zn also has been noted in a freshwater macrophyte (*Ceratophyllum demersum*; Aravind and Vara Prasad 2003) and toads (*Bufo arenarum*; Herkovitz and Perez-Coll 1990). Cadmium accumulation by *C. demersum* was reduced by 26% in the presence of Zn compared with exposure to Cd

alone. Furthermore, Zn accumulation from the Cd-Zn mixture increased 369% compared to when the plant was exposed to Zn alone. This supports Stewart's (1999) assertion that Cd and Zn share and compete for binding sites. Herkovitz and Perez-Coll (1990) reported that concurrent exposure to Cd and Zn reduced the teratogenic effects of Cd in toads and that exposure and accumulation of Zn prior to exposure to Cd also can reduce teratogenicity. These results indicate that Zn exposure plays a protective role against Cd exposure and toxicity.

The high ratios of Zn to Cd within the various incubation substrates and exposure solutions from the current study could have reduced the uptake of Cd because the two metals were competing for binding cites. The ratios of total Zn and Cd concentrations were 150:1 and 166:1 in the CF and DSP substrates, respectively, and ranged from 58:1 to 68:1 in the LS, MS, and HS substrates. The ratios of the bioavailable Zn and Cd concentrations were much lower in the CF and DSP substrates, 30:1 and 10:1, respectively; and only slightly lower in the LS, MS, and HS substrates, ranging from 46:1 to 57:1. The ratios of the total and bioavailable Zn and Cd concentrations in the exposure solutions were all the same and equal to 129:1. All ratios of Zn to Cd were greater than the 43:1 ratio reported by Stewart (1999).

However, red-eared slider embryos accumulated Cd and Zn from exposure sources. In the 2002 study, whole hatchlings and soft tissue from the CF, DSP, LPaint, MPaint and groups accumulated Cd and Zn to concentrations equal to or greater than the bioavailable concentrations of those metals in their respective exposure solutions. In 2003, only whole hatchlings and soft tissue from the CF group accumulated Zn to concentrations higher than the bioavailable concentration in the substrate. Perhaps the

reason so few adverse effects were seen as a result of Cd exposure and accumulation was that, in all experimental treatment groups from both years and the control group from the 2003 study, the ratios of whole hatchling Zn to Cd burdens were greater than 10. In fact, the ratios ranged from 12:1 in the LS group to 119:1 in the CF group from 2003 study. Oddly, the ratios in the control groups from the 2002 substrate and exposure solution studies were 10:1 and 9:1, respectively, and no adverse effects were observed in these hatchlings either. Undeveloped eggs from both years had the same ratio of Zn to Cd, equal to 21:1.

Although the Zn to Cd ratios were the same in undeveloped eggs from both years, the ratios in the control groups and CF groups from both years were remarkably different despite incubation on the same respective substrates both years. As mentioned previously, the ratios in the control groups from the 2002 substrate and exposure solution studies were 10:1 and 9:1, whereas the ratio in the control group from the 2003 substrate study was 75:1. The Zn to Cd ratio in the CF groups increased from 47:1 in 2002 too 119:1 in 2003. All experimental conditions were basically the same both years, except for the stirring of substrates with the addition of water in the 2003 study. Perhaps the bioturbation of the metals that occurred when the substrates were stirred altered the uptake of Cd and Zn enough to change the whole body ratios of Zn to Cd of the hatchlings.

Use of T. scripta *Eggs and Hatchlings as Biomonitors of Terrestrial Contamination*

The ability of an organism to accumulate contaminants in a dose-dependent fashion is essential for its consideration and use as a biomonitor. *T. scripta* eggshells, whole hatchlings, and hatchling components often accumulated Cd, Zn, and Pb in a dose-

dependent fashion from either the substrates or the exposure solutions and reflected metal bioavailability. For example, Cd, Zn, and Pb concentrations within eggshells from the 2002 substrate and exposure solution studies and the 2003 substrate study increased with increasing metal concentrations. Whole hatchlings from all three studies also exhibited a dose-dependent accumulation of Cd, Zn, and Pb during development. Furthermore, metal accumulation in the turtle shell and tissue was also dose-dependent, although to a lesser extent than observed in eggshells and whole hatchlings.

Although a dose response was apparent in the metal accumulation of many tissues, there were instances when that did not occur. For example, in the 2002 substrate study in which the DSP substrate had the higher total metal (9300 mg/kg vs. 3000 mg/kg) concentrations and the CF substrate the higher bioavailable metal concentrations (120 mg/kg vs. 10 mg/kg), no differences were observed between the metal concentrations found in the eggshells, whole hatchlings, and hatchling compartments from the two groups. Other instances include a lack of metal accumulation to levels significantly greater than those found in the control group. No significant accumulation, compared to the control group, occurred with regards to Cd in the turtle shell and soft tissue from the 2002 substrate and exposure solution studies and the hard tissue in the 2002 exposure solution study. There also was no significant accumulation of Zn and Pb, compared to the control, in the turtle shell from the 2002 substrate study.

The absence of dose-dependent accumulation in these cases can be attributed to many factors. Perhaps the differences in the bioavailable metal concentrations were not large enough to cause differences in metal accumulation. Another reason may be experimenter error in combination with small sample size. Inexperience with the

digestion method could have caused cross-contamination of samples and because sample sizes were so low (n=4 for each tissue type), any existing differences may not have appeared to be significant.

The accumulation of Cd, Zn, and Pb in hatchlings and eggshells during development was indicative of the bioavailable concentrations of those metals within the exposure regimes. Because the bioavailable portions of metals in contaminated substrates are the most critical in the exposure, accumulation, and toxicity to organisms (Basta and Gradwhol 2000), examining the metal levels in hatchlings and eggshells incubated on different substrates can help determine the relative bioavailability of the metals present.

The lack of significant effects concerning the physiological energetics of embryos, despite metal accumulation, indicated that the embryos were resilient to the metal exposure levels examined in the study. Furthermore, the lack of significant behavioral effects in light of sizeable metal burdens and changes in the physiological energetics of hatchling implies the continued resiliency of the turtles once they hatch. Hence, they are able to survive and function in the face of metal exposure, allowing for long term studies.

Thus, red-eared slider eggs and hatchlings exhibit several of the traits necessary for consideration and use as biomonitors of environmental metal contamination. First, eggs are oviposited in terrestrial nests (Congdon and Gibbons 1990) where metal exposure can occur if the surrounding substrate is contaminated. Second, eggs respond to the exposure by accumulating metals from the substrate, and hatchlings exhibit changes in RMRs resulting from exposure. The changes in the physiological energetics of the hatchlings were not coupled with changes in performance, such as reduced swim speed or

righting ability, suggesting red-eared slider hatchlings are resilient to contamination and suitable for long-term studies. Third, red-eared sliders are widely distributed (Ernst 1990), making their use as biomonitors possible across a broad range of sites and habitats.

Future Studies

The toxic effects of contaminants on red-eared slider turtles and other reptiles are not extensively studied (Gibbons et al. 2000). My study has provided valuable information on the accumulation of metals by red-eared slider turtle embryos during incubation and the effects that uptake has on resultant hatchlings. However, there are many more potential effects and avenues to be explored. First, to provide a more comprehensive picture of the partitioning of metals within a hatchling, specific tissue groups (liver, kidney, bone, shell, muscle, and blood) within the organisms could be evaluated for metal accumulation. A potential problem in such an evaluation would be the acquisition of adequate sample mass of the separate tissues, i.e., liver and kidney, for digestion and analysis and the ability to draw enough blood for analysis.

Second, there are several biomarkers of metal exposure and accumulation that were not examined in the current study. Two major ones are metallothionein and δ-aminolevulinic acid dehydratase (ALAD) activity. ALAD is an enzyme necessary for heme synthesis (Kelada et al. 2001) that is selectively inhibited by Pb (Goyer 1991). The amount of metallothionein present in an organism's system can indicate exposure to Cd, Zn, and other metals. Analysis of metal ion and metallothionein complexes and where they are localized within the body, coupled with determination of metal levels in various organs, can shed light on the regulation and potential detoxification of such metals.

Analysis of Pb levels and ALAD activity in the blood will reveal recent Pb accumulation and indicate potential negative effects on heme synthesis, a process for which ALAD is necessary.

Third, more information is needed on the effects of exposure to metal mixtures. In the current study, a mixture of Cd, Zn, and Pb was present in the incubation substrates and exposure solutions but little emphais was placed on the effects each metal had on the others. The interactions between Cd and Zn on bioavailability and toxicity of those metals has been documented and studied primarily in invertebrates. Insights on the interactions between Zn and Cd, as well as other metals, would be valuable when attempting to determine the potential effects metal mixture contamination in areas inhabited by wildlife. Experiments could involve incubation of eggs on substrates artificially or naturally contaminated with metal mixtures and single metals. Metal mixtures should be present in varying concentration ratios and the effects elicited by metal mixture exposure can be compared and contrasted with the effects elicited by single metal exposures.

Fourth, the current study was unable to determine the effect of embryonic metal exposure on the behavior of red-eared slider hatchlings. Swimming speed and the righting ability were not satisfactory measures of the effects of metal exposure on performance. It may be that no effects occurred, but the number of studies observing adverse effects in other organisms implies that the parameters may not have been examined adequately. Perhaps, when measuring swim speed, a tank with less depth should be used to prevent hatchlings from swimming between the front and back of the tank. This would allow for more accurate measurement of linear swim speed from one

end of the tank to the other. Assessment of righting ability probably would be more effective if the experimenter were not present in the room during timing. After the hatchlings were placed on their backs, they may have continued to be aware of the human presence, which may have caused them to remain in their shells on their backs.

Last, the phenomenon of maternal transfer deserves more attention. It has been suggested to occur in many egg-laying organisms, however, few studies have looked at the whole picture by analyzing metal levels in female turtles, their undeveloped eggs, and the resultant hatchlings and hatched eggshells. I recommend analyzing metal levels in each stage of the reproductive process in red-eared sliders from contaminated and reference sites and performing reciprocal transplant experiments with the eggs.

Summary

- ✓ *Trachemys scripta* eggs and embryos are capable of accumulating Cd, Zn, and Pb from incubation substrate and external application of metal solutions.
- ✓ Accumulation of metals within whole hatchlings, eggshell, turtle shell, and soft tissues was dependent on bioavailable fractions of metals rather than total fraction of metals.
- ✓ Preferential accumulation of metals occurred in the various tissues examined.
    - Exposure to increased bioavailable concentrations of metals resulted in eggshells accumulating the highest concentrations of all three metals, indicating that eggshells may play protective roles against metal exposure during development.
    - Exposure to the higher bioavailable metal concentrations examined resulted in Cd being accumulated to higher concentrations in the turtle shell compared to the soft tissue, no consistent pattern of Zn accumulation in either component, and Pb

being accumulated to higher concentrations in the soft tissue compared to the turtle shell.

- ✓ No consistent effects were observed on the RMRs of developing embryos, although exposure to the highest bioavailable concentrations of the metals resulted in decreased caloric consumption from the yolk sac.
- ✓ Hatchlings exposed to the highest bioavailable concentrations of metals had elevated RMRs after hatching.
- ✓ Swim speed and righting ability were not influenced by the metal exposures
- ✓ Overall, red-eared slider eggs and hatchlings may serve well as biomonitors of terrestrial metal contamination.

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APPENDIX

Atomic Absorption Spectrometry

Cadmium and Zn were analyzed via Flame Atomic Absorption (FLAA). Minimum detection limits for both elements were 10 and 2.5 µg/L, respectively. Cadmium concentrations were determined within the linear absorption range (Table 14). Standards for a five-point calibration curve were diluted from a certified 1000 mg/L Cd reference solution in 5% nitric acid purchased from Fisher Scientific. Three time-averaged measurements to the nearest one thousandth of a mg/L were performed for each sample and averaged. Each replicate was read for seven seconds with a read delay of 18 seconds between individual samples. The average concentrations of the liquid samples were then divided by the dry weight of the original sample to determine Cd concentration to the nearest one thousandth of a µg/g. All recommended conditions for Cd analysis were heeded except that for the lamp current, which was increased from 4 to 5 ohms (Table 14).

Zinc concentrations also were determined within the linear absorption range (Table 14). Standards for a five-point calibration curve were diluted from a certified 1000 mg/L Zn reference solution in 5% nitric acid purchased from Fisher Scientific. Three time-averaged measurements to the nearest one thousandth of a mg/L were performed for each sample and averaged. Each replicate was read for eight seconds with a read delay of 20 seconds between individual samples. The average concentrations of the liquid samples

were then divided by the dry weight of the original sample to determine Zn concentration. All recommended conditions for Zn analysis were heeded (Table 14).

The oxidant and fuel gases used for FLAA were air and ultra high purity acetylene respectively. Both the Cd and Zn lamps were Perkin Elmer Lumina Lamps. Calibration curves with correlation coefficients equal to or greater than 0.99900 were acceptable. Curves with correlation coefficients less than 0.99900 were unacceptable, and standards were remade and rerun to achieve this standard. All samples with Cd or Zn concentrations beyond respective calibration ranges were diluted manually and reanalyzed with the same respective method.

Lead was analyzed via background corrected Graphite Furnace Atomic Absorption Spectrometry (GFAA). The minimum detection limit was 0.4 µg/L. A five-point calibration curve ranged from zero to 100 µg/L. A standard solution of 100 µg/L Pb was diluted from a certified 1000 mg/L Pb reference solution in 5% nitric acid purchased from Fisher Scientific. The AAnalyst 700 generated the calibration curve by serially diluting the initial 100 µg/L standard solution to 75, 50, and 25 µg/L. Only calibration curves with correlation coefficients of 0.99900 or higher were accepted.

Recommended wavelength, slit width and lamp current were utilized (Table 14). The atomization site was a pyrolysis platform for which we used Perkin Elmer graphite tubes with integrated platforms.

Table 14. Atomic absorption spectrometry analysis conditions for Cd, Zn, and Pb.

Element	Method	Linear Range (mg/L)	Wavelength (nm)	Slit Width (nm)	Lamp Current (ohms)
Cd	FLAA	0-2	228.8	0.7	5
Zn	FLAA	0-1	213.9	0.7	15
Pb	GFAA	N/A	283.3	0.7	10

The method for Pb determination was a five-step process (Table 15). Steps 1 and 2 were drying steps during which the liquid portion of the sample evaporated. Step 3 was the pyrolysis step. During pyrolysis, the sample matrix is eliminated, ideally leaving behind only analyte (Pb). Step 4 is atomization and is the step during which the Pb concentration was determined. The read to determine Pb concentration time lasted the duration of the hold time for atomization. Step 5 was a clean out step during which any residue left behind was eliminated.

Table 15. Graphite furnace atomic absorption profile for Pb analysis.

Step	Temp. °C	Ramp Time (s)	Hold Time (s)
1	120	5	20
2	140	5	25
3	1200	5	30
4	1900	0	4
5	2500	1	3

Two replicates for each sample were performed. The instrument calculated the Pb concentration to the nearest tenth of a μg/L from the area of the absorption peak generated during step 4. The replicates were averaged to determine the Pb concentration of the liquid sample and then divided by the dry mass of the initial sample to determine its Pb concentration to 0.1 of a ng/g.

For each replicate of each sample, the instrument pipetted 20 μL of sample, 5 μL of diluent (5% nitric acid), and 5 μL of chemical modifier. The chemical modifier is used to stabilize the analyte during pyrolysis so that it is not atomized before reading occurred. The modifier we used was a 0.2% palladium nitrate solution purchased from SCP Science and diluted to 0.1%. The instrument diluted samples beyond the calibration range and reanalyzed them using the same method. If sample concentrations were still beyond the

range after being diluted by a factor of 2 then a factor of 4, they were diluted manually and reanalyzed.

The fuel gas for GFAA was ultra high purity argon. The Pb lamp was a Perkin Elmer Lumina Lamp.

VITA (1)

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Master of Science

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