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Metal Partitioning Among Tissues and Exoskeleton of Palaemonetes Pugio and Its Role in Depuration and Trophic Transfer.

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METAL PARTITIONING AMONG TISSUES AND EXOSKELETON OF *PALAEMONETES PUGIO* AND ITS ROLE IN DEPURATION AND TROPHIC TRANSFER

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ABSTRACT

The objective of this dissertation was to determine the partitioning of Cu, Zn and Cd between tissues and exoskeleton of the grass shrimp, *Palaemonetes pugio* Holthius, and to examine the consequences of this association. The role of the exoskeleton in the potential for depuration of metals through ecdysis was examined first. The portion eliminated with the exuviae varied for each metal;11% Cu, 18% Zn, and 26% Cd of the total intermolt body burden was associated with exuviae. Some fraction of Cu and Cd in the exoskeleton was likely reabsorbed before molting but excess Zn was likely depurated through excretion. The influence of salinity and source of exposure on metal partitioning among tissue and exoskeleton was then investigated. Grass shrimp were exposed to aqueous metals in 5, 18, and 30 ‰ seawater or fed metal-enriched algal epiphytes from the stems of *Spartina alterniflora*. Surface-adsorbed metals ranged from 0-20 % of the whole-body burden with the lowest values at 5 ‰. Metals associated with the exoskeleton matrix ranged from 7-45% with highest values for Zn and Cd. Tissue Cu and Zn burden showed very little variability among the salinity and dietary-exposure treatments likely due to physiological regulation. Finally, the role of partitioning among tissue and exoskeleton in the trophic transfer of metals to the predatory fish *Fundulus grandis* was examined. *F. grandis* were fed tissues, total exoskeleton (including the adsorbed metals), or exoskeleton matrix fractions of *P. pugio* separately for 10 d. Assimilation efficiencies of Cu and Cd associated with the exoskeleton matrix and tissues of *P. pugio* by *F. grandis* were relatively low and did not differ. Zn associated with the tissues was highly available, but exoskeleton matrix-bound Zn was essentially unavailable. Surface-adsorbed Cd and Zn were highly available to *F. grandis*. Surface-

adsorbed metals should be considered in future studies because they can be an important vector in food web transfer of metals. However, metals adsorbed to the epicuticle likely do not interact with receptor sites or contribute to toxicity and should be taken into account when determining toxicological endpoints.

CHAPTER 1

GENERAL INTRODUCTION

Crustaceans are important members of marine communities ranging from estuaries to the deep sea. Planktonic and benthic crustaceans are abundant, high-biomass invertebrates that exhibit high population consumption rates and account for a significant fraction of the flow of energy through all marine environments. Crustaceans, including copepods, amphipods, mysids, euphausiids, various decapods and many others, serve as principal prey for a large number of higher-level predators. Benthic and pelagic crustaceans have also been shown to have high contaminant body burdens of trace metals (Depledge *et al.* 1993; Watras and Bloom 1992) that may cause significant toxic effects and enhance the potential for the transfer of contaminants to higher trophic levels (Russell *et al.* 1999). Crustaceans are also frequently used as sentinel species at field monitoring sites (Peterson *et al.* 1996) and as model species in laboratory toxicological studies (Fisher and Foss 1993; Key and Fulton 1993; Klerks 1999).

Due to urban and agricultural runoff and the release of industrial and municipal waste, estuarine crustaceans are exposed to the impacts of trace-metal pollution including copper, zinc, and cadmium. Cu plays a key role in growth and in the oxygen carrying pigment, hemocyanin, (Hebel *et al.* 1997), and Zn is an essential cofactor in enzyme function in crustaceans (Bryan 1968). However, excess Cu and Zn are toxic, and sublethal concentrations may lead to metabolic and cellular disturbances (Hebel *et al.* 1997; Depledge *et al.* 1993; Bryan 1968). Because Cu and Zn are essential to metabolic function, they are subject to physiological regulation in crustaceans. On the other hand, Cd has no known physiological function, and Cd at low concentrations can induce toxic effects (Nimmo *et al.* 1977; Sprague 1986). Furthermore, Cd is chemically similar to Ca and can displace Ca uptake in crustaceans (Guerin and Stickle 1995; Wallace and Lopez

1997; Zanders and Rojas 1996). Toxic responses caused by Cd include tissue damage, abnormal development, reduced growth, and reduced fecundity (Nimmo *et al.* 1977).

Although crustaceans are highly variable in body size, habitat and ecological role, they share the presence of a chitinous exoskeleton that potentially allows partitioning of trace metals between the exoskeleton and soft tissues. Chitin, a polysaccharide composed of amino sugars, is a principal constituent of the inner procuticle layer of crustacean exoskeletons (Stevenson 1985) Cations bind to chitin by forming ionic bonds with nitrogen side groups within the chitin fibrils of the procuticle and epicuticle Relatively little is known of the role that partitioning with the exoskeleton plays in the bioaccumulation and fate of metals in crustaceans. In addition, the exoskeleton is subject to periodic molting and associated cycles of calcium deposition and release. Crustaceans harden the procuticle and epicuticle following molting by the deposition of calcium salts. Ca release from the exoskeleton occurs prior to molting, and Ca is stored in cells associated with the hepatopancreas while molting occurs (Zhuang and Ahearn 1996). Therefore, the distribution of metal cations, chemically similar to Ca (*e.g.*, Zn and Cd), among tissues and exoskeleton may vary throughout the molt cycle in a fashion similar to Ca. It is likely that deleterious cations become bound in this matrix of the exoskeleton in association with periodic Ca deposition. Some fraction of these cationic metals, however, may be subject to mobilization and release to the tissues along with Ca prior to molting. Other metals become associated with the exoskeleton through different mechanisms, but may also be subject to physiological variation associated the molt cycle. For example, Cu-containing proteins similar to the oxygen-carrier pigment hemocyanin are involved in cross-linking and hardening of the exoskeleton after molting (Terwilliger 1999). Thus,

metal distribution among tissues and the matrix of the exoskeleton probably varies with respect to metal species and crustacean physiological condition.

Many organisms precipitate metals in metal-rich granules to store and/or excrete essential and toxic metals (Brown 1982). Metal granules are insoluble, and therefore, unavailable to predators (Wang *et al.* 1999; Wallace and Lopez 1997). These mineral deposits are found both intracellularly and extracellularly and accrete Zn, Cu, Ca, Cd, Fe, and Pb. In crustaceans, these granules have been found in the hepatopancreas, midgut, digestive gland, malpighian tubules, and kidney and serve in the role of excretion (Brown 1982). However, localization in granules can vary with the molt cycle, and Cu-containing granules in the hepatopancreas are released during molting and influence compartmentalization within the tissues (Brown 1982; Bryan 1968). Following ecdysis, calcium-containing granules are used in exoskeleton construction (Greenaway and Farrelly 1991), and crustaceans may also sequester metals in granule form in the exoskeleton for detoxification or elimination with the molt. However, to my knowledge there are no reports that metals other than calcium are precipitated in granules in the exoskeleton.

Investigators frequently assume that crustaceans routinely depurate metals by molting (Bertine and Goldberg 1972; Khan *et al.* 1989; Reinfelder and Fisher 1994). Such generalizations may not always be warranted, and the fate of metals associated with molting appears to depend, at least partially, on the route of exposure to metals. Fowler *et al.* (1971) found that Zn accumulated from the dissolved phase associated with interstitial spaces in the exoskeleton and that up to 41% of the Zn body burden was lost with the molted exoskeleton. Fowler *et al.* (1970) found that Zn accumulated from food,

however, became localized on ligand-binding sites on the underside of the exoskeleton, and in long-term experiments, only 1% accumulated from food was eliminated with each molt. Much less is known about the importance of molting to the fate of Cu and Cd in crustaceans.

In addition to deposition within the chitin matrix, metals may directly bind to the outer surface of the epicuticle by adsorptive processes. Chitin and associated proteins have many hydroxyl, nitrogen, and sulfur-containing groups that may serve as ligand-binding sites for cationic metals (Stevenson 1986). Many such sites are likely to be quite common on the crustacean epicuticle and on the newly-exposed surfaces of exuviae, enhancing the potential for adsorption. Consequently, if adsorption of metals occurs directly to the epicuticle without association with biological tissues, adsorbed metals may not contribute directly to toxic effects. Ingestion of exuviae following molting is, however, common in many crustaceans, leading to the possibility that adsorbed metals may enter tissues by secondary processing after ingestion and absorption through the gut.

A limited number of studies have examined the partitioning of metals between soft tissues and chitinous exoskeletons. Fowler *et al.* (1970) found that 30-66% of the ^{65}Zn body burden was consistently contained in the exoskeletons of a euphausiid and a prawn. Metal concentrations have been reported to be higher in grass shrimp exoskeletons than in muscle tissue (Khan *et al.* 1989). Munger and Hare (1997) reported that 19% of the cadmium body burden was associated with the exoskeleton of a cladoceran. Reinfelder and Fisher (1994) observed that copepod exoskeletons contained 97% of their trace-metal burden. *Uca rapax*, a fiddler crab, was reported to accumulate 80% of its Cd burden in the carapace (Zanders and Rojas 1996). Of these, only Munger

and Hare (1997). Zanders and Rojas (1996) and Reinfelder and Fisher (1994) distinguished between the surface-sorbed and the matrix-bound fractions of the exoskeleton; however, none of the studies cited above were designed to quantify the amount of the metal burden that was superficially adsorbed to the epicuticle. Hare (1992) acknowledged that metals can be associated with the surface of aquatic insect exoskeletons, and reported that 25% of the Cu, 10% of the Zn and up to 40% of the Cd body burdens were associated with the surface of nymph *Hexagenia limbata* exoskeleton. Current knowledge appears to be insufficient to generalize the relative contribution of the adsorbed fraction to the total body burden in crustaceans.

A possible explanation for the discrepancy in reported exoskeleton metal burden is the influence of salinity on the binding affinities of metal cations (Libes 1992). Because seawater has a high concentration of ions, interactions between solutes occur that can influence the effective concentration or activity of an ion. The rate and degree at which a dissolved metal adsorbs to clay minerals and organic matter, such as the crustacean epicuticle, depends upon its elemental nature, concentrations of other solutes, and the abundance of particulate matter (Libes 1992). Salinity influences bioavailability (Guerin and Stickle 1995; Zanders and Rojas 1996) and may therefore influence the metal deposition within the chitinous matrix and uptake by the tissues and may thereby alter partitioning.

Partitioning among exoskeleton and tissue pools in crustaceans may also vary as a function of exposure route. Metals that enter with the ingestion of food (and are subsequently absorbed by the gut) or are absorbed via the gills are subject to physiological regulating mechanisms. The fate of such contaminants is to be stored in

various tissue and exoskeleton pools or ultimately metabolized or excreted (Fowler *et al.* 1971). Metals obtained from food will likely associate with the matrix of the exoskeleton only after absorption and transport through the circulatory system. Thus, the route of exposure should be considered when examining contaminant interactions with the exoskeleton.

Partitioning of metals in the crustacean exoskeleton may also greatly influence trophic transfer from crustacean prey to predators or to crustaceans that ingest their exuviae. Some predators reject the crustacean exoskeleton, and a high assimilation efficiency was observed for a predator feeding on metal-enriched crustacean prey perhaps because the predator of interest selectively ingested soft tissues (Smokorowski *et al.* 1998). Reinfelder and Fisher (1994) found a strong correlation between a predatory fish's absorption efficiency of metals and the non-exoskeleton tissues of copepod prey. Chitin degrading enzymes are extremely rare among fish and crustacean predators, and it is likely that metals bound in the matrix of exoskeleton at the time of ingestion are relatively unavailable for absorption. Alternatively, metals adsorbed to the exoskeleton may be readily available for absorption in the acidic digestive tract of predators and of crustaceans that ingest their own exuviae. It is imperative to understand the mechanisms and the partitioning of metals in order to predict trophic transfer and food-web effects, and ultimately the potential for human-health effects.

The overall objective of this dissertation research was to determine the association of Cu, Zn and Cd with exoskeleton of the grass shrimp, *Palaemonetes pugio* Holthius, including association through surface-adsorption. This study was designed to mimic natural conditions as closely as possible, and stable forms of Cu, Zn, and Cd were used

because the use of radiotracers may produce artifacts due to differences in biogeochemical behavior. For example, Paulson and Gendron (2001) found that the association of ^{64}Cu with organic particles was 70% higher than that of natural Cu. *P pugio* was used as a model crustacean due to its ecological relevance as an abundant resident of estuaries and as an important member of the salt-marsh food web (Kneib 1987). Furthermore, *P.pugio* is often exposed to elevated metals and can be a vector in bioaccumulation by higher trophic levels (Smith and Weis 1997; Khan *et al* 1989; Nimmo *et al.*, 1977). Another objective was to understand the role of the exoskeleton in the potential depuration of metals through ecdysis. Additionally, the effect of salinity and source of exposure on partitioning among tissue and exoskeleton was investigated. Finally, the ecological significance of the role of partitioning among the exoskeleton and tissues in the trophic transfer of metals was addressed. The following null hypotheses were tested in my dissertation:

- Ecdysis does not influence the Cu, Zn, and Cd body burdens in *Palaemonetes pugio*
- Salinity does not influence partitioning of Cu, Zn, and Cd between tissue and exoskeleton (both surface-adsorbed and matrix-sequestered fractions) in *P. pugio*
- The route of exposure does not influence partitioning of Cu, Zn and Cd between tissue and exoskeleton (both surface-adsorbed and matrix-sequestered fractions) in *P. pugio*.
- Partitioning between tissue and exoskeleton (both surface-adsorbed and matrix- sequestered fractions) does not influence the trophic transfer of Cu, Zn, and Cd to a fish predator *(Fundulus grandis)*.

The specific objective of the second chapter of this dissertation was to quantify the sequestration of Cu, Zn, and Cd in sublethal concentrations from the aqueous phase by the exoskeleton of grass shrimp and to determine what fraction of these trace metals are depurated through ecdysis. Elimination of metals in decapod crustaceans can be attributed to excretion, flux across the body surfaces and gills (Bryan 1968) as well as to molting. It has been suggested that the crustacean exoskeleton may sequester metals and contribute to elimination (depuration) through ecdysis (Reinfelder and Fisher 1994; Bertine and Goldberg 1972; Khan *et al.* 1989; Smokorowski *et al.* 1998). My study was designed to determine the role of ecdysis in the fate of Cu, Zn, and Cd in *P. pugio*.

Another topic addressed in Chapter 2 was the role of surface adsorption on exuviae shed in metal-enriched water Ligand-binding sites may be more common on the underside of the molted exoskeleton than on the exposed epicuticle. Therefore, after molting, adsorption to newly exposed binding sites could contribute to an increase in the concentrations of metals in exuviae cast in contaminated water. White and Rainbow (1984) found that exuviae of *Palaemon elegans* take up Cu, Zn, and Cd from the ambient medium, and it has been speculated that adsorption of metals onto the shed molt maybe an important phenomenon in the cycling of trace metals in aquatic systems (Wang *et al.* 1996). Additionally, many crustaceans, particularly in densely-populated farming conditions, ingest exuviae in order to compensate for the energetic loss of exuvial production (Guillaume 1997). Therefore, adsorption of metals from the aqueous phase by the exuviae was measured in the second chapter of dissertation because of its potential importance in aquaculture. This second chapter has been accepted for publication in *Marine Pollution Bulletin*.

The objective of Chapter 3 was to determine how the source of exposure (aqueous or dietary) and salinity influences the uptake and partitioning of Cu, Zn, and Cd among the tissues and exoskeleton (both surface-adsorbed and matrix-bound) of *P. pugio*. A variety of metals (i.e., Cu, Zn, Cd, Co, Se, Hg) associated with exoskeletons have been measured in diverse groups of crustaceans and authors report widely varying results (Zanders and Rojas 1996; Khan *et al.* 1989; Munger and Hare 1997; Depledge *et al.* 1993; Reinfelder and Fisher 1994; White and Rainbow 1984; Nimmo *et al.* 1977; Fowler *et al.* 1970; Bryan 1968). The highest reported values (97% of the total body Cd, Zn, and Co burden) of the exoskeleton are reported in marine planktonic copepods (Reinfelder and Fisher1994); however, a freshwater cladoceran was shown to have only 19% of the Cd burden in the exoskeleton (Munger and Hare 1997). Variability among these studies could be due to differences in physiology, metal-species behavior, salinity, and source of exposure. Because salinity influences metal speciation, and subsequently metal solubility and bioavailability (Guerin and Stickle 1995; Zanders and Rojas 1996), salinity effects on uptake and partitioning from aqueous exposure were examined by aqueous exposure at three salinities (5, 18, and 30‰).

In order to examine the effect of dietary exposure on partitioning, metal-exposed epiphytic algae associated with the stems of *Spartina alterniflora* were used as the food source because grass shrimp are know to ingest epiphytes (Morgan 1980) including those associated with *S. alterniflora* (Fleeger *et al.* 1999). *S. alterniflora* is instrumental in cycling trace metals in wetlands because metals are released through the salt glands in the leaves (Burke *et al.* 2000) and released metals may subsequently become available to epiphytes. Catallo *et al.* (1996) demonstrated that endosymbiotic yeast in *S. alterniflora*

are capable of assimilating Cu, Zn, Cd, and Ni, and perhaps other microbial symbionts may also be important in the cycling of trace metals in salt marshes through assimilation and trophic transfer to herbivores such as *P. pugio*.

A major objective of Chapter 3 was to quantify surface-adsorbed and matrix-bound metals under a range of uptake and salinity conditions. Although many authors (Reinfelder and Fisher 1994; Zanders and Rojas 1996; Smokerowski *et al.* 1998) acknowledge the potential significance of adsorption, no studies have attempted to directly quantify the distribution between metals adsorbed to the surface and metals sequestered within the chitin matrix in crustaceans. A strong chelator (EDTA) was used to remove surface-adsorbed metals in *P. pugio*. This process does not solubilize metals sequestered within the procuticle and served as the basis to distinguish between surface-adsorbed and matrix-sequestered metals.

The objective of Chapter 4 was to determine the role of the crustacean exoskeleton in the trophic transfer of metals from a crustacean (*P. pugio*) to a fish predator (*Fundulus grandis* Baird and Girard). *F. grandis* is an important estuarine resident, frequently used in toxicological studies (Smith and Weis 1997; Davis *et al.* 1998). *F. grandis* feeds on invertebrate prey including *P. pugio* (Kneib 1987). Assimilation efficiencies of metals by predators are dependent on the chemical species and the tissues in which they are adsorbed (Chen *et al.* 2000), and therefore, partitioning among tissues and exoskeleton of prey may influence bioavailability to higher trophic levels. The assimilation efficiency of metals (defined as the amount taken up by the tissues (Penry 1998) adsorbed to the surface of the exoskeleton, incorporated into the chitin matrix, and associated with the soft tissues were specifically measured and

compared. Grass shrimp exoskeleton, with or without removal of surface-adsorbed metals, or tissues were fed to *F. grandis* in separate gelatin capsules for 10 days. Previous authors have correlated assimilation efficiencies with the proportion of metals in soft tissues of crustacean prey (Munger and Hare 1997; Reinfelder and Fisher; 1994, Ni *et al.* 2000; Wang *et al.* 1999). However, direct measurements of metal assimilation from crustacean soft tissue or exoskeleton has not been reported. It has been suggested that metals associated with the exoskeleton are unavailable to higher trophic levels due to the indigestibly of chitin, but this presumption has not been directly tested. Furthermore, the assimilation of surface-adsorbed metals by higher trophic levels has not been determined. Surface-adsorbed metals may be an important vector in food web transfer because metals may be solublized in the gastric fluids of predators. Finally, the significance of this dissertation is discussed in the concluding chapter (Chapter 5).

CHAPER 2

THE CONTRIBUTION OF ECDYSIS TO THE FATE OF COPPER, ZINC AND CADMIUM IN GRASS SHRIMP, *PALAEMONETES PUGIO* HOLTHIUS*

*Reprinted from Marine Pollution Bulletin (in press) Keteles, K.A. and Fleeger, J.W .. The contribution of ecdysis to the fate of copper, zinc and cadmium in grass shrimp *Palaemonetes pugio* Hothius, with permission from Elsevier Science.

INTRODUCTION

There are several possible fates (*e.g.*, excretion, sequestration by metal-binding proteins) of trace metals after bioaccumulation in aquatic organisms (Hare 1992). In crustaceans, some fraction of the whole-body burden of trace metals is associated with the chitinous exoskeleton (Munger and Hare 1997; Zanders and Rojas 1996; Reinfelder and Fisher 1994; Depledge *et al.* 1993; Weeks *et al.* 1992; Khan *et al.* 1989; Nimmo *et al.* 1977; White and Rainbow 1984; Fowler *et al.* 1971; Bryan 1968). This relationship appears to be highly variable among taxa and metal species, but the fraction associated with the exoskeleton may represent a significant portion of the total body burden (reports cited above vary from 19-97%). To further complicate exoskeleton-metal associations, metals may either adsorb to the surface of the exoskeleton or bind to the inner exoskeleton matrix after uptake and transport through the hemolymph. The adsorbed fraction is rarely considered (Hare 1992). Also, as an important aspect of crustacean physiology, molting may influence metal concentrations and the distribution between the soft tissues and exoskeleton (Weeks *et al.* 1992). It has been suggested that the crustacean exoskeleton may sequester metals and contribute to elimination (depuration) through ecdysis (Reinfelder and Fisher 1994; Bertine and Goldberg 1972; Khan *et al.* 1989; Smokorowski *et al.* 1998).

The molt cycle, and its associated storage and deposition of calcium salts, may facilitate the sequestration of trace metals within the crustacean exoskeleton. Crustaceans harden the procuticle and epicuticle following molting by the deposition of calcium salts. Ca release from the exoskeleton occurs prior to molting (Stevenson 1985), and Ca in some crustaceans is stored in cells associated with the hepatopancreas and in the

gastroliths of the proventriculus while molting occurs (Adams *et al.*1982; Zhuang and Ahearn 1996). Metal cations (*e.g.*, zinc and cadmium) bind to chitin by forming ionic bonds with nitrogen side groups within the chitin fibrils of the procuticle. It is likely that cations become bound in this matrix of the exoskeleton in association with Ca deposition. Some fraction of these cationic metals, however, may be subject to mobilization and release to the tissues along with Ca prior to molting. Other metals become associated with the exoskeleton through different mechanisms, but may also be subject to physiological variation associated the molt cycle. For example, copper-containing proteins similar to the oxygen-carrier pigment hemocyanin are involved in cross-linking and hardening of the exoskeleton during molting (Terwilliger 1999).

A number of studies have examined the fate of Cu and Zn associated with the molt cycle, but report widely different results. Fowler *et al.* (1971) found that Zn accumulated from the dissolved phase associated with interstitial spaces in the exoskeleton and that up to 41% of the Zn body burden was lost with the molted exuviae. However, several investigators report much higher rates of retention of metals during ecdysis. Fowler *et al.* (1970) found that Zn accumulated from food became localized on ligand-binding sites on the underside of the exoskeleton, and in long-term experiments, only 1% accumulated from food was eliminated with each molt. Weeks *et al.* (1992) found negligible quantities of Cu and Zn in the exuviae of talitrid amphipods. Similarly, negligible levels of Cu were found in the exuviae of *Callinectes sapidus* (Engle 1987). Variable results may be due to differences in physiology between species and/or external metal concentrations.

Metals may also associate with the exoskeleton through adsorptive processes. Chitin and associated proteins have many hydroxyl, nitrogen, and sulfur-containing groups that may serve as ligand-binding sites for cationic metals. These sites may be quite common on the crustacean epicuticle, enhancing the potential for adsorption. Further, if molting occurs in contaminated water, metals may adsorb from the aqueous phase directly to the newly exposed surfaces of the procuticle of the exuviae (White and Rainbow 1984). The adsorbed metals may be bioaccumulated if the organism ingests its exuviae. Such adsorption of metals onto binding sites not associated with the epicuticle of exuviae may overestimate the role of ecdysis in the elimination of metals.

The purpose of this study was to quantify the sequestration of selected metals (Zn, Cu, Cd) in sublethal concentrations from the aqueous phase by the exoskeleton of the grass shrimp, *Palaemonetes pugio* Holthius and to determine what fraction of these trace metals are depurated through ecdysis. Also because of its potential importance in aquaculture (where a high percentage of crustacean exuviae are ingested), the adsorption of metals from the aqueous phase by the exuviae was measured.

METHODS

Exposure of Post-ecdysis Shrimp

In order to determine metal sequestration by the exoskeleton and depuration through ecdysis, grass shrimp were collected from a *Spartina alterniflora* salt marsh (salinity at the time of collection was 15 ‰) near Cocodrie, La. Grass shrimp from 22-28 mm in length from rostrum to telson were retained to maintain a consistent surface-area-to-volume relationship and molt frequency among individuals. Shrimp were acclimated to a salinity of 18 ‰ in the laboratory for 24 h prior to the experiment.

Single shrimp were placed in 50 low-density polyethylene (LDPE) acid-cleaned chambers in 18 ‰ Instant Ocean® (World Aquarium Systems) artificial seawater (ASW), exposed to aqueous sublethal mixtures of elemental Cd, Cu, and Zn (200 μg L^{-1} Cd, 1000 μg L^{-1} Zn, 500 μg L^{-1} Cu), and examined four times daily for molting. The sublethal mixture of the three metal concentrations was chosen based on unpublished data (Keteles, personal observation). Exuviae from shrimp that molted in metal-contaminated water were removed and analyzed for metal content. After 72 h, unmolted shrimp were removed and placed in LDPE acid-cleaned chambers containing clean 18 ‰ ASW without elevated metals; shrimp molted an average of 3.8 (± 0.35 SEM) days after being placed in uncontaminated water. After ecdysis in uncontaminated water, the exuviae and corresponding whole shrimp were frozen in liquid nitrogen and later analyzed for metal content. Metal concentrations were measured in six replicates of three randomly pooled whole shrimp and corresponding exuviae.

An estimate of percent depurated from a single molt was calculated based on the mass of metals retained in whole shrimp after molting compared to the mass of metals eliminated with the exuviae of the post-ecdysis shrimp. In order to determine the magnitude of the adsorption of metals onto exuviae, metal concentrations in exuviae cast in contaminated water were compared with exuviae cast in uncontaminated water and to concentrations in intermolt exoskeletons.

Exposure of Intermolt Shrimp

Metal concentrations were also measured in metal-exposed shrimp that either did not molt or were continuously exposed before and after a molt to determine the importance of the exoskeleton in the sequestration of metals and to compare to post-molt

metal-exposed shrimp. Twenty grass shrimp were exposed to the same sublethal mixture of metals (200 μg L^{-1} Cd, 1000 μg L^{-1} Zn, 500 μg L^{-1} Cu) in each of 6 replicate 4-L jars in 18 ‰ ASW and monitored daily for indications of molting. A small fraction (10%) of the grass shrimp in each jar molted. The low percentage of molts suggested that metal uptake in these shrimp would represent metal burdens under intermolt conditions. Following 72 h of exposure, shrimp were frozen in liquid nitrogen and exoskeletons (excluding legs) were excised. Metal concentrations were measured in the excised-exoskeletons and the remaining tissues of 10 pooled shrimp from each jar.

Intermolt vs. Post ecdysis Comparisons

Data from the post-ecdysis and intermolt experiments were used to calculate composite whole-body metal burdens in order to determine if body burdens were reduced after a single molt. Excised-exoskeleton metal concentrations of intermolt shrimp were compared with metal concentrations in the exuviae from post-ecdysis shrimp which molted in uncontaminated water.

Metal Analysis

Samples were placed in preweighed, acid-cleaned glass test tubes, weighed, and digested in 5 ml of concentrated trace metal grade HNO_3 (Fisher Scientific) at 90 C for 12 H. The acid containing digested samples were evaporated to about 1.5 ml, then diluted to ~25 ml and reweighed to determine volume. Blanks were prepared for each set of samples digested. Metal concentrations in each fraction were determined by Inductively Coupled Plasma (ICP) emission spectroscopy and corrected for interelement interference.

Statistical Methods

Whole-body metal burdens of post-ecdysis grass shrimp were compared with intermolt whole-body burdens using a t-test to determine if concentrations of Cu, Cd, and Zn were altered after a single molt. The concentrations of Cu, Zn, and Cd in the post-ecdysis grass shrimp exuviae produced in both contaminated and uncontaminated water were also compared with concentrations in the excised exoskeletons of intermolt grass shrimp using a t-test. Exuviae produced in uncontaminated water were compared with exuviae from contaminated water. In some instances, comparisons failed normality; if so the nonparametric Mann-Whitney Rank Sum Test was used. Sample size for each metal was 6 replicates, and all tests were conducted with Sigma Stat (Jandel Scientific®) software.

RESULTS

Whole-body Burdens of Intermolt and Post-ecdysis Shrimp

Trace metal whole-body burdens of intermolt *P. pugio* exposed to elevated metals averaged 94, 2.9, and 158 $\mu g\ g^{-1}$ for Cu, Cd, and Zn respectively (Figure 1). Metal concentrations in intermolt shrimp were compared to whole-body burdens in post-ecdysis shrimp (exposed to elevated metals for three days and allowed to molt in "clean" water) to estimate the retention of metals through a molt cycle. Results varied among the metals tested. Cu whole-body burdens in post-ecdysis shrimp averaged 80 $\mu g\ g^{-1}$ (Figure 1). Cu concentration did not decrease significantly after a single molt ($p=0.209$; power=1.000); 11% of the Cu intermolt body burden was associated with the exuviae (Figure 2). Cd whole-body burdens after ecdysis (Figure 1) averaged 1.7 $\mu g\ g^{-1}$ and Cd whole-body burdens decreased significantly after a single molt ($p=0.049$); 26% of the intermolt Cd

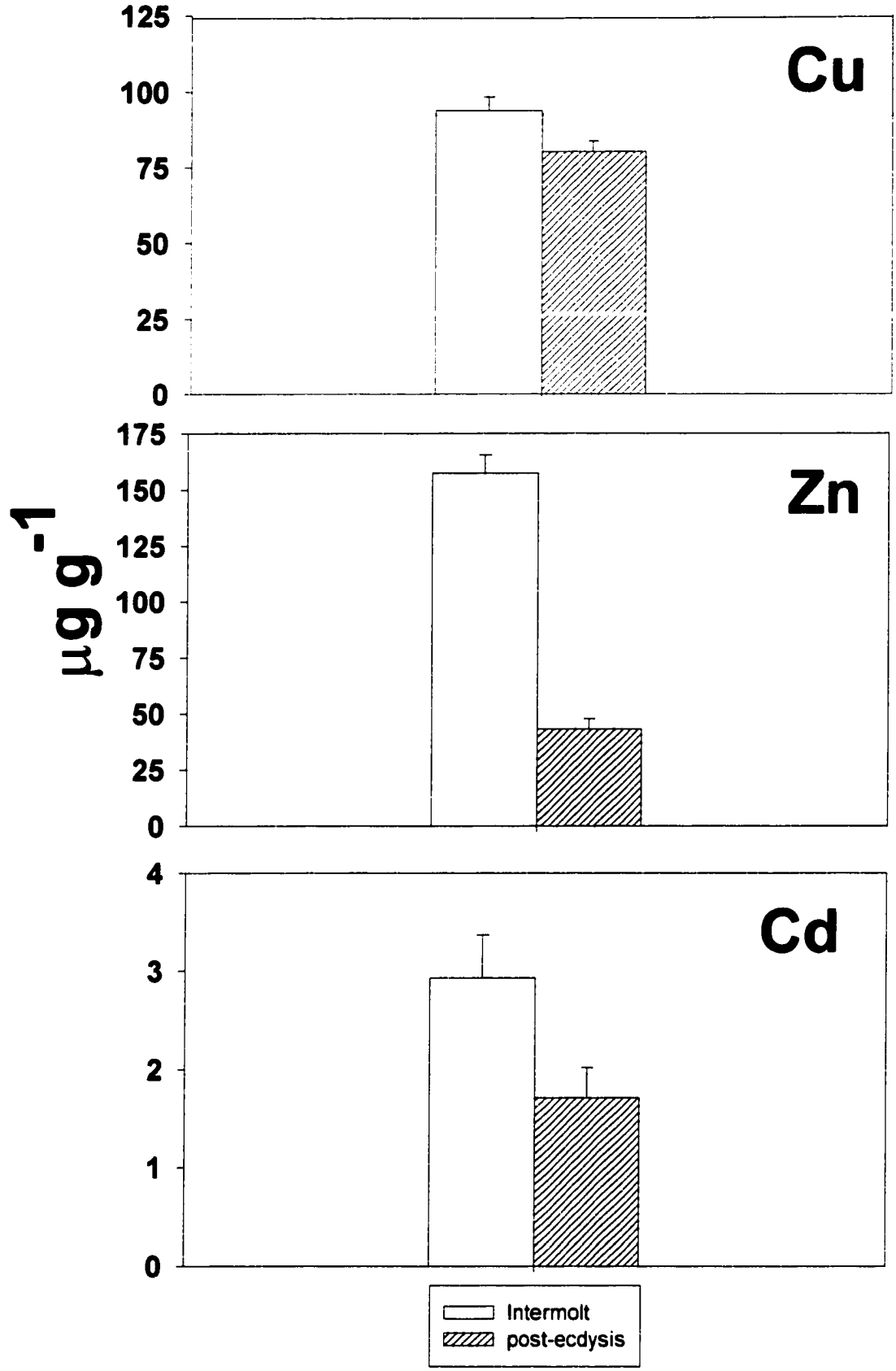

Figure 1. Mean whole-body burdens (µg g^{-1}) of Cu, Zn, and Cd of intermolt and post-ecdysis *Palaemonetes pugio* exposed to metal-enriched water. Post-ecdysis shrimp molted in uncontaminated water (n=6). Intermolt values were based on a composite shrimp, combining the total metals in the exoskeleton and tissues (n=6). Error bars represent standard error of the mean.

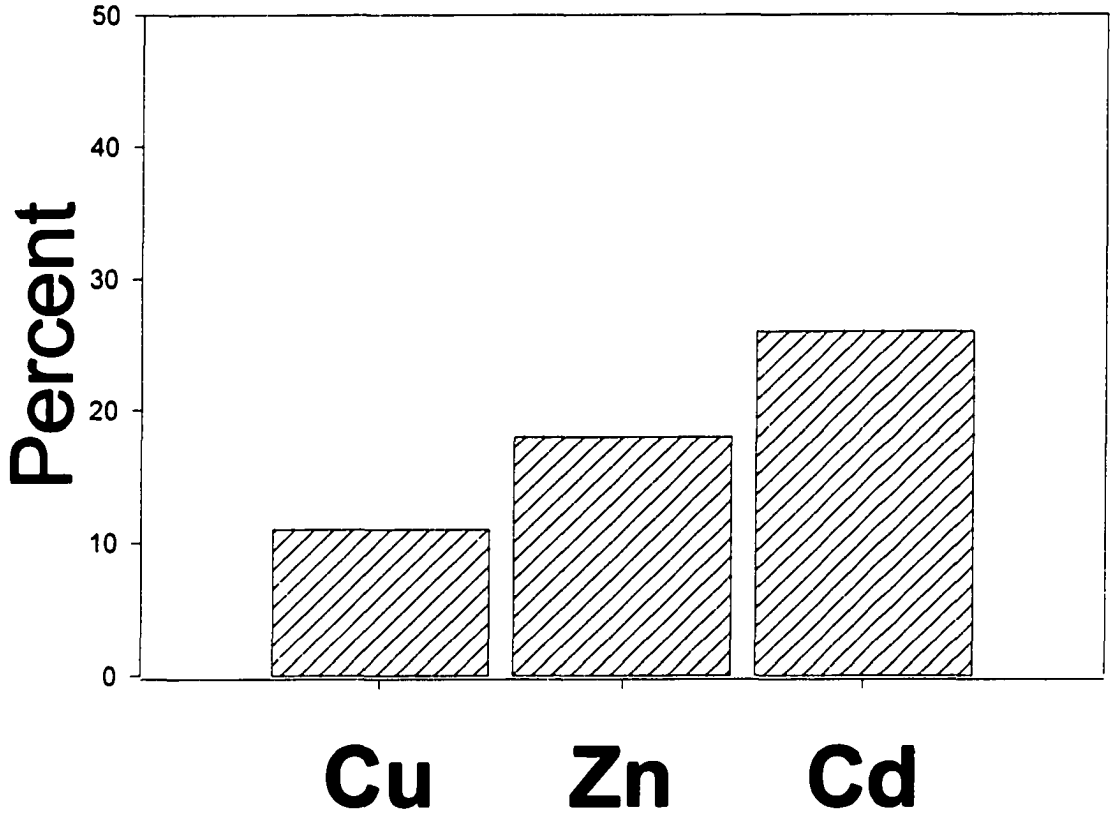

Figure 2. Depuration as a function of the relative percent of the whole-body burden that was eliminated with the exuviae in the grass shrimp, *Palaemonetes pugio*. Percent was calculated based upon the total mass (μg) of metal associated with the whole-body burden and the exuviae.

body burden was eliminated with the exuviae (Figure 2). Whole-body burdens of Zn in post-ecdysis shrimp averaged 43 $\mu g\ g^{-1}$ (Figure 1), which was significantly lower than intermolt concentrations ($p = <0.001$) even though only 18% of the intermolt body burden was associated with the exuviae (Figure 2).

Concentrations in Exoskeleton and Exuviae

Intermolt *P. pugio* stored a substantial portion of their total metal body burden in the exoskeleton. The exposure to elevated aqueous metals (500 $\mu g\ L^{-1}$ Cu, 1000 $\mu g\ L^{-1}$ Zn, and 200 $\mu g\ L^{-1}$) resulted in exoskeleton concentrations of 91 $\mu g\ g^{-1}$ Cu, 2.9 $\mu g\ g^{-1}$ Cd, and 220 $\mu g\ g^{-1}$ Zn (Figure 3) which represented 36, 52 and 40 % respectively of the total Cu, Zn, and Cd body burden (Figure 3). Metal concentrations of the exuviae produced after exposure to elevated metals but allowed to molt in clean water averaged 15, 0.8 , and 15 $\mu g\ g^{-1}$ Cu, Cd, and Zn respectively and were compared to the concentrations in excised exoskeletons of intermolt shrimp (Figure 3). With the exception of Cd, the intermolt exoskeleton concentrations were significantly higher than the concentrations in the exuviae ($p = 0.001$ for Cu, 0.061 for Cd (power=0.397), and <0.001 for Zn).

Adsorption onto Exuviae

Metal concentrations of exuviae produced in contaminated water averaged 198 $\mu g\ g^{-1}$ Cu, 36 $\mu g\ g^{-1}$ Cd, and 382 $\mu g\ g^{-1}$ Zn (Figure 3). Each was significantly higher than in exuviae produced by molting in uncontaminated water ($p = 0.001$ for Cu, 0.037 for Cd, and 0.007 for Zn), suggesting that metals adsorb to the exposed surfaces of the exuviae. When exuviae produced by molting in contaminated water were compared to excised intermolt exoskeletons, only exuvial Cd concentrations were significantly higher ($p=0.018$). Cu and Zn concentrations of exuvia cast in contaminated water were elevated

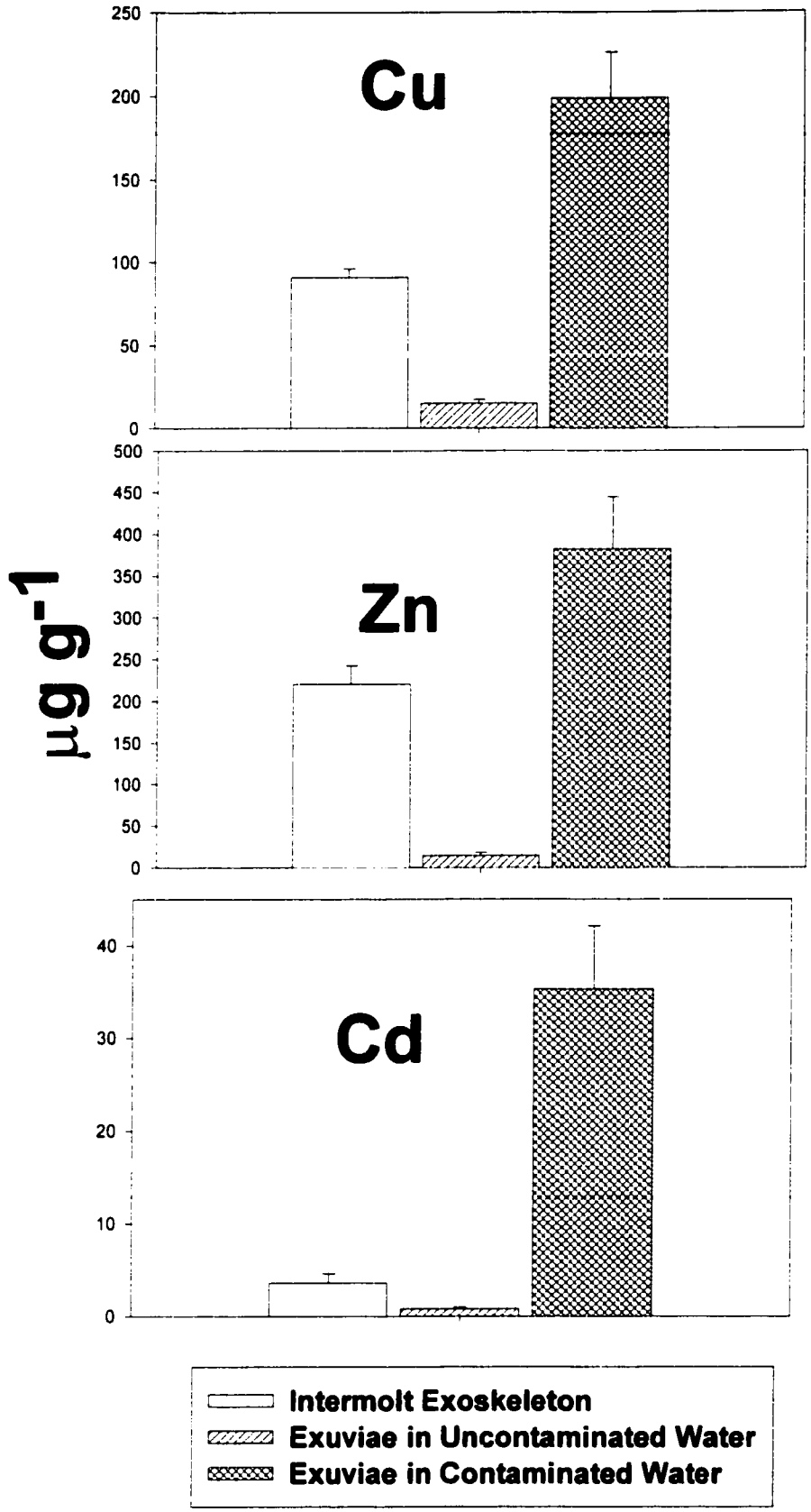

Figure 3. Mean Cu, Zn, and Cd concentrations of *Palaemonetes pugio* intermolt exoskeletons from metal-exposed unmolted shrimp (n=6), and post-ecdysis exuviae cast in metal-contaminated (n=6) and uncontaminated (n=6) water. Error bars represent the standard error of the mean.

compared to excised intermolt exoskeletons, but were not significant at the 0.05 level ($p=0.059$ for Cu and $p=0.087$ for Zn).

DISCUSSION

Our results were designed to determine the role of ecdysis in the fate of metals in *Palaemonetes pugio*. Elimination of metals in decapod crustaceans can be attributed to excretion via the urine and feces, flux across the body surfaces and gills (Bryan 1968), as well as with exuviae. Because the grass shrimp in our post-ecdysis experiment were held in seawater without elevated metals for an average of 3.8 d prior to molting, some metals taken up during the exposure period were likely excreted. If excretion was important to the fate of metals, our estimates of loss through the exuviae will be underestimated. Therefore, data on loss to exuviae were used in concert with whole-body burden comparisons in intermolt and post-ecdysis grass shrimp to determine if there was a potential for loss by a mechanism other than molting. Our results suggest that the potential role for depuration with the exuviae differs for Cu, Cd, and Zn.

Cu whole-body burdens of intermolt and post-ecdysis shrimp were not significantly different, and Cu concentration in exuviae was significantly lower than in the intermolt exoskeleton (Figures 1 and 3). These data suggest that grass shrimp conserve Cu by reabsorption from the exoskeleton prior to molting, and that relatively small amounts of Cu are excreted. Negligible quantities of Cu were associated with the exuviae in talitrid amphipods (Weeks *et al.* 1992) and the blue crab, *Callinectes sapidus*, (Engle 1987) further suggesting a conservation of Cu during the molt cycle. Research with the blue crab, *Callinectes sapidus* (Engle 1987), and the lobster, *Homarus*

gammarus (Hagerman 1983), showed that the distribution of Cu among tissues is altered just prior to ecdysis. Post-ecdysis *C. sapidus* may use stored Cu to synthesize hemocyanin (Engle 1987) and *P. pugio* may similarly utilize Cu conserved during ecdysis in hemocyanin production. Furthermore, the crustacean exoskeleton is formed by hypodermis cells, and hemolymph proteins similar to hemocyanin are instrumental in the synthesis of the new exoskeleton (Terwilliger 1999). Cu containing prophenoloxidases cross-link and harden the exoskeleton during ecdysis.

Our results for Cd suggest that intermolt exoskeletons of *P.pugio* sequester a relatively large fraction (40%) of the total Cd whole-body burden. Similar results were found by Khan *et al.* (1989) in *P.pugio* collected from a contaminated site. The total Cd whole-body burdens in *P. pugio* were significantly lower in post-ecdysis than in intermolt shrimp in our study (Figure 1). Additionally, there was no difference between exuviae and intermolt exoskeleton Cd concentrations (Figure 3), and 26 % of the intermolt Cd body burden was associated with the exuviae (Figure 2). Taken together, these data suggest that loss to the exuviae substantially contributed to Cd depuration, but do not exclude some loss by other mechanisms. A 50% loss of Cd by excretion was measured in pink shrimp, *Penaeus duararum*, in 7 to 10 days (Nimmo *et al.* 1977). Furthermore, it is possible that some Cd was mobilized and transported from the exoskeleton to tissues prior to ecdysis. Nimmo *et al.* (1977) found that when transfered to Cd-free water following exposure, Cd levels in pink shrimp exoskeleton decreased, while the concentration in muscle increased. Sensitivity to metal toxicity has been attributed to the uptake of ions from the external medium during the post-molt period in the shore crab, *Carcinus maenas* (Scott-Fordsmand and Depledge 1997); however, the results of our

study suggest that high mortality of molting crustaceans during toxicity bioassays could be attributed to an increase in metal levels by redistribution to tissues preceding ecdysis.

Zn whole-body burdens were significantly lower in post-ecdysis *P. pugio* than in the intermolt grass shrimp (Figure 1). Moreover, exuvial Zn concentrations were significantly lower than that of intermolt exoskeletons (Figure 3) and 18% of the Zn whole-body burden was associated with the exuviae (Figure 2). Therefore, it appears that Zn was depurated, but our data suggest that excretion is probably more important to the fate of Zn than the loss with exuviae. Fowler *et al.* (1971) reported that molting accounted only for a 1% loss of ^{65}Zn from the initial body burden over five months for a euphausiid, yet 96% of the initial body burden was eliminated, suggesting excretion was the primary route of loss. Large losses of Zn in decapod crustaceans have been attributed to excretion across the gills (Bryan 1968). Decapod crustaceans are typically effective regulators of Zn content; however, Zn flux can vary with external Zn concentrations, temperature, and between individuals (White and Rainbow 1984). Alkaline phosphatase, a Zn metalloenzyme, has been found to be associated with the new exoskeleton of a freshwater crayfish before ecdysis (Bryan 1968), and it is possible that molt physiology may influence Zn flux in crustaceans.

Significant adsorption to exuviae occurred from aqueous metals as suggested by the elevated levels of Cu, Cd, and Zn associated with molted exuviae in metal-enriched water (Figure 3). Cd concentrations of exuviae cast in contaminated water were also significantly higher than intermolt exoskeletons. Chitin and associated proteins have many hydroxyl, nitrogen, and sulfur-containing functional groups that may serve as ligand-binding sites for cationic metals (Stevenson 1985). Many such sites are likely to

be quite common on the crustacean epicuticle, enhancing the potential for adsorption. These ligand-binding sites may be even more common on the underside of the molted exoskeleton. Therefore, after molting, new surfaces for adsorption could contribute to the increase in the concentrations of exuviae cast in contaminated water. White and Rainbow (1984) also found that crustacean exuviae take up Zn, Cu, and Cd from the ambient medium. The adsorption of metals onto the shed molt maybe an important phenomenon in the cycling of trace metals in aquatic systems (Wang *et al.* 1996). Additionally, many crustaceans, particularly in densely-populated farming conditions, ingest exuviae in order to recycle nitrogen and compensate for the energetic loss of exuvial production (Guillaume 1997). In fact, the molted exoskeletons are incorporated into the calculated food budgets of commercial shrimp production (Dr. R. Portier, personal communication). Elevated levels of metals associated with molted exoskeleton due to adsorptive processes under contaminated conditions, as a result of the ingestion of exuviae, may be an important source of metal bioaccumulation by crustaceans.

Our work suggests that a substantial portion of the whole-body burdens of Cu, Zn and Cd are associated with the exoskeleton (36, 52 and 40%, respectively) and that the molt cycle is important to the fate and distribution of metals in *P. pugio*. Ecdysis contributes to reductions in Cd burdens while Zn appears to be largely depurated by excretion. Most Cu associated with the exoskeleton appears to be redistributed to the tissues before molting and is therefore retained after a molt. Furthermore, metals appear to adsorb to the surface of *P. pugio* exuviae when cast in metal-enriched water. Such findings may have important implications for crustacean-metal interactions. First, metals may not exert a toxic effect when associated with the exoskeleton. This suggests that

critical-body residue predictions of toxicity derived in other taxa may be inaccurate in crustaceans and that crustaceans may not serve as reliable biomonitors. Our data also suggest that tissue levels vary over a molt cycle, possibly influencing the timing of toxic effects. Second, metal bioavailability in tissues and exoskeleton may differ, influencing trophic transfer of metals to predators.

CHAPTER 3

METAL UPTAKE AND PARTITIONING AMONG TISSUES AND EXOSKELETON OF THE GRASS SHRIMP, *PALAEMONETES PUGIO*

INTRODUCTION

Trace metals have been shown to associate with both tissues and the chitinous exoskeleton in crustaceans. Measurements of a variety of metals (*e.g.*, Cu, Zn, Cd, Co, Se, Hg) associated with exoskeletons have been made in at least eight species of crustaceans in groups as diverse as decapods and copepods (Zanders and Rojas 1996; Khan *et al.* 1989; Munger and Hare 1997; Depledge *et al.* 1993; Reinfelder and Fisher 1994; White and Rainbow 1984; Nimmo *et al.* 1977; Fowler *et al.* 1970; Bryan 1968). Several studies (Zanders and Rojas 1996; Khan *et al.* 1989; Reinfelder and Fisher 1994; Fowler *et al.* 1970; Depledge *et al.* 1993; Nimmo *et al.* 1977) have found higher concentrations of Cd and Zn in the exoskeleton compared to whole-body tissues (or specific tissues such as muscle). The highest reported values (97% of the total body Cd, Zn, and Co burden) of the exoskeleton have come from marine planktonic copepods (Reinfelder and Fisher1994); however, a freshwater cladoceran was shown to have 19% of its Cd burden in the exoskeleton (Munger and Hare 1997). Of the metals studied, Cu appears to have the lowest fraction associated with the exoskeleton (Depledge *et al.* 1993). Variability among these studies could be due to differences in physiology, metal-species behavior, salinity, and source of exposure.

Metals may associate with the exoskeleton in two ways. First, the deposition of calcium salts following molting may facilitate the sequestration of trace metals within the procuticle (or matrix) of the exoskeleton (Adams 1981, Stevenson 1985). White and Rainbow (1984) found that zinc concentration in the cuticle varied with degree of calcification in a fashion closely related to the molt cycle. Cations bind to chitin by forming ionic bonds with nitrogen side groups within the chitin fibrils of the procuticle.

Some fraction of these cationic metals, however, may be subject to mobilization and release to the tissues along with Ca prior to molting. Other metals become associated with the exoskeleton through different mechanisms, but may also be subject to physiological variation associated with the molt cycle. For example, copper-containing proteins similar to the oxygen-carrier pigment hemocyanin are involved in cross-linking and hardening of the exoskeleton during molting (Terwilliger 1999).

The second method of metal association with the exoskeleton is through an adsorptive process. Chitin and associated proteins have many hydroxyl, nitrogen, and sulfur-containing groups that could serve as ligand-binding sites for cationic metals (Stevenson 1985). Such sites are likely to be quite common on the crustacean epicuticle, enhancing the potential for surface adsorption. It appears that much of the zinc associated with the exoskeleton of some decapod crustaceans is adsorbed to the surface, and metal concentration of the exoskeleton increases when the concentration of the ambient water increases (Bryan 1968). Adsorption to exuviae is know to occur from metal-enriched water (Keteles and Fleeger, in press). If significant adsorption on the epicuticle occurs, surface area may influence the quantity of adsorbed contaminant per individual crustacean, and body size may therefore contribute to species-specific differences in adsorption. Exoskeleton chemical composition also differs among crustaceans, adding to the potential for species-specific differences in contaminant partitioning. Furthermore, adsorption of contaminants likely occurs directly to the epicuticle without association with biological tissues, and such adsorbed substances may not contribute to toxic effects. Although many authors (Reinfelder and Fisher 1994; Zanders and Rojas 1996; Smokerowski *et al.* 1998) acknowledge the potential significance of adsorption, no

studies have attempted to directly quantify the distribution between metals adsorbed to the surface and metals sequestered within the crustacean chitin matrix in the procuticle, although studies have show that 7-40% is adsorbed to the exoskeleton of aquatic nymphs (Hare 1992).

Partitioning among these various exoskeleton and tissue pools in crustaceans may vary as a function of exposure route (Fowler *et al.* 1970). Contaminants that enter with the ingestion of food, and are subsequently absorbed by the gut, may be subject to physiological regulating mechanisms (Rainbow 1995). The fate of such contaminants is to be stored in various tissue and exoskeleton pools or ultimately metabolized or excreted (Fowler *et al.* 1970). Generally, tissue concentrations of Zn and Cu appear to be tightly regulated in crustaceans (Bryan 1968; Rainbow 1995) at least at low exposure concentrations; Cd does not appear to be regulated (Rainbow 1995). Metals obtained from food will likely associate with the chitin matrix of the exoskeleton only after absorption and transport through the circulatory system. Alternatively, some fraction of dissolved metals may rapidly reach equilibrium directly by adsorption to the surface of exoskeletons without first entering tissues and being subject to regulatory mechanisms.

The objective of this study was to determine how the source of exposure (aqueous or dietary) effects uptake and partitioning of Cu, Zn, and Cd between the tissues, chitin matrix in the procuticle, and the surface of the epicuticle in *Palaemonetes pugio* Holthius. Epiphytic algae associated with the stems of *Spartina alterniflora* were used as the food source. Grass shrimp are known to ingest epiphytes (Morgan 1980) including those associated with *S. alterniflora* (Fleeger *et al.* 1999). Because salinity influences metal

speciation, salinity effects on uptake and partitioning from aqueous exposure were also examined.

METHODS

Aqueous Exposure with Varying Salinity

In order to determine metal bioconcentration and partitioning between exoskeleton and soft tissues, grass shrimp were collected from a *Spartina alterniflora* salt marsh (at 10 ‰ and 27°C) near Cocodrie, La on May 21, 1999. An additional collection was made on August 26, 1999 at the same site (at 15 ‰ and 29.4° C). Grass shrimp were subsequently exposed to an aqueous sublethal mixture of Cd, Cu, Zn (200 μg L^{-1} Cd, 1000 μg L^{-1} Zn, 500 μg L^{-1} Cu) in artificial seawater (ASW) comprised of ultra clean water (Milli-Q) and Instant Ocean sea salts for three days. This sublethal mixture of the three metal concentrations was used in previous experiments and was found to lead to metal uptake without elevates mortality (Keteles and Fleeger, in press). Experiments were conducted at 5, 18 and 30 ‰ , and shrimp were step-wise acclimated at a rate change of 5 ‰ per day to the desired salinity. Twenty grass shrimp per replicate were exposed to the metal mixture in 3 L of ASW in acid-cleaned 4-L glass jars. Four replicate jars were used at 5 and 30‰ because of the limited number of shrimp available (collected on August 26, 1999); six replicates were used at 18‰ (collected on May 21, 1999). Jars were covered with plastic wrap to prevent evaporation, and each was aerated by airstone to maintain dissolved oxygen at saturation. Twenty shrimp were also placed in one jar with ASW (no metals amended) of the appropriate salinity for each experiment to determine background burdens. Shrimp were selected on the basis of size (all were 22-28 mm in length from rostrum to telson) to maintain a consistent surface-area-to-volume

relationship and molt frequency, and shrimp were monitored daily for molting during the course of the experiment. Approximately, 10% of the grass shrimp molted during the course of the experiment.

Dietary Exposure

Grass shrimp were later collected at the same sampling site on May 6, 2000 (at 15‰ and 23° C) and acclimated to 18 ‰. P. *pugio* (again ranging from 22-28 mm) were fed Zn, Cu, and Cd-enriched epiphytic microalgae to determine the uptake and partitioning of metals from dietary exposure. Epiphytes associated with *S. alterniflora* stems were exposed to Cd, Cu, and Zn concentrations of 200, 500 and 1000 $\mu g\ L^{-1}$ respectively amended in 18‰ clean ASW and placed under cool white lights (14:8 light: dark cycle). Epiphyte exposure was conducted in staggered batches of one-day intervals to maintain a consistent exposure of epiphytes to be fed to shrimp. Following three days of exposure, stems were removed, rinsed with ethylenediaminetetraacetate (EDTA) to remove metals adsorbed to the surfaces of algal cells and stems. A subsample of epiphytes scraped from three stems with a razor blade was frozen and retained for analysis of metals to determine uptake by the microalgae.

Five groups of 20 shrimp were placed in 3-L acid-cleaned glass fish bowls and allowed to feed daily on either metal-enriched or uncontaminated epiphytic microalgae (control) for three days in 2 L of 18‰ clean ASW. Five epiphyte laden stems (approximately 10 cm in length) were held vertically by plastic mesh placed at the bottom of each fish bowl. Shrimp were allowed to feed *ad libitum* on epiphytes each day for 12 h after which stems (and remaining microalgae) were removed. Water was replaced daily

to limit the aqueous exposure from metals depurated from the shrimp or microalgae during the course of the experiment.

Post-exposure Methods

Grass shrimp from each experiment described above were removed following exposure and sacrificed by freezing in liquid nitrogen. Half (ten) of the grass shrimp from each jar were rinsed three times for 20 min with 20 ml of 10 mM EDTA to chelate and remove metals adsorbed to the surface of the epicuticle. Exoskeletons were peeled from remaining tissues (excluding legs and antennae) of the EDTA-rinsed (referred to hereafter as exoskeleton matrix) and the unrinsed (referred to hereafter as total exoskeleton) shrimp. Cu, Zn, and Cd were measured in the exoskeleton and soft tissues of both the EDTA-rinsed and the unrinsed shrimp in aggregate, *i.e.* measurements were made on 10 pooled exoskeletons or tissue from a single jar. The dry mass of exoskeletons and tissues from each aggregate was weighed with a Mettler balance (model pm460, ± 1 μg).

Metal concentrations (in $\mu g\ g^{-1}$) were measured in three shrimp compartments: total exoskeleton, exoskeleton matrix and tissue. Whole-body burdens were estimated by summing tissue and total exoskeleton values. For tissues and exoskeleton, the mass of each metal (per shrimp) was estimated from the concentration and appropriate body mass. Metals adsorbed onto the epicuticle surface were determined by calculating the difference in mass (per shrimp) of each metal in the unrinsed exoskeletons and exoskeletons rinsed with EDTA. Partitioning of metals was also measured in control grass shrimp (not exposed to elevated metals levels) in order estimate background burdens.

Enrichment Factors

Cu, Zn, and Cd concentrations in shrimp not exposed to elevated metals (control shrimp) were subtracted from the concentrations in the tissue and exoskeleton compartments of metal-exposed shrimp to calculate metal bioconcentration and bioaccumulation factors. The degree of concentration of Cu, Zn, and Cd in the shrimp was calculated as an enrichment factor for the tissues and exoskeleton at each salinity and for both sources of exposure. Enrichment factors of dietary (bioaccumulation factor) and water-only (bioconcentration factor) exposure for tissue, total exoskeleton, and exoskeleton matrix were determined and compared. Bioconcentration factors of the three compartments were also compared for each salinity.

Enrichment factors are defined as:

Bioconcentration Factor

$$\frac{\text{metal concentration in biogenic material (corrected for background)}}{\text{metal concentration in seawater}}$$

Bioaccumulation Factor

$$\frac{\text{metal concentration in biogenic material (corrected for background)}}{\text{metal concentration in food.}}$$

Metal Analysis

Shrimp tissues or exoskeletons were placed in preweighed, acid-cleaned glass test tubes, weighed, and digested in 5 ml of concentrated trace-metal-grade HNO_3 (Fisher Scientific) at 90° C for 12 h. The acid-containing digested samples were evaporated to about 1.5 ml, then diluted to ~25 ml and reweighed to determine volume. Blanks were prepared for each set of samples digested. Cu, Zn, and Cd concentrations in each fraction

were determined by Inductively Coupled Plasma (ICP) emission spectroscopy and corrected for interelement interference.

Statistical Methods

The effects of source (dietary and aqueous exposure) and salinity on metal partitioning were addressed in two ways. First, metal burden in tissues and total exoskeleton was analyzed by one-way ANOVA. Due to lack of independence, the difference between tissues and total exoskeleton ($\mu g\ shrimp^{-1}$) from each replicate was calculated. This difference was compared at each salinity to determine if salinity influenced the fate of metals. In a separate one-way ANOVA, source (dietary vs. 18‰ aqueous uptake) effects on the difference between tissue and total exoskeleton burdens were also compared. Second, two-way ANOVA's were conducted to determine if salinity or source altered partitioning of metals associated only with the exoskeleton. Metal burdens ($\mu g\ shrimp^{-1}$) of the exoskeleton of EDTA-rinsed and unrinsed shrimp were treated as a main effect. Salinity and source (dietary vs. 18 ‰ aqueous uptake) were the other main effect in separate two-way ANOVA's. Interactions were also examined. Significant differences ($p<0.05$) were analyzed with Tukey's post-ANOVA analysis.

RESULTS

Metal Concentrations

Control shrimp used in both experiments contained relatively high background whole-body burdens of Zn and Cu. Background Cd was, however, differed substantially between aqueous and dietary-exposure experiments, perhaps because shrimp were collected at different times. However, whole-body burdens after exposure to elevated

metals from food or water were always higher than in shrimp not exposed to elevated metals (Figure 4).

When exposed to elevated aqueous metals for three days, grass shrimp Cu, Zn, and Cd whole- body concentrations averaged 98, 159, and 3.9 $\mu g\ g^{-1}$ respectively across all three salinities (Figure 4). Generally, tissue and total exoskeleton concentrations were broadly overlapping for each metal (data not reported) and served as the basis for estimating enrichment factors and mass calculations used to calculate metal partitioning.

S. alterniflora epiphytes accumulated metals, and metal-enriched epiphyte concentrations of Cu, Zn and Cd were 25 , 38 and 11 $\mu g\ g^{-1}$ respectively (Figure 5). Whole-body burdens in grass shrimp exposed to metals from ingestion of epiphytic microalgae for three days averaged 74, 99, and 3.4$\mu g\ g^{-1}$ for Cu, Zn, and Cd, respectively (Figure 4).

Bioconcentration and Bioaccumulation

Enrichment factors were calculated for tissue, total exoskeleton and exoskeleton matrix compartments from aqueous exposure at three salinities and from dietary exposure. Bioconcentration factors (from aqueous exposure) ranged from 4.4-102.8 for Cu, 0-160.7 for Zn, and 8.6-31.1 for Cd (Figure 6). Bioconcentration factors for Zn tended to be higher at 30 ‰ than at 5 or 18 ‰; Cu bioconcentration factors tended to be lower at 30 ‰ than at 5 or 18 ‰ . Tissue and total exoskeleton bioconcentration factors were variable among metal species and condition. Exoskeleton Zn bioconcentration was higher than tissue bioconcentration at each salinity; however, Cd and Cu bioconcentration of the tissue and total exoskeleton varied with salinity. Rinsing exoskeletons with EDTA decreased bioconcentration factors by 0-50%. At 5‰, rinsed Cu exoskeleton

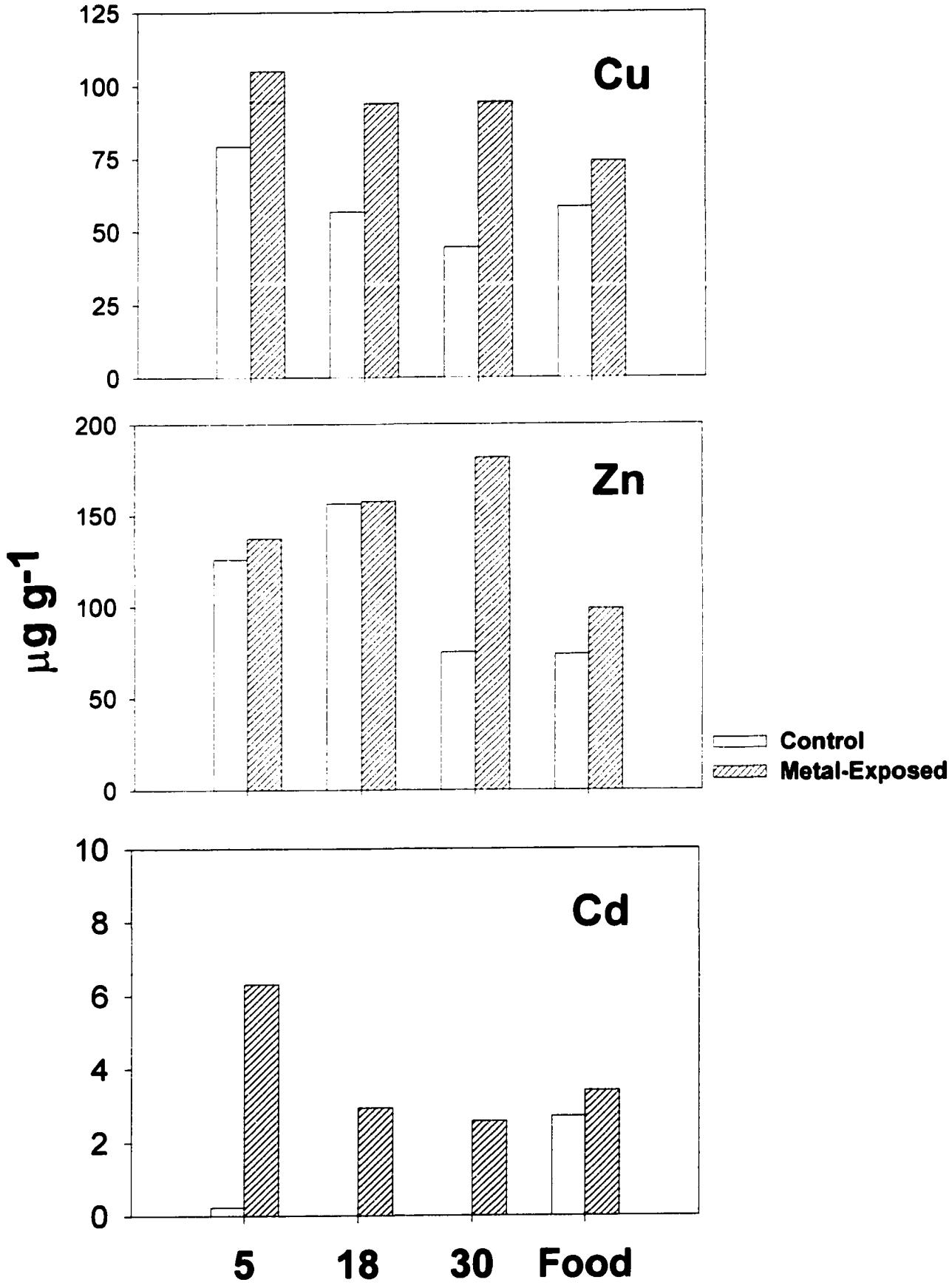

Figure 4. Cu (a), Zn (b), and Cd (c) whole-body burdens ($\mu g \cdot g^{-1}$) in *P. pugio* whole-body (tissue and total exoskeleton) are represented in control and metal-exposed grass shrimp from three salinities (aqueous exposure) and exposure from food.

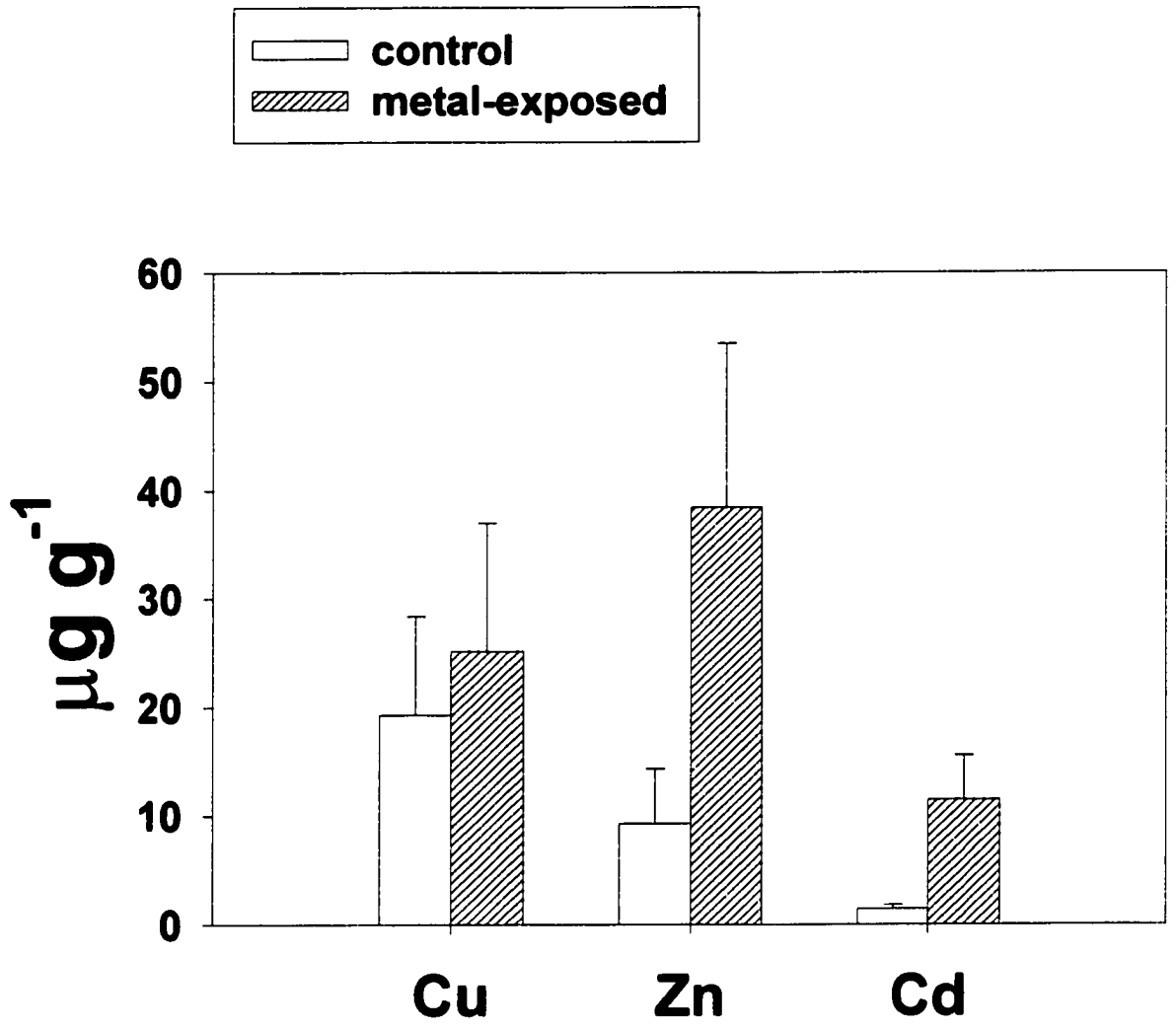

Figure 5. Epiphyte metal concentrations ($\mu g\ g^{-1}$) from *Spartina alterniflora* stems either exposed or not exposed (control) to elevated concentrations of Cu, Zn, and Cd. N=3, error bars represent standard error of the mean.

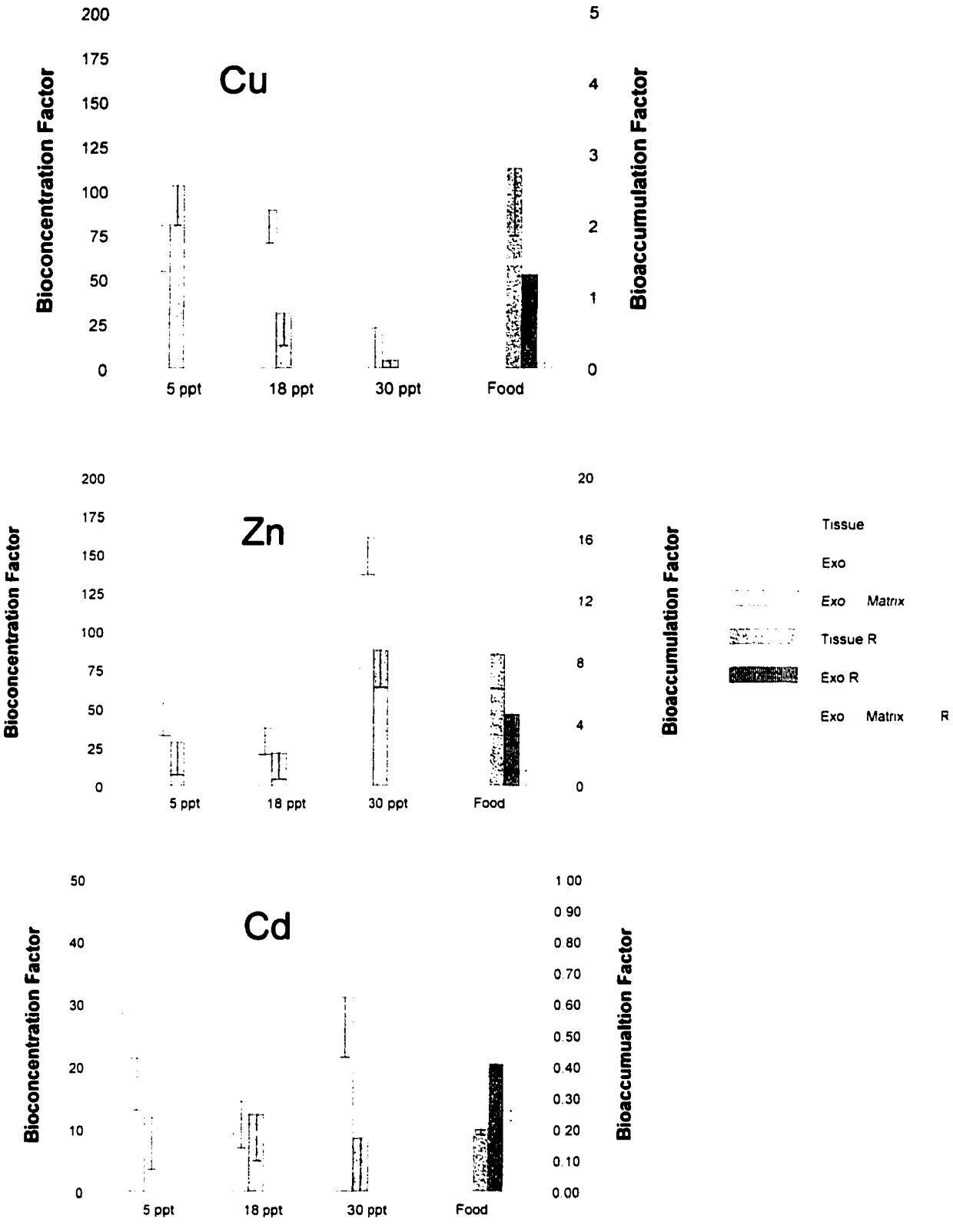

Figure 6. Cu (a), Zn (b), and Cd (c) enrichment in *P. pugio.* Exoskeleton - adsorbed, total exoskeleton, and tissue values are represented across three salinities (bioconcentration) and from dietary exposure (bioaccumulation, right Y axis (R)). (N=4, 5, 30, and Food; N=6 18) Error bars represent standard error of the mean.

bioconcentration was higher than unrinsed, probably because of background variability among different collections of shrimp. Therefore, Cu adsorption to exoskeleton was considered negligible at 5‰.

Bioaccumulation factors (from dietary exposure) averaged 1.39 for Cu, 0.29 for Cd, and 4.70 for Zn (Figure 6). Cu and Zn bioaccumulation factors were higher in tissues than in exoskeleton; bioaccumulation of Cd however was higher in the exoskeleton. The EDTA rinse decreased Zn and Cu bioaccumulation by about 90%, suggesting that a significant fraction of exoskeleton Cu uptake was by adsorption. EDTA rinse decreased Cd bioaccumulation by about 30%.

Body Mass

Over both experiments, the exoskeleton of *P. pugio* averaged 37 % of the total dry weight of grass shrimp. The mean mass of the shrimp used in these experiments ranged from 30-50 mg dry wt shrimp^{-1} and a one-way ANOVA suggested that mass did not vary among the four salinity or source treatments ($p=0.127$). Therefore, we felt justified in comparing the metal mass (on a per shrimp basis) among experiments because we used a limited size range of grass shrimp, and because no differences in body mass were detected among exposure experiments.

Whole-body Comparisons

Across all salinity and source treatments, the total mass of Cu averaged about 3.2 μg shrimp^{-1} (Table 1). A range of 62-68 % of the whole-body Cu burden was associated with the tissues across the three salinity treatments from aqueous exposure, and 65% of the total body burden was associated with the tissues from dietary exposure. Seventeen to thirty-two percent of the whole-body Cu burden was associated with the exoskeleton

Table 1. The whole-body mass and percentage of the whole-body burden in *P. pugio* associated with the tissue, exoskeleton matrix, and adsorbed fractions of Cu, Zn, and Cd for each salinity treatment and dietary exposure.

Cu	µg shrimp^{-1}	Tissue (%)	Exoskeleton Matrix (%)	Exoskeleton Adsorbed (%)
5‰	3.91	68	32	0
18‰	4.49	64	19	17
30‰	1.30	62	17	20
Diet	3.27	65	17	16

Zn	µg shrimp^{-1}	Tissue (%)	Exoskeleton Matrix (%)	Exoskeleton Adsorbed (%)
5‰	4.92	65	32	3
18‰	7.57	48	45	6
30‰	3.18	54	39	7
Diet	4.36	58	24	18

Cd	µg shrimp^{-1}	Tissue (%)	Exoskeleton Matrix (%)	Exoskeleton Adsorbed (%)
5‰	0.29	54	43	4
18‰	0.14	60	24	16
30‰	0.10	77	7	16
Diet	0.15	34	41	25

matrix in aqueous exposure, and 17% of the whole-body Cu burden was associated with the exoskeleton matrix after exposure from food. Zero to twenty percent of the whole-body Cu burden was adsorbed to the exoskeleton after aqueous exposure and 16% was adsorbed to the surface after exposure from food. No Cu was associated with the adsorbed fraction at 5‰.

Across all salinity and source treatments, the total mass of Zn averaged about 5.0 μg shrimp^{-1} (Table 1). A range of 48-65 % of the whole-body Zn burden was associated with the tissues across salinity treatments from aqueous exposure, and 58% of the total body burden was associated with the tissues from dietary exposure. Thirty-two to forty-five percent of the whole-body Zn burden was associated with the exoskeleton matrix in aqueous exposure, and 18 % of the whole-body Zn burden was associated with the exoskeleton matrix after dietary exposure. Three to seven percent of the whole-body Zn burden was adsorbed to the exoskeleton after aqueous exposure and 24% was adsorbed to the surface after dietary exposure. The smallest fraction (3%) was associated with the adsorbed fraction at 5 ‰.

Across all salinity and source treatments, the total mass of Cd averaged about 0.17 μg shrimp^{-1} (Table 1). A range of 54-77 % of the whole-body Cd burden was associated with the tissues across salinity treatments from aqueous exposure, and 34% of the total body burden was associated with the tissues from dietary exposure. Seven to forty-three percent of the whole-body Cd burden was associated with the exoskeleton matrix in aqueous exposure, and 34% of the whole-body Cd burden was associated with the exoskeleton matrix after dietary exposure. Four to sixteen percent of the whole-body Cd burden was adsorbed to the exoskeleton after aqueous exposure and 25% was adsorbed

to the surface after dietary exposure. The lowest percent (4%) was associated with the adsorbed fraction at 5‰.

Tissue - Exoskeleton Comparisons

Mean tissue Cu ranged from 0.80-2.86 μg shrimp^{-1}, and mean total exoskeleton Cu ranged from 0.50-1.63 μg shrimp^{-1} (Figure 7). Salinity did not influence the difference between tissue and exoskeleton Cu mass (p=0.5390) (Table 2), but source of exposure did (p=0.0081, Table 2). The difference between tissue and total exoskeleton Cu in aqueous exposure (1.30 μg shrimp^{-1}) was greater than in dietary exposure (0.95 μg shrimp^{-1}). Neither salinity nor source significantly influenced the difference among the tissue and total exoskeleton compartments of Zn mass (p=0.2675 for salinity; p=0.2474 for source) or Cd mass (p=0.1565 for salinity, p=0.0952 for source; Table 2). Mean tissue Zn was 2.8 (± 0.4 standard error of the mean; this convention will be used in all values that follow) μg shrimp^{-1} and mean total exoskeleton Zn was 2.2(±0.6) μg shrimp^{-1} (Figure 7). Tissue Cd averaged 0.09 (± 0.02) μg shrimp^{-1} and total exoskeleton Cd averaged 0.08 (±0.02) μg shrimp^{-1} (Figure 7) .

Intra-Exoskeleton Comparisons

Two-way ANOVA showed that salinity affected the Cu exoskeleton metal mass such that exoskeleton Cu mass at 5 and 18‰ was greater than at 30 ‰ (p=0.0020, Table 3). Additionally, exoskeletons had significantly more Zn mass at 18 ‰ than at either 5 or 30 ‰ (p=0.0006). Salinity did not significantly influence Cd exoskeleton mass (p=0.2861). The EDTA rinse reduced exoskeleton Cu mass (total exoskeleton Cu burden was significantly higher than the exoskeleton matrix burden; p=0.0165, Table 3). Total exoskeleton Cu averaged 1.3 (± 0.2) and exoskeleton matrix values averaged 0.9 (±0.1)

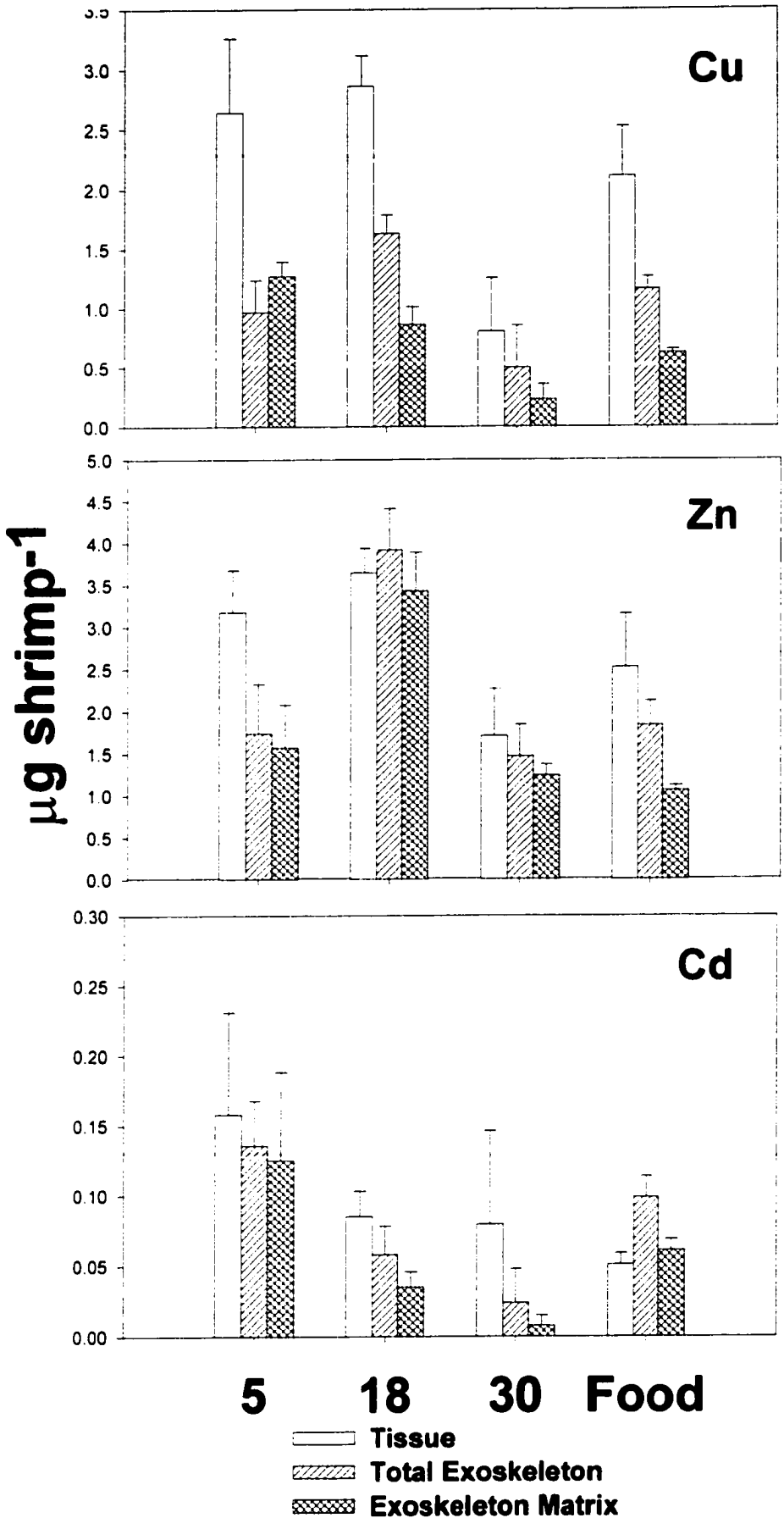

Figure 7. Cu (a), Zn (b), and Cd (c) burdens (µg shrimp^{-1}) in *P.pugio*. Tissue, total exoskeleton, and exoskeleton - adsorbed values are from dietary and aqueous exposure (three salinities). N=4 5, 30 and food; n=6 18 and error bars represent standard error of the mean.

Table 2. One-way ANOVA comparisons of tissue - total exoskeleton mass for salinity (5, 18, and 30 ‰) and source (water vs dietary) for Cu, Zn, and Cd.

Metal	Source (p)	Salinity (p)
Cu	Water > Food (0.0081)	0.5390
Zn	0.2474	0.2675
Cd	0.0952	0.1565

Table 3. Two-way ANOVA comparisons of Cu, Zn, and Cd exoskeleton mass of EDTA rinsed and unrinsed exoskeletons across salinities (5, 18, and 30 ‰)

Metal (model p)	Salinity (p)	Rinse (p)	Rinse * Salinity (p)
Cu (0.0233)	18>30, 5>30 (0.0020)	Unrinsed > Rinsed (0.0165)	0.0919
Zn (0.0345)	18>5 : 18> 30 (0.0006)	0.7020	0.9173
Cd (0.5085)	0.2861	0.9011	0.5084

μg shrimp^{-1} (Figure 7). The EDTA rinse did not reduce the exoskeleton Zn (p=0.7020) or Cd (0.9011) mass. Interactions between salinity and rinsing were not significant for Cu (p=0.0919), Zn (p=0.9173) or Cd (p=0.5084).

Exoskeleton Zn and Cu masses were significantly higher when the source of exposure was from water (p = 0.0012 for Zn, p= 0.0044 for Cu; Table 4). Source did not influence Cd exoskeleton mass (p=0.0694). The EDTA rinse significantly reduced the exoskeleton Cu mass (p=0.005), but rinsing did not significantly reduce Zn or Cd mass. Total Cu exoskeleton mass was significantly higher (1.4 μg shrimp^{-1}) than exoskeleton matrix mass (0.7 μg shrimp^{-1}) (Figure 7). Interactions between salinity and rinsing were not significant for Cu (p=0.4024), Zn (p=0.7754) or Cd (p=853, Table 2).

DISCUSSION

Experiments with *Palaemonetes pugio* were conducted over a 72 h exposure to elevated metals, and resulted in uptake of Cu, Zn, and Cd in tissues and exoskeleton (surface adsorption and deposition within the chitinous exoskeleton matrix). Most grass shrimp did not molt during the exposure period, but it is possible that metals, at least in the tissues and exoskeleton matrix, did not reach equilibrium. Metals adsorbed to the surface of the epicuticle in relatively small crustaceans like *P. pugio* probably equilibrium quickly (White and Rainbow 1984). Previous work by Nimmo *et al.* (1977) suggests that Cd whole-body concentrations reach equilibrium in *P. pugio* in no more than seven days. However, Zn and Cu may reach equilibrium sooner because they are tightly regulated in decapods (Bryan 1968). Because tissue and internal exoskeleton matrix Cd concentrations may not have reached equilibrium in my experiments, the proportion adsorbed to the surface may decrease over time if the concentration in the tissues and

Table 4. Two-way ANOVA comparisons of Cu, Zn, and Cd exoskeleton mass of EDTA rinsed and unrinsed exoskeletons from dietary and aqueous (18 ‰) exposure.

Metal (model p)	Source (p)	Rinse (p)	Rinse * Source (p)
Cu (0.0142)	Water > Food (0.0044)	Unrinsed > Rinsed (0.005)	0.4042
Zn (0.1244)	Water > Food (0.0012)	0.2188	0.7754
Cd (0.3366)	0.0694	0.0684	0.8352

exoskeleton matrix increase over time. Therefore, if the tissues did not reach equilibrium after 96 h, the exoskeleton-adsorbed metals could inflate the proportion associated with the exoskeleton. However, unpublished data (Keteles) show that field-collected *P. pugio* have a similarly high fraction of the total metal burden associated with the adsorbed, suggesting these short-term exposures generate realistic estimates of exoskeleton values. Nevertheless, my results appear to be the first to quantify the fraction of metals adsorbed to the surface of the crustacean epicuticle.

The rate and degree at which a dissolved metal adsorbs to organic matter, such as the crustacean epicuticle, depends upon concentrations of other solutes (Ca^{2+}, Na^{+}, Cl^{-}, etc.), among other factors (Libes 1992). Salinity greatly influenced adsorbed metal uptake in *P. pugio*. The fraction of adsorbed metal body-burdens was very low at 5 ‰ (0-4% of the total for Cu, Zn, and Cd) perhaps because metals are likely to be in the free ionic state under conditions of low salinity. Ninety-seven percent of the Cd, Se, Co, Zn body burden was associated with the exoskeleton of marine zooplankton (Reinfelder and Fisher 1994), whereas, only 19% of the Cd burden was on the exoskeleton of freshwater zooplankton (Munger and Hare 1997), further suggesting that salinity may contribute to differences in exoskeleton metal content. At salinities of 18 and 30 ‰, the adsorbed fraction was consistently high and ranged from 16-20% of the whole-body burden for Cu, 6-18% for Zn, and 16-25% for Cd (Table 1). Rinsing with EDTA, a metal chelator, reduced exoskeleton bioconcentration and biomagnification factors of all three metals by 0-50% (Figure 6). Furthermore, the EDTA rinse significantly reduced the mass of Cu associated with the exoskeleton (Table 3 and 4). Although, the EDTA rinse did not significantly reduce the body burdens of Zn and Cd (Table 3 and 4), relatively high levels

of Zn and Cd were found in the exoskeleton matrix, and thus, the proportion of adsorbed Cd and Zn were low relative to the total exoskeleton. Similarly, Hare (1992) reported that the proportion of externally bound Cd on an aquatic insect, *Hexagenia limbata*, decreased with increasing internal exoskeleton accumulation.

Metals also bioaccumulated and bioconcentrated in the internal exoskeleton matrix relative to controls, and metals associated with the interior of the exoskeleton ranged from 7-45% of the total metal body burden. Zn and Cd were particularly enriched in the exoskeleton matrix (Figure 7). A Zn metalloenzyme has been reported within the exoskeleton of the freshwater crayfish, *Cambarus virilis* (Bryan 1968). Because Cd is chemically similar to Ca, deposition of Cd in the exoskeleton could occur as grass shrimp harden the procuticle and epicuticle by the deposition of calcium salts (Stevenson 1985). Depledge *et al.* (1993) found high levels of Cd in the exoskeleton of the benthic crab, *Dorippe granulata*, and Khan *et al.* (1989) similarly found high levels of Cd in the exoskeleton of *P. pugio*.

Metals externally adsorbed to the surface of the epicuticle likely have a very different potential for impact on the grass shrimp compared with metals integrated into the exoskeleton matrix. At least a large fraction of the metals removed by EDTA likely adsorbed directly from the aqueous solution. Adsorbed metals, therefore, do not have the opportunity to interact with receptor sites and cause toxic effects. On the other hand, matrix-bound metals must be transported to the exoskeleton through the circulatory system and may interact with tissues (Bryan 1968; Fowler *et. al* 1970). This suggests that adsorbed metals inflate estimates of metal body burden in crustaceans. As a result, critical body residue predictions of toxicity for marine crustaceans of a size similar to *P.*

pugio may be underestimated by as much as 15-20% (about the fraction adsorbed in salinities greater than 5‰). Furthermore, predictions concerning bioavailability of metals to crustaceans which do not account for the adsorbed fraction may be overestimated, and crustaceans may represent poor choices in biomonitoring programs (Langston and Spence 1995). Routine removal of adsorbed metals with a strong chelator, such as ETDA, before chemical analysis may provide a more accurate estimate of total metal burden. Finally, very little is known concerning the role of surface-adsorbed metals in trophic transfer of metals to higher trophic levels. Because adsorbed metals represent an important part of the whole-body burden, surface-adsorbed metals could be an important vector to higher trophic levels. In fact surface-adsorbed metals may be easily desorbed in the digestive tract of a predator.

It has often been suggested that crustaceans store toxic metals in the exoskeleton and thereby reduce exposure to the organism (Depledge *et al.* 1993; Khan *et al.* 1989; Reinfelder and Fisher 1994). However, once associated with the exoskeleton matrix, both essential (Cu, Zn) and nonessential (Cd) metals maybe remobilized during the molt cycle for storage in tissues for reabsorption following ecdysis. Therefore, tissue metal concentrations in crustaceans have been shown to vary before and after ecdysis (Depledge *et al.* 1993; Nimmo *et al.* 1977). My previous work suggests large differences among metals in the potential for depuration in *P. pugio* through ecdysis; most of the exoskeleton Cu was retained in the tissues during a molt, and although a significant fraction of the exoskeleton Cd was eliminated with the exuviae, some appears to have entered tissues (Keteles and Fleeger, in press). This remobilization of metals from the

exoskeleton to tissues during molting may cause spikes in tissue concentrations, perhaps exceeding the critical body residue and causing mortality.

The proportion of the total Cu and Zn body burden in *P. pugio* associated with tissues was relatively high and showed little variability among the salinity and dietary-exposure treatments. Tissues contained the majority of the Cu and Zn burden (Table 4). Zn and Cu are physiologically regulated by crustaceans (Bryan 1968; Rainbow 1995; Hebel *et al.* 1997). For example, Cu plays a key role in the oxygen-binding pigment, hemocyanin. However, exposure to excess Cu activates detoxification mechanisms that lead to increases in granule formation to aid in excretion (Hebel *et al.* 1997). Similarly, Zn is an essential cofactor in enzymatic function, although excess zinc is excreted (Bryan 1968). Therefore, regulation of Zn and Cu in the tissues may reduce tissue variation when exposed to elevated Zn and Cu. Cd tissue burden, however, ranged from 34-77 % of the whole body burden (Table 4). Cd is a nonessential toxic metal and is not physiologically regulated (Smokerowski *et al.* 1998). Therefore, Cd tissue concentrations may be more variable depending on exposure regime and stage of the molt cycle.

When metal exposure was through diet, the removal of adsorbed Zn and Cu decreased bioaccumulation values by up to 90% and removal decreased Cd by about 30%. Additionally, 16% of total Cu, 24 % of total Zn, and 25 % of total Cd burdens were adsorbed to the exoskeleton when the source of exposure was dietary. These values generally exceeded surface adsorption associated with aqueous exposure. Several factors may have contributed to this observation. Metals may have leached from the epiphytic algae or *Spartina* stems, been released by sloppy feeding by the shrimp or excreted by the shrimp into the water, and then adsorbed onto the exoskeleton from the aqueous medium.

Metal concentrations were not measured in the water containing epiphytes and grass shrimp (although water was replaced daily to minimize aqueous exposure). Wang *et al.* (1996) demonstrated that copepod grazing on microalgae enhanced the release of metals into the dissolved phase. Alternatively, *S. alterniflora* have been shown to take up metals and then release dissolved metals through salt glands in the leaves (Burke *et al.* 2000). Although salt glands appear to be more common in leaves than stems, it is possible that dissolved metals were released directly from the stems of the living *S. alterniflora*, and subsequently may have adsorbed to the epicuticle of the grass shrimp during feeding activities. Furthermore, grass shrimp constantly move up and down stems while scraping epiphytes, and metal-enriched debris (epiphytes, exopolymers, etc) from the stems may have adhered to the epicuticle of the grass shrimp.

The route of exposure significantly influenced partitioning of Cu between the exoskeleton and tissues in *P. pugio*. Cu and Zn bioaccumulation factors in the dietary exposure experiment were higher in tissue than in exoskeleton. Fowler *et al.* (1970) also found that more Zn was associated with tissues when uptake was from food due to direct absorption across the gut wall and binding to tissue proteins associated with a regulatory process. More Cd was associated with the exoskeleton when the source of exposure was dietary (41%) versus aqueous exposure (24%) (Table 3). Reinfelder and Fisher (1994) hypothesized that the overwhelming association of Cd with the exoskeleton of herbivorous copepods was accumulated from ingested food by rapid mobilization from the gut and deposition in the exoskeleton as a means of detoxification or storage. Because seawater has a high concentration of ions, interactions between solutes occur that can alter the effective concentration (or activity) of an ion (Libes 1992). Metals are more

likely in the dissolved state at low salinities, and are, therefore, more bioavailable and toxic (Guerin and Stickle 1995). However, Zn had higher bioconcentration factors at 30‰ than at 5 and 18‰ (Figure 6). Zn flux in crustaceans can vary with external Zn concentrations, temperature, and between individuals (White and Rainbow 1984). The background body burdens of the shrimp used in the 5 and 18 ‰ exposures were initially high (Figure 4), and it is possible that these high background burdens activated Zn regulatory mechanisms. Zn is bound to hemolymph proteins, and excess Zn increases the concentration of unbound zinc in the hemolymph, and is usually removed rapidly by excretion (Bryan 1968). Another possible explanation for the apparent decrease in Zn bioconcentration at low salinities could be due to a reduction in apparent water permeability (APW) (Rainbow 1995). At low salinities, many euryhaline crustaceans decrease their AWP, causing a low uptake of free ions. For example, the estaurine crab, *Carcinous maenas*, from areas of high salinity exhibited lower uptake rates of Zn at lower salinities than at high salinities due to changes in APW (Rainbow 1995).

The results of this study differentiate between superficially-adsorbed and internally sequestered metals in the exoskeleton of *P. pugio*. Surface-adsorbed metals were shown to be an important part of the body burden and their presence can lead to elevated body burden values presumably without causing toxic responses. Exoskeleton bioconcentration and bioaccumulation factors increased by 0-50% due to surface adsorption to the epicuticle. Furthermore, salinity was shown to influence the surface adsorption of metals on the exoskeleton; low salinity reduced the amount of adsorbed metals. The exoskeleton of *P. pugio* is not highly calcified nor waxy compared to other decapod groups. Therefore, due to the differences in chemical composition of

exoskeletons, such as increase in calcification or waxes, there may be differences in adsorption values for different crustacean taxa. Also, surface-area to body volume may influence the relative importance of adsorption, and adsorbed metals may be a higher percentage of the body burden of very small crustaceans (larvae, meiofauna and zooplankton). Future work may compare a broader range of crustaceans from different habitats. Nevertheless, the crustacean exoskeleton must be considered to understand crustacean-metal interactions.

CHAPTER 4

TROPHIC TRANSFER OF CU, ZN, AND CD ASSOCIATED WITH EXOSKELETON AND TISSUES OF THE GRASS SHRIMP, *PALAEMONETES PUGIO*, TO A PREDATORY FISH, *FUNDULUS GRANDIS*

INTRODUCTION

Diet in fishes has been shown to be a predominant pathway for trace-metal uptake, particularly Cd (Langston and Spence 1995; Watras and Bloom 1992). Crustaceans, an important source of prey for fishes, are exposed to heavy metals from food, sediment and aqueous phases, and crustacean trace-metal body burdens are often elevated, which may facilitate contaminant transfer via the food chain (Watras and Bloom 1992; King *et al.*, 1992). However, the bioavailability of metals to a predator depends on the chemical form and binding within the prey's tissues as well as food quality (Langston and Spence 1995).

Crustaceans all share the presence of a chitinous exoskeleton, and metal cations may associate with the chitin matrix by forming ionic bonds with nitrogen side groups within the procuticle (Stevens 1985). Metals may thus be deposited within the exoskeleton matrix associated with the deposition of metal salts. Additionally, a Zn metalloenzyme (Bryan 1968) and Cu-containing proteins (Twelliger 1999) are involved in the tanning of the exoskeleton. It was shown in Chapter 3 of this dissertation that a significant portion of the total metal-body burden may be associated with the chitinous exoskeleton in the grass shrimp, and estimates of the exoskeleton burden in other crustaceans are reported as high as 97% of the whole-body metal burden (Reinfelder and Fisher 1994a). Low absorption efficiencies of metals by the fishes *Menidia menidia* and *M. beryllina* fed copepod prey have been attributed to the proportion of metals associated with the exoskeleton and the indigestibility of chitin (Reinfelder and Fisher 1994a). High assimilation efficiencies in zooplanktivorous mysids were attributed to the selective ingestion the soft tissues rather than exoskeleton (Smokerowski *et. al.* 1998). However,

most fish consume crustacean prey whole, and little is known about the bioavailibility of metals sequestered within the exoskeleton.

Metals may also associate with the arthropod exoskeleton through an adsorptive process by binding externally to the surface of epicuticle (Langston and Spence 1995; Hare 1992). For example, it has been suggested that much of the Zn associated with the exoskeleton of some decapod crustaceans is externally adsorbed because the metal concentration of the exoskeleton increases with increasing concentration in the ambient water (Bryan 1968). Similarly, Munger and Hare (1997) suggested that Cd associated with the exoskeleton of a freshwater cladoceran was externally bound because Cd was rapidly lost when transferred to Cd-free media. The adsorbed fraction of the total metal burden of *Palaemonetes pugio* was measured at 18‰ salinity in Chapter 3 of this dissertation, and averaged 17, 6, and 16% of the total Cu, Zn and Cd burdens respectively. Furthermore, adsorbed metals increased on the exuviae shed in metal-enriched water as reported in Chapter 2. Many authors (Reinfelder and Fisher 1994a; Munger and Hare 1997; Zanders and Rojas 1996; Smokerowski *et al.* 1998; Hare 1992) acknowledge the presence of surface-adsorbed metals in crustaceans and aquatic insects, but the bioavailability of this fraction to higher trophic levels has not been determined. Because metals may be solublized in the gastric fluids of predators, surface-adsorbed metals may be an important source in the transfer of metals to higher trophic levels.

The objective of this portion of my dissertation was to determine the role of the crustacean exoskeleton in the trophic transfer of metals from a common and abundant estuarine crustacean prey (*Palaemonetes pugio* Holthius) to a fish predator (*Fundulus grandis* Baird and Girard). *P. pugio* is generally classified as a detritovore/ominvore and

is a frequent prey of larger fish species including species in the genus *Fundulus* (Knieb 1987). *P. pugio* is also frequently used in toxicological studies (Klerks 1999; Wallace and Lopez 1997; Smith and Weis 1997; Davis *et al.* 1998). Fishes in the genus *Fundulus* are important estuarine residents (Wainright *et al.* 2000; Rozas and Lasalle 1990; Kneib 1986) which prey upon invertebrates, and are also frequently used in toxicological studies (Smith and Weis 1997; Davis *et al.* 1998; Vandenhurk *et al.* 1998). The assimilation of metals adsorbed to the surface of the grass shrimp exoskeleton, incorporated into the chitin matrix, and associated with the soft tissues by *F. grandis* were compared.

METHODS

Approximately 800 *P. pugio* were collected from a *Spartina alterniflora* salt marsh near Cocodrie, La on May 6, 2000 (at 15 ‰ and 23° C) and June 11, 2000 (15 ‰ and 29 ° C). Half were subsequently exposed to an aqueous sublethal mixture of Cd, Cu, Zn (200 μg L^{-1} Cd, 1000 μg L^{-1} Zn, 500 μg L^{-1} Cu) in artificial seawater (ASW) comprised of ultra clean water (Milli-Q) and Instant Ocean sea salts for three days in acid-cleaned, low-density polyethylene (LDPE) 36-L plastic chambers. The remaining shrimp were placed in ASW without amended metals. Chambers were aerated to maintain high levels of dissolved oxygen. Grass shrimp were selected on the basis of size (all were 22-28 mm in length from rostrum to telson) to maintain a consistent surface-area- to-volume relationship and molt frequency, and shrimp were monitored daily for molting during the course of the exposure. Very few grass shrimp molted during the exposure period (<10%).

Post Exposure

After 72 h, grass shrimp were removed from the tubs and frozen in liquid nitrogen. Half of the metal-exposed and unexposed grass shrimp were rinsed three times for 20 min with 20 ml of 10 mM ethylenediaminetetraacetate (EDTA) to chelate and remove metals adsorbed to the surface of the epicuticle. Exoskeletons were excised from remaining tissues (excluding legs and antennae) of the EDTA-rinsed (referred to as "exoskeleton matrix"hereafter) and the unrinsed (referred to as "total exoskeleton" hereafter) grass shrimp. The mass (wet weight) of exoskeletons and tissues were determined with a Mettler balance (model number pm460, ± 1 μg). Either total exoskeleton, exoskeleton matrix, or tissues from three shrimp were mixed with 2-ml Knox gelatin (following Wallace and Lopez 1997) and 1g dissolved fish food (TetraMin). The mixture was then placed in acid-cleaned small plastic conical molds to yield pellets (containing the appropriate fraction of three grass shrimp, fish food; approximate weight was 2.5 g) palatable to fish.

Trophic Transfer

Fundulus grandis (ranging from 3- 4 g wet weight) were obtained from a bait shop and maintained for ten days in 18 ‰ ASW. Five replicate 2.5-L glass fish bowls were used to house single fish, in 18 ‰ ASW. Fish were offered pellets containing either the tissue, exoskeleton matrix, or total exoskeleton fraction of grass shrimp either exposed or unexposed to elevated metals. Each fish was fed a single pellet containing the appropriate fraction of three grass shrimp daily for 10 d. Unconsumed shrimp pellets were removed daily and accounted for by mass. Water was replaced every other day in

all fish bowls to minimize ammonia and dissolved metal accumulation as a result of excretion or leaching from fecal pellets.

Samples (whole fish and three replicate subsamples of each gelatin pellet treatment) for metal analysis were placed in preweighed, acid-cleaned glass test tubes, weighed, and digested in 5 ml of concentrated trace-metal grade HNO_3 (Fisher Scientific) at 90° C for 12 h. The acid- containing digested samples were evaporated to about 1.5 ml, then diluted to ~25 ml and reweighed to determine volume. Blanks were prepared for each set of samples digested. Metal concentrations in each fraction were determined by Inductively Coupled Plasma (ICP) emission spectroscopy and corrected for interelement interference.

For tissues and exoskeleton treatments, the mass of each metal (per grass shrimp) was estimated from the concentrations and total body mass. Metals adsorbed onto the epicuticle surface were determined by calculating the difference in mass (per grass shrimp) of each metal in the unrinsed exoskeletons and exoskeletons rinsed with EDTA of both the metal-exposed and unexposed grass shrimp. Metal body burdens in *F. grandis* were measured and corrected for background body burdens. Assimilation efficiencies (AEs) were also calculated based on the total mass of each metal in fish tissue and the total mass of metal consumed by the fish as follows:

$$AE = \frac{\text{Fish Tissue Metal}(\mu g\ fish^{-1})}{\text{Ingested Metals}\ (\mu g\ fish^{-1})} * 100.$$

Statistical Methods

The concentrations of each metal in fish tissues were compared for each metal with separate two-way ANOVAs in which main effects were exposure (metal-exposed or

unexposed shrimp) and grass shrimp fraction (total exoskeleton or tissues). AEs from the two shrimp fractions (total exoskeleton or tissues) of grass shrimp exposed or unexposed to elevated metals were also compared for Cu and Zn using similar two-way ANOVAs. Significant differences were analyzed with Tukey's post-ANOVA analysis. AEs for fish fed grass shrimp not exposed to elevated Cd could not be calculated because concentrations were below detection limits. Therefore, Cd AEs in fish fed metal-exposed grass shrimp total exoskeleton and tissues were compared using a t-test. Concentrations of fish tissues and AEs of the total exoskeleton (adsorbed + matrix) and exoskeleton rinsed with EDTA (metals only in the matrix form) of shrimp exposed to elevated metals were compared with t-tests to determine if removing the adsorbed fraction influenced trophic tranfer.

RESULTS

Shrimp Pellet Concentrations

Cu concentrations of the food pellets containing exoskeleton matrix, total exoskeleton, and tissues of grass shrimp not exposed to elevated metals averaged 19.1 (± 4.3 standard error of the mean; this convention will be used in values that follow), 21.2 (± 2.8), and 11.3 (± 0.8) $\mu g\ g^{-1}$ wet weight, respectively (Figure 8). Based on the total mass of Cu associated with the fractions (3.1, 4.3, 5.1 μg, respectively), 13% was adsorbed to the surface of the exoskeleton, 33% was incorporated in the exoskeleton matrix, and 54% of Cu was associated with the tissues of grass shrimp not exposed to elevated metals (Table 5). Cu concentrations of the pellets containing exoskeleton matrix, total exoskeleton, and tissues of grass shrimp exposed to elevated metals averaged 20.0 (± 2.7), 34.9 (± 4.16), and 19.1 (± 0.7) $\mu g\ g^{-1}$ wet weight, respectively

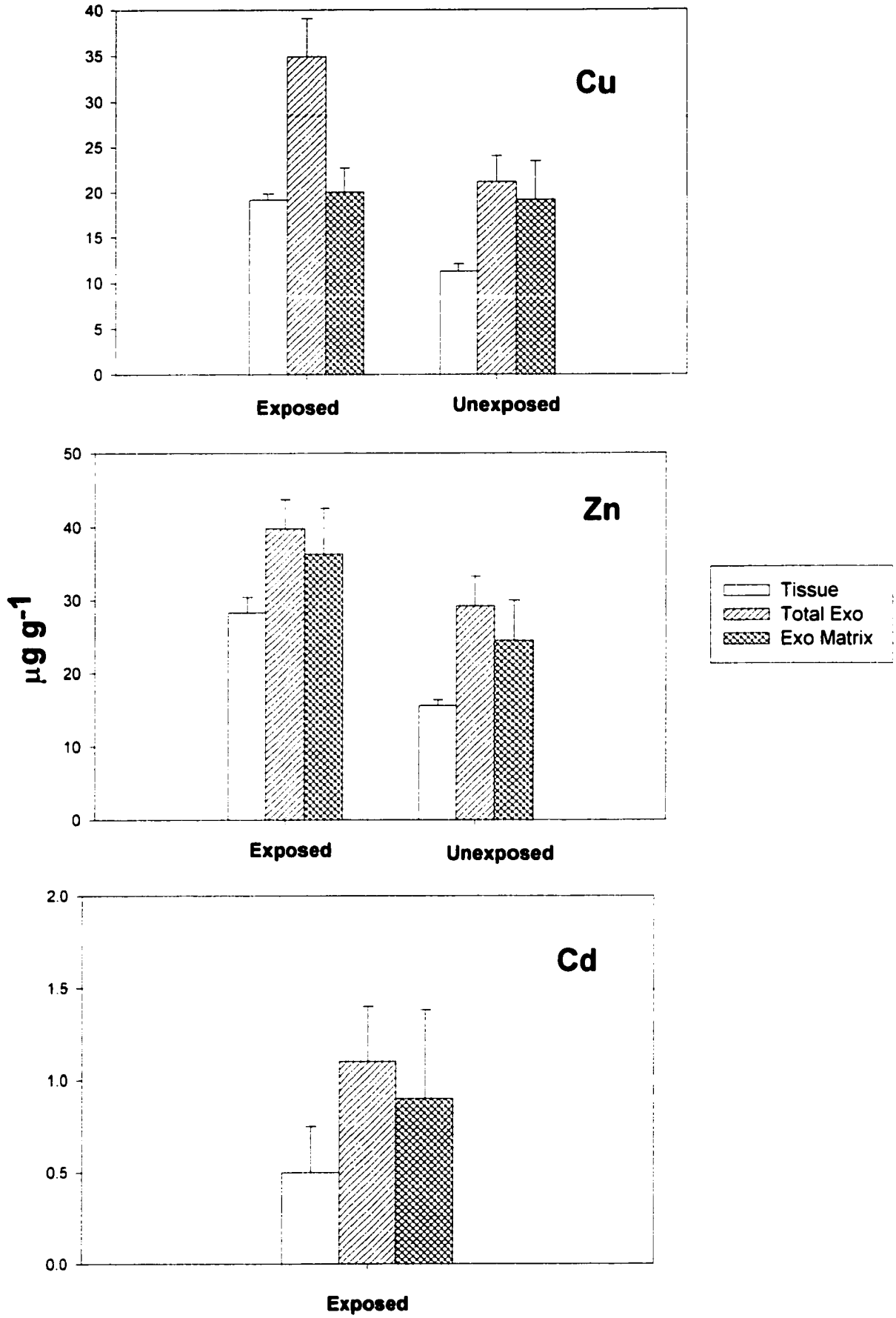

Figure 8. Concentrations (µg g^{-1} wet weight) of (a)Cu, (b) Zn, and (c)Cd in tissue, total exoskeleton, and exoskeleton matrix of metal-exposed and unexposed grass shrimp, *Palaemonetes pugio*. N=3 and error bars represent standard error of the mean.

Table 5. Partitioning of metals into exoskeleton matrix, surface-adsorbed, or tissues of shrimp exposed or unexposed to Cu, Zn, and Cd expressed as a percentage of the whole body burden. N.D.= no data, due to detection limits.

	Exposed Cu	Unexposed Cu	Exposed Zn	Unexposed Zn	Exposed Cd	Unexposed Cd
Matrix	22	33	30	36	52	N.D.
Adsorbed	23	13	8	14	7	N.D.
Tissue	55	54	61	54	41	N.D.

(Figure 8). Based on the total mass of Cu associated with the fractions (3.7, 7.6, 9.2 μg, respectively), 23% of grass shrimp Cu was adsorbed to the surface of the exoskeleton, 22% was incorporated in the exoskeleton matrix, and 55% was associated with the tissues (Table 5).

Food pellet Zn concentrations of exoskeleton matrix, total exoskeleton and tissues of grass shrimp not exposed to elevated metals averaged 24.5 (± 5.4), 29.2(± 4.0), and 15.6 (± 0.8) μg g^{-1} wet weight, respectively (Figure 8). Based on the total mass of Zn associated with the fractions (4.3, 6.0, 6.5 μg respectively) 14% was adsorbed to the surface of the exoskeleton, 36% was incorporated in the exoskeleton matrix, and 54% was associated with the tissues of grass shrimp not exposed to elevated metals (Table 5). Pellet Zn concentrations of exoskeleton matrix, total exoskeleton and tissues of grass shrimp exposed to elevated metals averaged 36.3 (± 6.3), 39.8 (± 4.0), and 28.3 (± 2.1) μg g^{-1} wet weight, respectively (Figure 8). Based on the total mass of Zn associated with the fractions (6.9, 8.7, 14.0 μg respectively), 8% of the Zn mass associated with a grass shrimp was adsorbed to the exoskeleton surface, 30% was incorporated into the exoskeleton matrix, and 61 % was associated with the tissues (Table 5).

Concentrations of Cd in pellets containing exoskeleton matrix, total exoskeleton and tissues of grass shrimp not exposed to elevated metals were below detection limits. Cd concentrations in food pellets containing exoskeleton matrix, total exoskeleton and tissues of grass shrimp exposed to elevated metals averaged 0.90 (± 0.48), 1.10 (± 0.31), and 0.50 (± 0.25) μg g^{-1} wet weight, respectively (Figure 8). Based on the total mass of Cd associated with the fractions (0.20, 0.23, 0.25 μg, respectively), Cd adsorbed to the

surface, incorporated in the matrix, and sequestered in the tissues represented, 7, 41, and 54% respectively of the total mass of Cd per grass shrimp (Table 5).

Metal Concentration in Fish Tissue

Fish tissue metal concentrations were corrected for background body burdens in *F. grandis*. Corrected-Cu concentrations in *F. grandis* tissues after feeding on shrimp pellets for 10 d ranged from 0.01 to 0.16 $\mu g\ g^{-1}$ wet weight across the three shrimp fractions (exoskeleton matrix, total exoskeleton, or tissues) in fish that ingested shrimp not exposed to elevated Cu. Corrected-Cu concentrations were typically higher (0.33-0.49 $\mu g\ g^{-1}$ wet weight across the three shrimp fractions) in fish that ingested shrimp exposed to elevated metals (Figure 9). Rinsing with EDTA, to create an exoskeleton with only matrix-bound Cu, did not reduce the mean fish tissue concentrations, suggesting little or no uptake from the adsorbed fraction. Corrected-Zn concentrations in *F. grandis* ranged from 0-25.0 $\mu g\ g^{-1}$ wet weight across the three shrimp fractions in fish that ingested shrimp not exposed to elevated Zn. Corrected-Zn concentrations were relatively higher in fish that ingested shrimp exposed to elevated metals and ranged from 0.6-33.3 $\mu g\ g^{-1}$ wet weight across the three shrimp fractions (Figure 9). Rinsing with EDTA reduced corrected-Zn concentrations by about 90% compared to total exoskeleton, suggesting a high uptake of adsorbed Zn. Corrected-Cd concentrations in *F. grandis* tissues ranged from 0.002-0.04 $\mu g\ g^{-1}$ wet weight in fish that ingested shrimp not exposed to elevated Cd. Corrected-Cd concentrations were typically higher (0.023-0.073 $\mu g\ g^{-1}$ wet weight across the three shrimp fractions) in fish that ingested shrimp exposed to elevated metals (Figure 9). Rinsing with EDTA reduced fish tissue concentrations by about 70% suggesting a high uptake of adsorbed Cd.

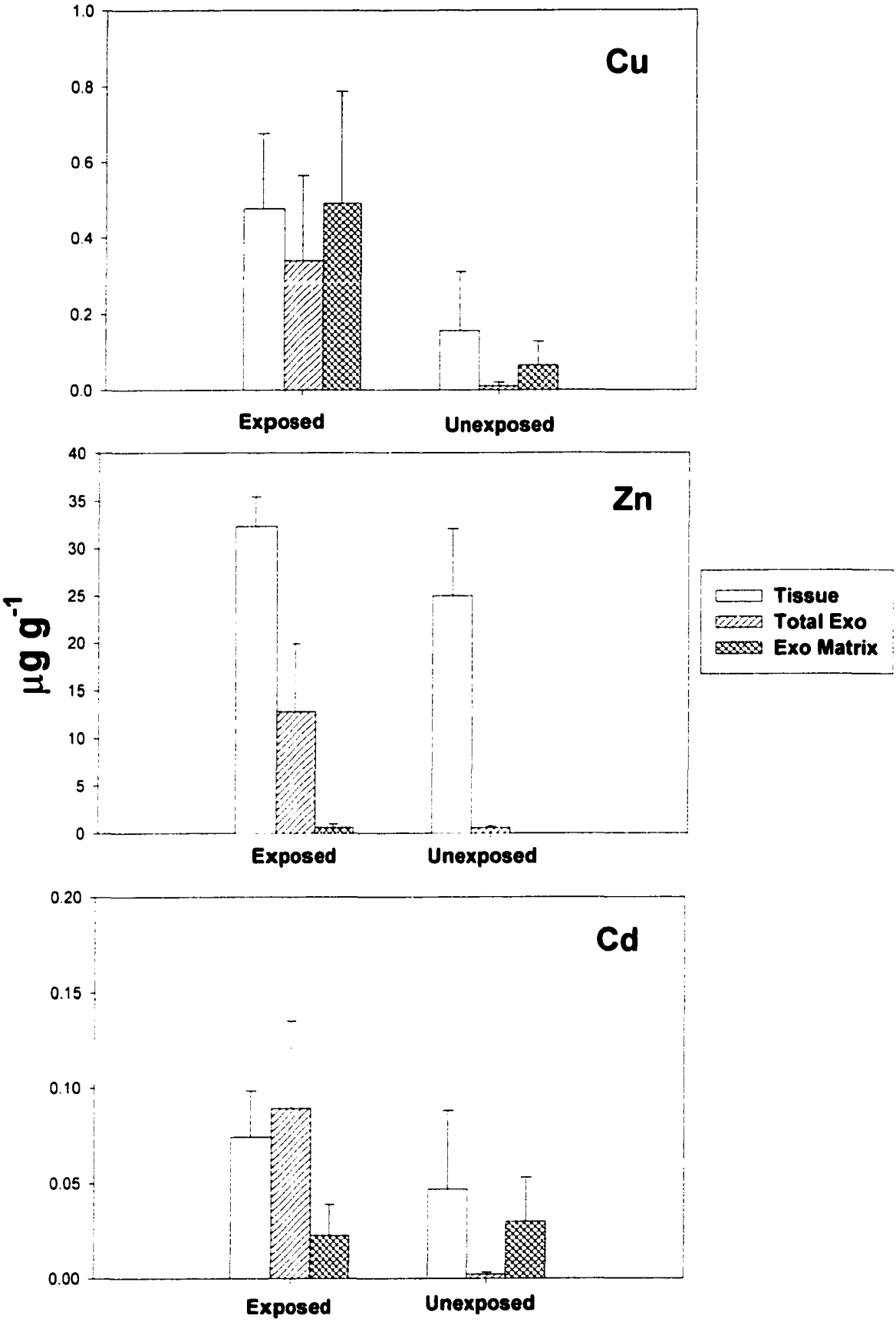

Figure 9. *Fundulus grandis* whole-body corrected concentrations (µg g^{-1} wet weight) of (a)Cu, (b) Zn, and (c) Cd fed either tissue, total exoskeleton, or exoskeleton matrix of metal-exposed and unexposed *P. pugio*. N=5 and error bars represent the standard error of the mean.

Two-way ANOVAs showed that fish-tissue metal concentrations were significantly elevated for Zn and Cd when fed shrimp exposed to metals compared to fish fed shrimp not exposed to elevated metals (Table 6). Shrimp fraction did not influence fish tissue corrected concentrations for Cu and Cd. However, shrimp fraction did influence the corrected Zn concentrations of fish tissue, and fish fed shrimp tissue had higher corrected concentrations of Zn than fish fed total exoskeleton ($p=<0.001$). Interactions were not significant for any of the metals. t-tests showed that mean Cu, Zn and Cd tissue corrected concentrations of fish fed total exoskeleton and exoskeleton matrix of shrimp exposed to elevated metals were not significantly different ($p=0.790$, 0.080, and 0.221, respectively; Table 7).

Assimilation Efficiencies (AEs)

F. grandis Cu AEs after feeding on shrimp pellets for 10 days ranged from 0.13-1.46% across the three fractions of shrimp not exposed to elevated metals (Figure 10). Cu AEs of fish that ingested fractions of shrimp exposed to elevated metals ranged from 1.2-5. %. The EDTA rinse did not decrease mean fish Cu AE, suggesting a very low assimilation of adsorbed Cu. Zn AEs ranged from 0.01-133.4% in fish that ingested shrimp fractions not exposed to elevated metals (Figure 10). Tissue AEs probably exceeded 100% due to background variability among the shrimp or *F. grandis*. Zn AEs of fish which ingested fractions of shrimp exposed to elevated metals ranged from 2.4-94.6%. Highest AEs were always from the ingestion of tissue. The EDTA rinse reduced Zn AE by about 50%, suggesting a high assimilation of adsorbed Zn. Because Cd concentrations in shrimp were undetectable, AEs were calculated only for fish fed fractions of shrimp exposed to elevated metals, and values ranged from 6.7-12.2% across

Table 6. Two-way ANOVA comparisons of Cu, Zn, and Cd body burdens (μg g^{-1} wet weight) of *F. grandis* fed *P. pugio* fractions (total exoskeleton and tissues) that were either exposed or unexposed to elevated metals. Values are P values from separate ANOVAs (n=6).

Metal	Treatment (metal-exposed vs. no metals)	Fraction (total exo, tissues)	Interaction Term
Cu	0.069	0.611	0.835
Zn	0.018 metal-exposed > no metals	<0.001 tissue > total exo	0.154
Cd	0.032 metal-exposed > no metals	0.897	0.565

Table 7. t-test comparisons of *F. grandis* concentrations of fish that were fed either exoskeleton matrix (EDTA rinsed) or total exoskeleton of *P. pugio* exposed to elevated metals (n=5).

Metal	P value
Cu	0.790
Zn	0.080
Cd	0.221

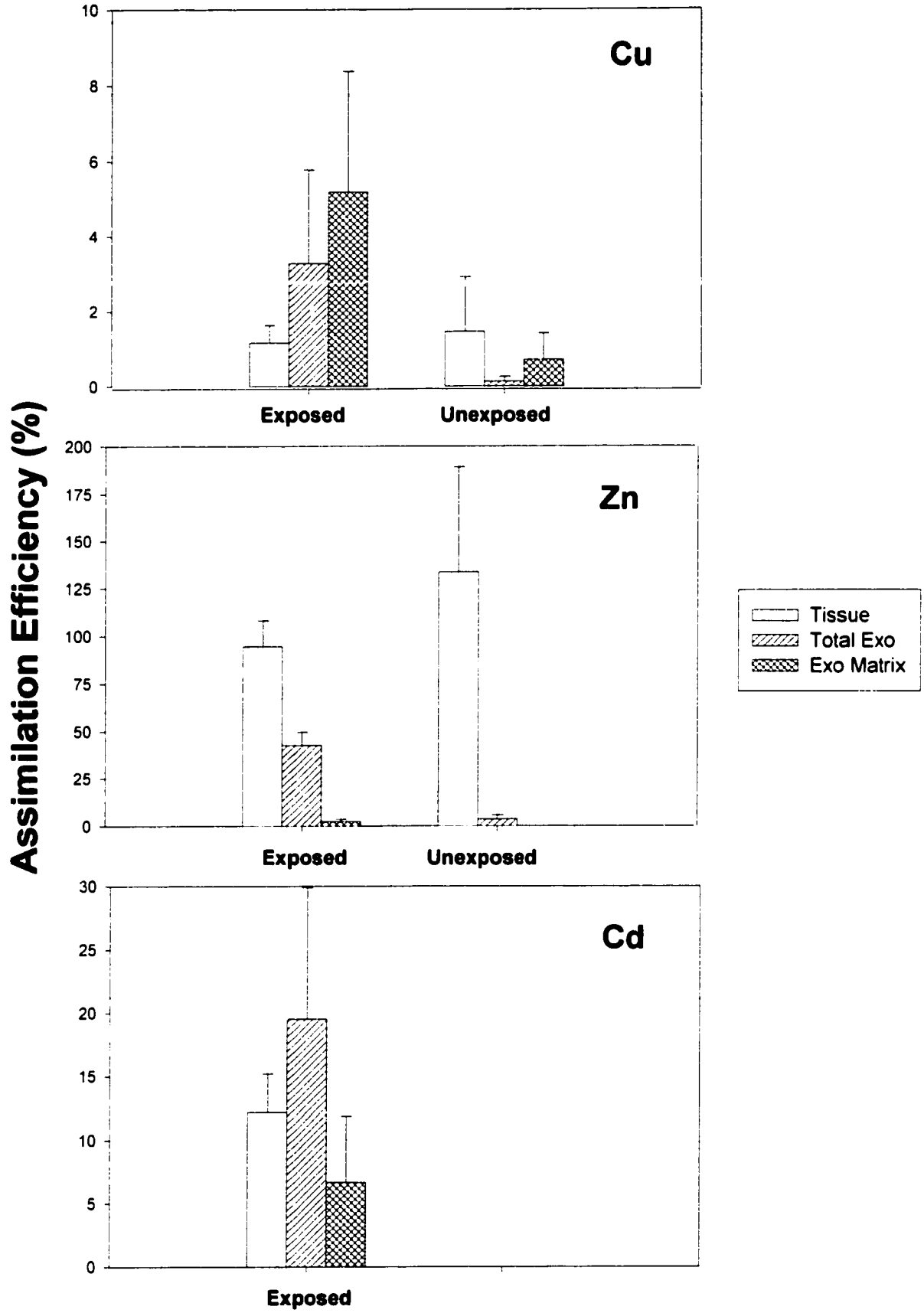

Figure 10. *F. grandis* (a)Cu, (b) Zn, and (c)Cd assimilation efficiencies fed either tissue, total exoskeleton, or exoskeleton matrix of metal-exposed and unexposed *P.pugio*. N=5 and error bars represent standard error of the mean.

the shrimp fractions (Figure 10). Rinsing reduced Cd AEs by about 30% suggesting that some adsorbed Cd was assimilated.

Two-way ANOVAs showed that Cu and Zn *F.grandis* AEs were not significantly different among fish fed shrimp exposed or not exposed to elevated metals (Table 8). Shrimp fraction did not significanlty influence fish AEs of Cu and Cd (Table 8). Zn AE, however, was influenced by shrimp fraction (Table 8). Zn AEs were higher in fish fed shrimp tissues than shrimp fed total exoskeleton. Interactions were not significant for either metal. A t-test showed that Zn AEs were reduced by EDTA rinsing (Table 9). Cu and Cd AEs from total exoskeleton and exoskeleton matrix of shrimp exposed to elevated metals were not significantly different (Table 9).

Percent Contribution

Using the total mass of metals associated with a whole shrimp and the measured mass of metals assimilated from each fraction, the contribution (in %) of a hypothetical composite shrimp to *F. grandis* metal-body burden was estimated (Table 10). Adsorbed metals were estimated using the difference between total exoskeleton and exoskeleton matrix values. According to these calculations, if a fish consumes a whole shrimp, 51% and 49% of the fish Cu burden could be attributed to the exoskeleton matrix and tissues of the shrimp, respectively. The surface-adsorbed fraction did not contribute to the fish Cu burden. Of the Zn assimilated by *F. grandis*, 33, 2, and 65% could be attributed to the adsorbed, exoskeleton matrix, and tissues of the shrimp prey, respectively. For Cd, 38, 15, and 41% of the fish burden could be attributed to the adsorbed, exoskeleton matrix and tissues of the shrimp respectively.

Table 8. Two-way ANOVA comparisons of Cu and Zn, assimilation efficiencies of *F. grandis* fed *P. pugio* fractions (total exoskeleton and tissues) that were either exposed or unexposed to elevated metals (n=5). t-test comparison of Cd assimilation efficiency of *F.grandis* fed *P.pugio* total exoskeleton or tissues (n=5). Values are P values from separate ANOVAs. N.D.= no data, due to detection limits.

Metal	Treatment (metal-exposed vs. no metals)	Fraction (total exo, tissues)	Interaction
Cu	0.318	0.792	0.557
Zn	0.991	0.021 tissue > total exo	0.284
Cd	N.D.	0.557	N.D

Table 9. t-test comparisons of *F. grandis* assimilation efficiencies that were fed either exoskeleton matrix (EDTA rinsed) or total exoskeleton of *P. pugio* exposed to elevated metals (n=5).

Metal	P value
Cu	0.841
Zn	0.008 total exo>exo matrix
Cd	0.300

Table 10. Percent of the metals in the exoskeleton matrix, surface-adsorbed, and tissues of *P. pugio* estimated to contribute to the body burden of *F. grandis* if whole shrimp were ingested.

	Cu (%)	Zn (%)	Cd (%)
Matrix	51	2	15
Adsorbed	0	33	38
Tissue	49	65	41

DISCUSSION

Metal uptake and assimilation efficiency by the predatory fish, *F. grandis*, from a crustacean prey, *P. pugio*, varied among metal species and with the distribution of metals within the prey. These experiments utilized three shrimp fractions (tissues, total exoskeleton, and exoskeleton matrix) fed separately to *F. grandis* for 10 days, and thus directly compared metal uptake and assimilation from each fraction. Uptake by a predator is a product of the available metals from its prey and the efficiency by which metals are assimilated. Grass-shrimp tissues were an important source in the uptake of all metals examined in *F. grandis*. Tissues comprised 55, 61, and 41% of the Cu, Zn, and Cd total prey body-burdens, respectively, and *F. grandis* AEs of metals from exclusive ingestion of shrimp tissues tended to be as high or higher than the exoskeleton fractions. Consequently, 49, 65, and 41% of the Cu, Zn, and Cd, respectively, accumulated by *F. grandis* would be attributed to the tissues if whole *P. pugio* were ingested. Furthermore, a distinction was made between metals adsorbed to the surface of the exoskeleton versus those sequestered within the exoskeleton matrix. The exoskeleton matrix did not contribute substantially to the Zn (2%) or Cd (15%) burden of *F. grandis*. Although the matrix contained 30% of the shrimp total Zn body burden, AE of the matrix-bound Zn was very low. The exoskeleton matrix comprised a relatively small fraction of the total shrimp body burden of Cu (22%), but a high AE of Cu from the exoskeleton matrix (relative to the AE of tissue Cu) suggested that the matrix would contribute 51% of Cu assimilated if whole grass shrimp were ingested. The AEs of surface-adsorbed metals ranged from 0-50%. Although the adsorbed proportion of the shrimp total Zn and Cd burden was relatively low (8 and 7%, respectively), *F. grandis* AEs from the adsorbed

fraction were high compared to the other fractions. The contributions of the adsorbed fraction were estimated to be 33% of the total Zn and 38% of the total Cd accumulated by *F. grandis* if consuming whole shrimp. The surface-adsorbed Cu did not appear, however, to contribute to the Cu burden of *F. grandis* relative to the exoskeleton matrix and tissues because AEs of Cu were very low.

Of the three metals examined, Zn assimilation by *F. grandis* was most strongly influenced by its distribution among shrimp exoskeleton and tissue fractions. Tissue and surface-adsorbed Zn were highly bioavailable, whereas Zn bound to the exoskeleton matrix was essentially not bioavailable to *F. grandis*. Previous authors have found that metals dissolved in the cytosol of tissues are more available to higher trophic levels than metals precipitated in insoluble granules (Reinfelder and Fisher 1994b; Wallace and Lopez 1997; Wang *et al.* 1999). The observed high AE suggests that shrimp-tissue Zn is in a soluble form, whereas the low AE of matrix Zn suggests that Zn is in an highly insoluble form within the exoskeleton matrix of *P. pugio*. Zn adsorbed to the surface of the exoskeleton maybe is a surprisingly important pathway of uptake by *F. grandis*. Surface-adsorbed Zn was likely readily mobilized in the digestive tract of *F. grandis* by the digestive juices and absorbed across the intestinal wall (Langston and Spence, 1995). Wang *et al.* (1999) found that Zn associated with the soft tissues of copepods did not influence uptake by barnacles and suggested that Zn associated with the exoskeleton was assimilated by barnacles. In contrast, Reinfelder and Fisher (1994a) found a correlation between Zn associated with the soft tissues of copepod and assimilation by fishes in the genus *Menidi*a. However, these correlations were indirectly inferred from the AEs and the proportion associated with the soft tissues of the copepod prey and were not based on

direct measurements of assimilation of separate fractions. Differences in digestive physiology of the predator, metal solubility in the prey tissues and exoskeleton, and assimilation of the surface-adsorbed fraction may lead to differences in reported metal AEs.

An unexpected result of this study was the high AE of Cu from the exoskeleton matrix (exoskeleton matrix AEs were higher than from the tissues). Although the exoskeleton is not generally subject to digestion, the binding of metals to relatively digestible proteins in the exoskeleton procuticle may control Cu bioavailability. Metals associated with soluble proteins are available to higher trophic levels (Reinfelder and Fisher 1991) and may be present in the exoskeleton. Phenoloxidases, Cu-containing proteins similar to the hemolymph proteins in the tissues (hemocyanin), are also present in the crustacean exoskeleton (Terrwilliger 1999). Therefore, the similarity in Cu AEs of the exoskeleton and tissue may be the result of the similarity in digestibility of the Cu-containing proteins associated with the tissues and exoskeleton. To my knowledge, no studies have been conducted to examine the assimilation of Cu from prey hemocyanin by consumers. My calculations estimate that Cu associated with the matrix of the exoskeleton and tissues contribute about equally to the total Cu body burden of *F. grandis* (51% and 49% respectively, Table 10). The surface-adsorbed Cu does not appear to contribute to *F. grandis* Cu burden relative to the exoskeleton matrix and tissues. It is not clear if this result is due to a different mechanism for binding surface-adsorbed Cu compared to Zn and Cd, or if the fate of surface-adsorbed Cu is different from other sources after assimilation.

My measurements of Cd AEs in *F. grandis* (7-20%) were slightly lower than those reported by Ni *et al.* (2000) for mudskipper, *Periophthalmus cantonensis*, and glassy, *Ambassis urotaenia*, fishes (10-33%), yet slightly higher than those reported by Reinfelder and Fisher (1994a) for silversides (2.7%). Furthermore, the mean AEs by *F.grandis* of Cd in the total exoskeleton and tissues were not distinguishable statistically. Ni *et al.* (2000) did not find a correlation between assimilation of Cd by the glassy and the distribution between exoskeleton and tissue of zooplanktonic prey. Nevertheless, a significant correlation between Cd assimilation and metal distribution in zooplanktonic prey tissues was found for the mudskipper (Ni *et al.* 2000), and Reinfelder and Fisher (1994) found a correlation between the percent of the metals in the tissues of copepod prey and the AEs of Cd by silversides. Although these correlations are based upon indirect evidence, the assimilation of exoskeleton-bound metals may vary because of differences in digestion processes among fishes (*i.e.*, differences in gut pH, gut passage time). Furthermore, the surface-adsorbed Cd was highly available to *F. grandis*. The 15% contribution of the adsorbed fraction suggests that surface-adsorbed Cd is desorbed in the gut of *F.grandis* (see alsoLangston and Spence 1995). Variation in the adsorbed fraction may account for the variability reported in the assimilation of Cd associated with the exoskeleton (Ni *et al.* 2000; Reinfelder and Fisher 1994a), and is generally a neglected component of trophic transfer.

Fishes generally have been found to regulate essential elements such as Cu and Zn (Langston and Spence 1995). For example, Cu concentrations of *Pleuronectes platessa* remained unchanged regardless of exposure concentrations over a range of sublethal conditions (Saward *et al.* 1975). In addition to physiological regulation, low body burdens

may be due to low uptake because dietary Cu is likely complexed by mucus present in the gut wall and egested (Handy 1986). AEs of *F.grandis* for Cu, Zn, and Cd were very different from each other, with highest AEs for Zn and lowest for Cu. The generally low assimilation efficiencies (AE < 2%) of Cu by *F. grandis* may be the result of physiological regulation or low adsorption across the gut wall. The Zn uptake pathway is similar to Cu, and may result in competition for binding sites, which could possibly account for the difference in AE of the two metals. Zn AE of 70% are considered typical for fishes, and Zn AE in the present study was similarly high (from 50-100%), but varied among shrimp fractions, suggesting that excess Zn was not regulated by *F. grandis* efficiently as was Cu, or that this species may have high metabolic requirements of Zn. Wang *et al.* (1999) also reported high Zn AEs in barnacles (93%) ingesting copepod prey. Other authors have reported that Zn is more bioavailable to fishes than other elements including Cu and Cd (Reinfelder and Fisher 1994a; Cross *et al.* 1975). These observations suggest that there may be interactions between metal species by processes such as substitution and competition for binding sites between metals, which may limit uptake (Zyadah and Abdel-Baky 2000). Zn has been shown to reduce Cu body burdens in rainbow trout (Mount *et al.* 1994), and Zn may also inhibit Cd uptake (Goyer 1995). Therefore, the high Zn body burdens may have reduced the assimilation efficiencies of Cu and Cd by *F. grandis*.

Variability of metal uptake could be due to physiological regulation and could result in the under or over estimation of the true proportion of metals assimilated by *F. grandis*. An alternative approach to estimate metal uptake would be to measure absorption efficiency (the fraction of the ingested metal that is taken up across the gut wall, Penry 1998). Absorption efficiency is generally measured using the portion of the

metals egested in short-term, pulse-chase experiments. Using absorption efficiencies in concert with the assimilation efficiencies should provide a more accurate estimation of bioaccumulation from the different fractions of *P. pugio*. In this study *F. grandis* feces were collected every other day, and subsequently analyzed for metal content in an attempt to measure absorption efficiency. However, metal concentrations in the feces were extremely high, probably due to the scavenging of metals that were excreted into the water. In order to obtain an accurate estimate of adsorption efficiencies, radionuclides utilized in pulse-chase experiments would be useful; however the short half life of ^{64}Cu (half-life = 12.7 h) makes radionuclide work with Cu generally problematic. Furthermore, this study was designed to mimic natural conditions as closely as possible, and the use of radiotracers may cause artifacts such as differences in biogeochemical behavior of the radioisotopes from the naturally occurring stable isotopes. Paulson and Gendron (2001) found that the association of ^{64}Cu with organic particles was 70% higher than that of natural Cu, suggesting that measurements of assimilation efficiency using ^{64}Cu may overestimated true rates of uptake.

Biota are known to be an important influence on the cycling of metals in the environment through mechanisms such as sequestration and trophic transfer (Wang and Fisher 1995). Because crustaceans are abundant and widespread, it is important to understand how trace-metal distributions among tissues and exoskeletons of crustacean prey influence transfer to higher trophic levels. The findings of this study suggest that the bioavailability of metals associated with the tissues and exoskeleton (both incorporated within the chitin matrix and surface-adsorbed) of *P. pugio* to *F. grandis* varied among metals. Zn associated with the tissues was shown to be highly available to *F. grandis*,

whereas there was no difference in AE of Cu and Cd associated with either tissues or total exoskeleton. Furthermore, Cd and Zn adsorbed to the surface of shrimp exoskeleton potentially contribute a high fraction of the total metal uptake of *F. grandis* from grass shrimp. The assimilation of metals associated with the different fractions of the grass shrimp may be a function of chemical speciation (solubility) of the metals and the digestive physiology of the predator. Variation among crustacean prey exoskeleton and size (surface-area-to-body-volume ratio) may result in variation in the surface-adsorbed fraction and therefore may contribute to the large variability among metal AE reported in other studies (Reinfelder and Fisher 1994a; Ni *et al* 2000; Wang *et al* 1999; Smokerowski *et al.* 1998) that have examined the uptake of metals from crustacean prey. It is often presumed that metals associated with the exoskeleton are unavailable to higher trophic levels; however, the results of this study indicate that the adsorbed exoskeleton-associated metals can be important an vector in the transfer of metals to higher trophic levels.

CHAPTER 5

SUMMARY AND CONCLUSIONS

Although bioaccumulation of trace metals by crustaceans has been investigated in numerous studies, relatively little research has been done to clarify the role of the exoskeleton in the distribution and sequestration of trace metals, or to determine how various abiotic and biotic factors influence this role. The limited studies that have examined the association of metals with the crustacean exoskeleton report varying results, and a major goal of this dissertation was to determine if molt cycle, salinity, and route of exposure explain the discrepancies in the reported values. Furthermore, although many authors acknowledge the presence of surface-adsorbed metals on the crustacean epicuticle (Munger and Hare 1997; Reinfelder and Fisher 1994b; Zanders and Rojas 1996), to my knowledge no researchers have made quantitative measurements of the contribution of this fraction to the whole-body burden in any crustacean. Therefore, in my dissertation the fraction of the whole-body burden that was associated with the tissues, adsorbed to the surface of the exoskeleton, and associated with the matrix of the exoskeleton were each quantified. The exoskeleton fractions were shown to vary under different conditions. Finally, the ecological relevance of the partitioning between exoskeleton and tissues was demonstrated by examination of trophic transfer to a predatory fish. It has often been presumed that exoskeleton-associated metals are unavailable to predators due to the indigestibility of chitin. A few studies have attempted to determine indirect correlations between metal assimilation efficiencies in predators and the proportion of total body burden associated with soft tissues of crustacean prey, but no direct measurement of trophic- transfer efficiency has yet been published.

Results of the second chapter of this dissertation suggested that molt-cycle stage is important to the fate and distribution of Cu, Zn, and Cd in *P. pugio*. Furthermore, the

fate of exoskeleton-associated metals varied for each metal species examined. Most of the whole-body-Cu burden appeared to be retained during a single molt; exoskeleton-associated Cu may therefore be reabsorbed before ecdysis. This conservation of Cu may be associated with the presence of Cu-containing proteins, prophenoloxidases, that cross link and harden the exoskeleton during ecdysis. Total Cd body burden was significantly reduced by a single molt; however, some fraction of the Cd burden in the exoskeleton was likely reabsorbed into the tissues. Some Cd was probably mobilized and transported to the tissues during molting because a relatively low fraction (25%) of the total burden was lost with a single molt. If such mobilization and transport is common within many crustaceans, the redistribution of Cd into the tissues may be the cause of the frequently observed increase in the mortality of molting crustaceans. Finally, my data demonstrated that post-ecdysis Zn whole-body burdens were significantly lower than the burdens of intermolt grass shrimp, but relatively little Zn was associated with the exuviae. Therefore, Zn appeared to be depurated by *P. pugio* through a physiological mechanism other than ecdysis.

Another finding presented in Chapter 2 was that metals adsorbed onto the surface of exuviae shed in metal-enriched water. A substantial quantity of metals adsorbed onto the exuviae are likely due to an increase in available binding sites caused by the exposure of the underside of the molted exoskeleton. Because many crustaceans consume their exuviae, elevated metals associated with exuvial adsorption may be an important pathway of uptake in crustaceans.

These findings may have important implications with regard to metal fate in crustaceans. Metals may not exert toxic effects while retained within the exoskeleton and

in fact, it is often presumed that metals are stored in the exoskeleton for potential depuration through ecdysis. However, this study demonstrated that there is variation in the distribution of different metal species before and after ecdysis. Furthermore, it appears that some fraction of the total metal burden is readsorbed before ecdysis and is not depurated. If metals are redistributed before ecdysis, tissue concentrations may vary widely over a molt cycle. Metals reabsorbed into the tissues may cause a body burden that exceeds critical body residues, leading to consequent mortality. This sudden increase in tissue concentrations at specific times in the molt cycle may reduce the usefulness of crustaceans as test organisms in toxicological bioassays because their pattern of toxicity may not be representative of non-molting animals. Future work should be done to further determine the fate of toxic metals during ecdysis and if (and by how much) tissue burdens increase as a result of redistribution from the exoskeleton. The correlation of critical body residues and tissue-reabsorption may explain why there is an apparent increase in mortality among crustaceans associated with ecdysis.

The results presented in Chapter 3 of this dissertation differentiated between surface-adsorbed metals and metals internally sequestered in the matrix of the exoskeleton. Surface-adsorbed metals led to increases in whole-body burden values and an apparent increase (0-50%) in metal bioconcentration and bioaccumulation factors, presumably without causing toxic responses. Adsorbed Cu, Zn, and Cd were as high as 20, 18, and 25%, respectively of the whole-body burden, in *P. pugio*. Therefore, estimated crustacean body burdens that induce mortality (the so called "critical body residues") may be inflated if the absorbed metals are included in the body burden measurement. Salinity was shown to influence the adsorption of metals onto the

exoskeleton (at 5‰ only 0–4% of the whole-body burden of Cu, Zn, and Cd was due to the adsorbed fraction). Additionally, differences in the chemical composition, such as the degree of calcification and the amount of waxes on the exoskeleton, may lead to differences in surface adsorption among different crustacean taxa. Furthermore, surface-area-to-body volume ratio may influence the relative importance of adsorption, and the adsorbed metals may represent a higher portion of the total-body burden in smaller crustaceans, particularly larvae, zooplankton and meiofauna. These factors may contribute to the variation of the apparent bioaccumulation of metals among crustaceans. Future work should include the measurement of the adsorbed fraction across different taxa of varying size classes with different exoskeleton chemical compositions to more broadly characterize its importance.

Metals associated with the exoskeleton matrix also varied with each metal examined and ranged from 7-45% of the whole-body burden. Zn and Cd levels were particularly high within the exoskeleton matrix, perhaps due to their chemical similarity with Ca. Cu and Zn tissue burdens showed very little variability among dietary and aqueous exposure treatments, presumably because they are physiologically regulated. Cd tissue burdens varied across different treatments possibly because Cd has no known metabolic function and is not regulated. The chemical form of stored metals associated with the exoskeleton matrix is currently unknown. Although metal-detoxifying granules are reported in the digestive tissue of crustaceans, to my knowledge there are no reports of metal-containing granules in the crustacean exoskeleton with the exception of Ca (Greenaway and Farrelly 1991). Therefore, it is possible that the substitution of metals with Ca could result in the production of insoluble granules rich in Cd and Zn.

Knowledge of the chemical form of metals is necessary to explain toxic effects to the organism because insoluble granules render metals nontoxic. However, Cu in the matrix may have been associated with proteins. More work should be done to determine the distribution and chemical behavior of metals within the procuticle and chitin matrix.

The accumulation of metals in the tissues, exoskeleton matrix, and total exoskeleton of *P. pugio* from aqueous (as a function of salinity) and dietary exposure varied for each metal examined. Zn was chosen here as a graphic benchmark with which to compare the other metals examined because of its prevalence in the literature and it is a regulated, essential metal (Brian 1968; White and Rainbow 1984; Fowler *et al.* 1971; Wang *et al.* 1999). Bioconcentration of Cd in the exoskeleton fractions (matrix and total exoskeleton) always exceeded that of Zn regardless of salinity (Figure 11). Cu and Cd bioconcentration in the tissues and exoskeleton (matrix and total exoskeleton) were higher than that of Zn at 30 ‰. Zn bioaccumulation and bioconcentration at 18‰ were also compared with the other metals, again as an important benchmark. Cu and Cd bioaccumulation factors were always lower than bioaccumlation factors (Figure 12). However, Cu bioconcentration factors were consistently higher than Zn bioconcentration factors for tissues, total exoskeleton, and exoskeleton matrix. Cd bioconcentration exceeded Zn only in the tissues. This variation in the uptake and partitioning of each metal may influence critical body residues and the toxicity of mixtures. Future work should compare the accumulation of additional metals in the exoskeleton and tissues of *P. pugio* and other crustaceans.

Chapter 4 addressed the ecological relevance of the partitioning of metals between the exoskeleton and tissues in *P. pugio*. The metal distribution between tissues and

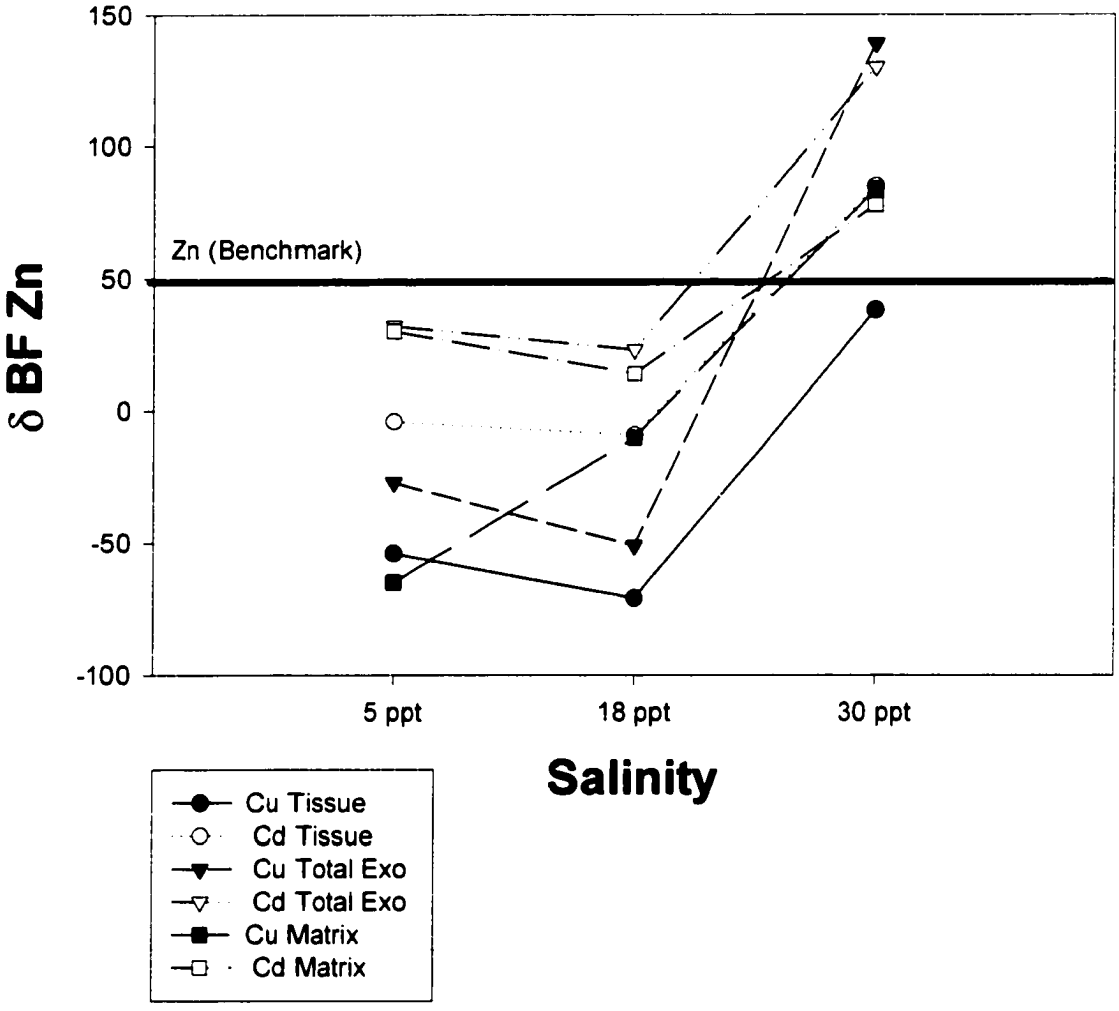

Figure 11. The differences in Cu and Cd bioconcentration factors using Zn as a benchmark (solid line) of tissues, total exoskeleton, and exoskeleton matrix for *Palaemonetes pugio* plotted across salinity exposures. The y axis represents the difference between Zn and Cu bioconcentration factors and Zn and Cd bioconcentration factos

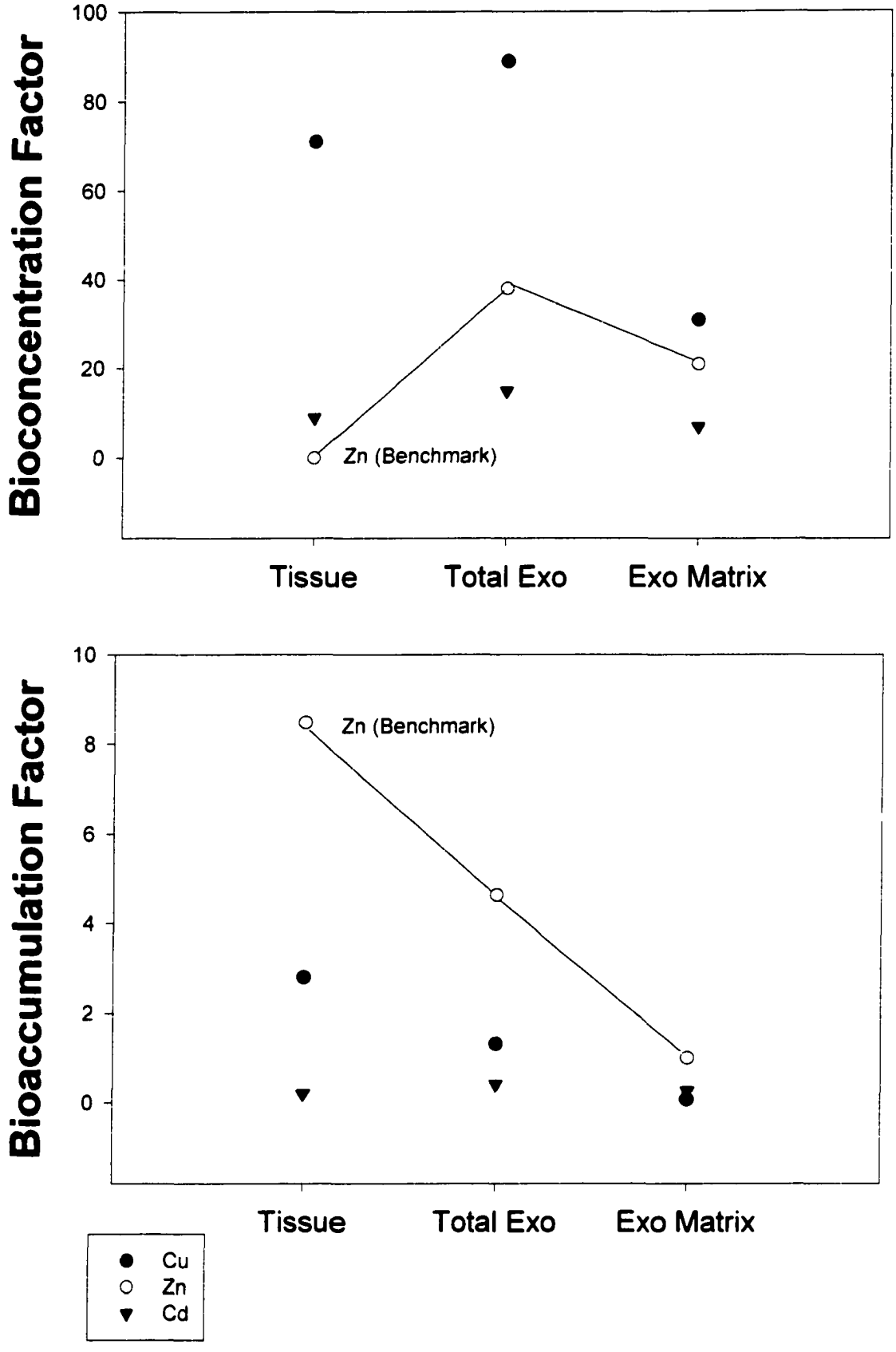

Figure 12. Cu, Zn (benchmark, solid line), and Cd bioaccumulation and bioconcentration factors in the tissues, total exoskeleton, and exoskeleton matrix of *Palaemonetes pugio*.

exoskeleton was found to influence trophic transfer to the predatory fish *Fundulus grandis*. The assimilation efficiencies by *F. grandis* of metals adsorbed to the exoskeleton surface, accumulated within the matrix, or associated with the tissues varied among metal species. It is often presumed that metals associated with the exoskeleton are unavailable to higher trophic levels, but the results of Chapter 4 indicated that exoskeleton-associated metals, particularly the surface-adsorbed metals, can be an important vector in trophic transfer to a predator that consumes whole crustacean prey. *F. grandis* assimilation efficiencies from the different fractions varied for each metal. The assimilation efficiencies of Cu and Cd associated with the exoskeleton matrix were not different from the tissues of *P. pugio* and ranged from 1-20% . If *F. grandis* were to consume whole shrimp, approximately 51 and 15% of the Cu and Cd, respectively, accumulated could be attributed to the exoskeleton matrix; whereas, 49 and 41%, respectively, was estimated to be contributed by shrimp tissues. Furthermore, Zn associated with the grass shrimp tissues was highly available (near 100%), while Zn sequestered in the exoskeleton matrix was essentially unavailable to *F. grandis*. Hypothetically, 65% of the Zn accumulated by *F. grandis* was associated with the tissues of whole shrimp, yet only 2% was estimated to be accumulated from the exoskeleton matrix. This study also concluded that Zn and Cd adsorbed to the surface of the exoskeleton are highly bioavailable to *F. grandis*. Thirty-three percent of the Zn and 38% of the Cd accumulated by *F. grandis* from hypothetical whole shrimp was estimated to be adsorbed to the exoskeleton surface.

The assimilation of Cu, Zn, and Cd from the different shrimp fractions by *F. grandis* varied greatly for each metal. Zn was again chosen as a benchmark to compare

AE with the other metals studied due to its prevalence in the literature and it is regulated. Assimilation efficiencies of Cu and Cd associated with the exoskeleton matrix were slightly higher than the assimilation of matrix Zn, although all matrix AEs were generally low (Figure 13). Zn AEs were much higher than Cd and Cu for tissues and total exoskeleton. Although surface-adsorbed Zn was highly bioavailable to *F. grandis*, adsorbed Cu was probably not readily assimilated. Therefore, the mixture of metals may contribute the variation of assimilation efficiencies from the tissues, total exoskeleton and exoskeleton matrix. The assimilation of other metals associated with the tissues and exoskeleton fractions should be compared in future investigations with crustaceans.

Metal surface-adsorption also varied with salinity and for each metal. Surface-adsorption also may vary due to differences in exoskeleton composition, surface-area-to-body volume ratio, and exposure time. Such differences likely lead to variations in assimilation efficiency by predators consuming crustacean prey. Therefore, in order to predict trace-metal fate in aquatic ecosystems through trophic transfer to predators that consume whole crustacean prey, an improved knowledge of surface-adsorbed metals is required. More studies are needed with different types of crustacean prey because exoskeleton composition varies and partitioning with the exoskeleton may also vary. Other metals may have a different affinity with the exoskeleton than those examined here, and should be compared in order to determine the bioavailability to predators. This study was conducted on one species of fish, and other fishes may have physiological differences (*i.e.*, gut passage time, gut pH) that will influence the assimilation of metals from crustacean prey. The assimilation of exoskeleton-associated metals by different species of fishes would provide additional insight. Future studies may utilize radiotracers in

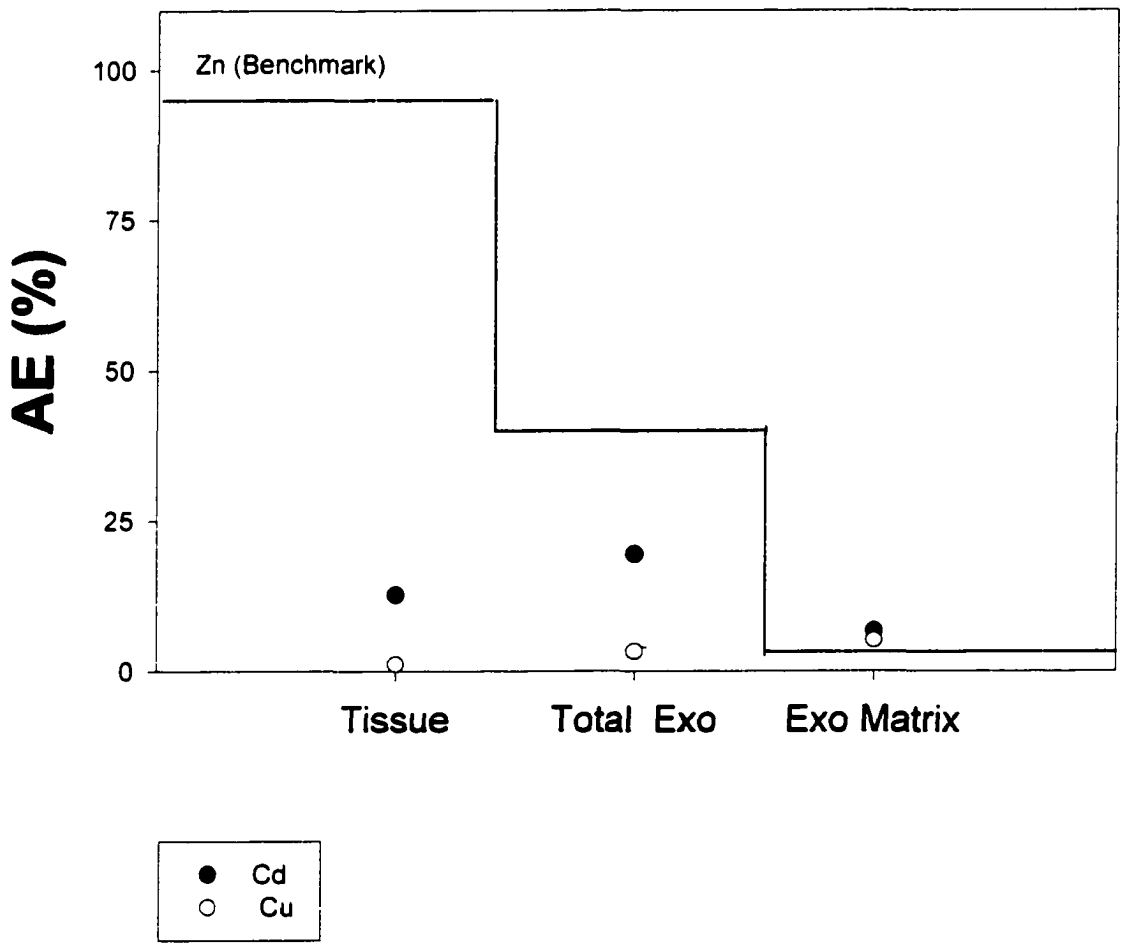

Figure 13. *Fundulus grandis* assimilation efficiencies of Cd and Cu from tissues, total exoskeleton, and exoskeleton matrix of *Palaemonetes pugio* compared to Zn (solid line) as a benchmark.

order to measure adsorption efficiencies and a thorough mass balance study would further elucidate metal fates. The assimilation of metals associated with the exoskeleton and tissues may be a function of solubility of metals. X-ray emission analysis of exoskeletons of crustaceans exposed to elevated metals would be useful to determine if various metals are stored in insoluble granules in the exoskeleton, and may be necessary to explain differences in assimilation efficiencies of the three metals studied.

The results of this dissertation have important implications with regard to how investigations using crustaceans exposed to trace metals should be conducted. The presence of surface-bound metals associated with the exoskeleton suggest that whole-body burden estimates that do not first remove such metals are greater than metal burdens that are actually experienced in the tissues. Thus, estimates of body burdens which cause mortality, such critical-body-residues, may be higher than what tissues actually experience and may be inflated. Surface-adsorbed metals may also lead to variability in biomonitoring data based on whole-body burdens due to different degrees of surface-adsorption on crustaceans of different sizes and from different habitats of varying salinity. Therefore, in order to obtain accurate correlations between tissue residues and toxic effects, results of this dissertation suggest that crustaceans should be rinsed with a strong chelator such as EDTA prior to analysis to remove surface-adsorbed metals. On the contrary, absorbed metals contribute a large fraction of the trophic transfer of metals to predators. Investigations designed to model the trophic transfer of metals from crustacean prey should include the surface-adsorbed metals as part of the whole-body burden. Thus, the goals of the project of interest involving crustaceans and metal-body burdens should be considered before a specific technique is chosen.

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DOCTORAL EXAMINATION AND DISSERTATION REPORT

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Title of Dissertation: Metal Partitioning among Tissues and Exoskeleton of Palaemonetes pugio and Its Role in Depuration and Trophic Transfer

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