BEHAVIORAL AND SUBCELLULAR APPROACH TO UNDERSTANDING METAL TOXICITY IN FIDDLER CRABS

by

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Abstract

Behavioral and Subcellular Approach to Understanding Metal Toxicity in Fiddler Crabs

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Behavioral toxicities of Cd and Ag were examined in the fiddler crab, *Uca pugnax* at 5ppt and 30ppt salinities. Changes in competitive foraging (scooping on mud surface and on a protein-rich patch) and dominance (ability to control patch and percent successful attacks) of both dominant and submissive crabs were examined prior to and after exposure of dominant crabs to series of μmolar concentrations of Cd and Ag.

Results show that exposure of crabs to metal concentrations ≤22.3μM had no observable adverse effects on competitive behaviors. At 44.5μM Cd or Ag and greater, competitive behavior of the dominant crab was drastically diminished as compared with pre-exposure, while that of submissive crabs improved remarkably. The protective effect of salinity against metal toxicity was first observed at the lowest observable adverse effect level (LOAEL) of 44.5μM Cd or Ag. The ability of fiddler crabs to tolerate Cd to a greater extent than Ag (88.9- 44.5μM) at 5ppt, but tolerance to a greater Ag concentration (148.0-88.9μM) at 30ppt suggests that Ag is more toxic than Cd at low salinity, but it is less toxic than Cd at high salinity. This shows that salinity plays an important role in determining metal toxicity, presumably, by altering metal speciation.

The combined toxicity of Cd and Ag was examined by exposing dominant crabs to Cd and Ag in mixtures at the two salinities. At LOAEL, results suggest that the combined toxicity of Cd and Ag is additive at 5ppt, but may be synergistic at 30ppt.

Analysis of tissue burden showed that the bioaccumulation of Cd and Ag increased on a whole body basis as well as in all subcellular fractions analyzed (i.e. organelles, enzymes, metallothioneins and insolubles) with the increase in metal exposure concentration. Unlike Cd, bioaccumulation of Ag was not affected by salinity. This may because neutral Ag chloro complexes are bioavailable whereas Cd chloro complexes are not. This study demonstrates that the toxicity of Cd and Ag is dependent on the physicochemical condition (e.g. speciation), and on metal ability to bind to intracellular fractions, specifically enzymes.

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§ Chapter One §

INTRODUCTION

A vast number of pollutants are continuously released into the environment with coastal waterways and estuaries often being the ultimate sink for these contaminants. For instance, the widespread use of metals for industrial purposes has resulted in increased loadings of metals in estuaries (Hornberger *et al.* 1999), and in metal concentrations elevated above natural background (Gallagher *et al.* 1997). Because many metals are toxic to aquatic organisms even in trace quantities in abiotic (sediments) and biotic (animals) matrices, this can lead to ecosystem-wide implications (Caussy *et al.* 2003, Hornberger *et al.* 1999).

Estuaries have a low biodiversity due to stress associated with fluctuations in salinity, temperature, acidity, oxygen consumption, etc. (Jha 2004, Kautsky 1998). Estuarine invertebrates, which constitute more than 90% of estuarine species play an important role in ecosystem functioning and are impacted by these stresses (Jha 2004). The impact of these natural stresses can be further amplified by exposure to anthropogenic contaminants (i.e., metals and organics). This exposure can have important impacts on the growth, reproduction and survival of sensitive estuarine organisms (Hebel *et al.* 1997, Kautsky 1998). In order to understand the extent of these impacts, it is necessary to develop short-term biological tests that can provide ecologically relevant measures of toxicity (i.e., beyond LC-50 studies) (Kautsky 1998).

The research described in this dissertation focuses on understanding the relationship between metal exposure and various indices of behavioral toxicity and bioaccumulation in the fiddler crab *Uca pugnax*. The influence of salinity, a major physical factor in estuarine environment, was also taken into considerations because of its influence on metal toxicity and speciation.

It is important to appreciate however, that simulating natural conditions under a laboratory setting is difficult, if not impossible to attain, and that using of short-term sublethal metal toxicity tests only helps to elucidate possible mechanisms of metal uptake and effects. Additionally, because of the suite of pollutants (and their combinations) organisms are exposed under field conditions, results from laboratory studies can only approximate those occurring in nature (Kautsky 1998).

In the last few decades, the introduction of Cd into the ecological systems has increased dramatically (Laws 1993). Aquatic ecosystems are particularly sensitive to Cd pollution; many plants and animals absorb it efficiently and concentrate it within their tissues (De Mora *et al.* 2004, Hopkins *et al.* 2004, Siboni *et al.* 2004). This leads to the bioaccumulation of this element in the food web (Burger and Campbell 2004). Due to its high affinity to sulfhydral groups, Cd exerts its toxicity by binding to enzymes, thus inhibiting their activities (Berman 1980). It can also interact with phospholipid monolayers of cell membranes, altering their structure even at very low concentrations (Domouhtsidou *et al.* 2004).

Ag is a trace metal with no known biological function (Bell and Kramer 1999, Fisher and Wang 1998). It is one of the most toxic metals to aquatic organisms, surpassed only by mercury (Ratte 1999). Once released into the environment, it can change its speciation by combining with other dissolved ligands such as sulfide, chloride and dissolved organic carbon (Hogstrand and Wood 1998). Depending on Ag species, the toxicity of Ag ranges through several orders of magnitude (Purcell and Peters 1998). The free Ag ion is the most readily available form of the metal to aquatic organisms (Morgan *et al.* 2004, Ratte 1999). Ag exhibits a very high affinity for sulfhydral groups, which

leads to its strong association with proteins (Bell and Kramer 1999). Ag can exert its toxicity by inhibiting gill enzymes responsible of osmoregulation in aquatic organisms (Bell and Kramer 1999), and by blocking the activities of membranes (Morgan *et al.* 2004, Wood *et al.* 1999).

For estuarine organisms, salinity plays an important role in determining metal toxicity. Studies show that accumulation of Cd increases with decreases in salinity (Legras *et al.* 2000, Verslycke *et al.* 2003). Part of the explanation for the increased toxicity of Cd at low salinity may result from increased stress on animals that are not adapted to low salinity (McLeese *et al.* 1987). Cieluch *et al.* 2004 showed that green crabs *Carcinus maenas* showed an increase in their osmoregulatory activities at low salinities which may lead to an increase in the uptake of Cd. However, a more important factor is the change in salinity. As salinity increases, the concentration of chloride ions increase, resulting in a decrease in the presence of the free toxic Cd ions (Wildgust and Jones 1998) (see Table 1 below). It is generally accepted that organisms can accumulate only free Cd ions (Cd^{+2}), whereas Cd chloro-complexes are not taken up (Peakall and Burger 2003). As with Cd, the free Ag ion (Ag^{+}) is also the most toxic form of the metal (Hogstrand *et al.* 1996). Salinity greatly affects the chemical speciation of Ag. As chloride concentration increases with increasing salinity, the activity of Ag ions decreases, and the available Ag chloro-complex becomes the predominant dissolved Ag species (Peakall and Burger 2003, Wood *et al.* 1999) (see table below). Salinity is therefore a major factor in controlling the accumulation and hence toxicity of these two metals (and other metals) in estuarine ecosystems.

Table 1: Influence of salinity on Cd and Ag speciation relative to chloro complexes

Salinity	$[Cd^{+}]$ / $[Cd_t]$ complexes*	$[Ag^{+}]$ / [Ag-Cl] complexes**
5	0.20	0.12
10	0.13	0.03
15	0.10	0.014
20	0.08	0.007
25	0.06	0.004
35	0.03	0.0003

* Adapted from Ward and Kramer, 2002
** Adapted from Engel and Fowler, 1979

The research presented in this dissertation focuses on the effects of Cd and Ag exposure on the foraging and dominance behaviors of the salt marsh fiddler crab *Uca pugnax*. To account for the extreme salinity change in their estuarine environment, crabs were exposed to dissolved metals at low and high salinities. Additionally, because estuarine organisms are almost never exposed to one particular metal at a time in their environment, the effect of exposure to metal mixtures (Cd and Ag) was also investigated. To determine the impacts of Cd and Ag on crabs, various indices of accumulation and behavioral toxicity were investigated. Subcellular fractions (such as organelles, enzymes, metallothioneins and granules) were isolated, and were analyzed for metals. The enzyme and organelle fractions are considered to be sensitive to metal exposure (i.e., metal-sensitive fractions) (Wallace *et al.* 2003). Because of their vulnerability to metals, it is suspected that there may be a relationship between behavioral toxicity and the binding of metals to these fractions. A field investigation was to verify that metal pollution of the

environment might alter foraging and dominance behavior of fiddler crabs. For this study, the behavior of fiddler crabs from polluted and unpolluted sites were compared and metal analyses on their subcellular fractions were conducted.

Behavioral studies were conducted in the laboratory using a plastic box containing a thin layer of mud. In order to promote competition between crabs, a protein-rich patch placed in the center of the mud. Briefly, behavioral studies consisted of two video taping sessions, a 'pre-exposure' assessment of behavior (Trial 1) and a 'post-exposure' assessment of behavior (Trial 2). The purpose of Trial 1 was to (1) determine which of the two crabs was dominant and (2) quantify feeding and dominance behaviors of both 'submissive' and 'dominance' crabs. Subsequent to Trial 1, dominant crabs were exposed to metals. Following this exposure, crabs were re-paired and video taped for a second session (Trial 2). This trial was conducted to assess the effect of metal exposure on dominant crabs.

In order to determine if behavior could be used to assess the relative health of different filed populations of fiddler crabs, behavioral studies on Meredith (impacted) and Tuckerton (clean) crabs were conducted as described in Trial 1 above. These crabs were not exposed to any metal in the laboratory.

Following Trial 2 of behavioral studies, crabs from both the lab and field study were analyzed for metal content. In bioaccumulation studies, crabs were subjected to differential centrifugation after being homogenized. Cytosol fractions were subjected to heat in order to separate heat-labile and heat-stable proteins. Before being analyzed for metal, all fractions were digested using standard sample preparation procedure.

This study showed that exposure of crabs to a Cd (or Ag) solution at a concentration >44.5μM (and to a mixture of 22.3μM of Cd and Ag) resulted in a decrease in the foraging and dominance parameters of dominant crabs and in an increase in those of submissives crabs. This reveals the interactive nature of dyadic (one-on-one) relationship between dominant and submissive crabs. It shows that foraging behavior is dependent on the dominance status of fiddler crabs. This study also showed that foraging behavior is more sensitive to metal exposure than dominance. Metal analyses of various subcellular fractions suggested that there might be a relationship between behavioral changes in fiddler crabs and increased bioaccumulation of Cd and Ag to enzyme. These findings were supported by results obtained from the field investigation where Meredith (polluted site) crabs with higher level of metals (Cd, Ag, Cu, Ni, and Se) in their enzyme fractions forage far less than those collected from Tuckerton (unpolluted) site.

§ Chapter Two §

USE OF BEHAVIOR IN PREDICTING METAL TOXICITY

I. The Field of Environmental Toxicology

A. Environmental toxicology:

Environmental toxicology or ecotoxicology is the quantitative study of the effects of exposure to pollutants on plants and animals (Duffus 1980). It is related directly to the subject of water pollution, since ecotoxicological studies reveal the quantitative relationship between the concentrations of pollutants found in water and their effects on aquatic organisms (Laws 1993). Investigations in physiological toxicity study the effects of pollutants on a vast number of physiological and biochemical variables (Cohn and Robert 1996, Weber and Spieler 1992). In the past, most attention was focused on acute toxicities of pollutants. However, laboratory research in toxicology has progressed far beyond simple measures of mortality to include behavioral changes in organisms, as well as modifications in growth and reproduction, as a means to evaluate the effects of sublethal exposure to pollutants (Hebel *et al.* 1997). This alternative strategy involves the use of behavioral endpoints to assess the impact of pollutants on the environment since, frequently, behavioral effects precede physiologic and biochemical effects (Cory 1996, Duffard and Duffard 1996, Laws 1993).

B. Ecological risk assessment:

Water is the most important medium in natural and socioeconomic systems (Helma *et al.* 1996). As a consequence of the increased anthropogenic pollution of water, the need for biomonitoring to assess anthropogenic impacts on the aquatic environment is growing (Burger *et al.* 2003). Ecological risk assessment serves to estimate the likelihood that an exposure will lead to an unacceptable adverse health response (Baker *et al.* 2003). Assessing environmental quality is essential for managing and regulating the

environment, and for setting of safe limits of tolerance in marine and freshwater ecosystems to protect aquatic life (Baker *et al.* 2003, Suter 1990).

The basic approach in risk assessment is the dose-response relationship where the increase in exposure concentration leads to an increase in severity of health effects (Baker *et al.* 2003, Burger *et al.* 2003). Recently, considerable attention has been devoted to the methodology of ecological risk assessment (Peakall and Burger 2003, Robson 2003). Burger *et al.* (2003) suggested an additional step in the dose-response experimental process in which the distribution of metals inside studied organisms is examined. Hence, a relationship between exposure concentrations and levels of metals in target sites can be established. This is essential for providing insights into the use of 'lowest observable adverse effect level' (LOAEL) and 'no observed adverse effect level' (NOAEL) for risk assessment (Burger *et al.* 2003).

Basic to any ecological risk assessment is an assessment of organisms' health, behavior, and interactions as well as an understanding of the structure and function of the existing population, communities and ecosystems (Attrill and Depledge 1997, Burger *et al.* 1991). Assessing risk for most habitats entails basic ecological research on the structure and function of ecosystems as well as monitoring of these systems. Since one cannot monitor every organism or every function of an ecosystem, an important step in monitoring is selecting indicator organisms (Burger 1994, Kautsky 1998). Once an indicator species is selected for ecological risk assessment, it is followed by choosing quantifiable variables (e.g. growth rate, reproductive rate, behavioral changes, survival of offspring and pollutants bioaccumulation). Ecological risk assessment covers a range from single species to ecosystem effects. The examination of effects of pollutants on

single species has been well developed and constitutes part of the field of ecotoxicology (Okkerman *et al.* 1991).

C. The use of behavioral studies in risk assessment:

The rationale for behavioral studies consists of four main points. First, behavioral changes can be used to assess the health of aquatic ecosystems. It is possible to measure directly the amounts of pollutants in an aquatic system, but it is really the effects of these pollutants on the behavior of organisms that should be used to indicate exposure of organisms to toxic levels of substances in the environment (Needleman 1995). Such behavioral effects may be used as warning signal that an extensive or permanent damage to biota could occur if there is continued exposure to these pollutants (Peakall 1996). Second, behavioral patterns are known to be highly sensitive to the changes in the steady state of an organism. This sensitivity is one of the key values for its use in studying sublethal toxicity. Third, the behavior of an organism represents the final integrated result of a diversity of physiological and biochemical processes. Hence, behavioral parameters can be generally used to comprehend changes in physiological and/or biochemical parameters. Last, modification in behavior patterns due to exposure of organisms to environmental pollutants, even at concentration considerably below the lethal level, can be determinantal to the survival of a species, and can have the most important impact on estuarine community (Cohn 1996, Wallace *et al.* 2000).

Modification in behavioral patterns involves a wide variety of effects, such as alterations in migratory behavior, reproduction, foraging behavior, dominance, predator avoidance and so forth (Hinkle-Conn *et al.* 1998, Klein and Lincer 1974, Vernberg *et al.* 1974, Weis *et al.* 2001). For example, salmon migrate from the ocean back to the freshwater stream where they were born in order to spawn. The chemical substances that

guide this migration have been shown to include certain volatile organic compounds. The presence of chemicals in the polluted water even at low concentrations level may completely disrupt the migratory patterns of these organisms and, through this behavioral modification, effectively eliminate the species (Laws 1993). Study showed that exposure of flagfish *Jordanella floridae* to sublethal concentrations of chemical pollutants (cadmium and zinc) inhibited reproduction (U.S. EPA 1976). Lake trout *Salvelinus namaycush* forages on fingerling rainbow trout *Oncorhynchus mykiss*. In a laboratory experiment, chronic exposure of *S. namaycush* to sublethal concentrations of cadmium resulted in a dose-dependent decrease in foraging rate (Scherer *et al.* 1997). When social dominance behavior was examined in juvenile rainbow trout, subordinate fish exhibited a higher accumulation rate of pollutant (copper) in gill and liver tissues than dominant trout (Sloman *et al.* 2002). Predator avoidance is essential to the survival of organisms. Fiddler crabs *Uca pugnax* showed disturbed motor coordination after their habitat was sprayed by Dieldrin. These changes resulted in the crabs' inability to escape predators, which was reflected by unusual numbers of egrets and raccoons feeding on the crabs (Klein and Lincer 1974). A recent prey-capture study by Wallace *et al.* demonstrated that grass shrimp *Palaemonetes pugio* that were fed field Cd-contaminated oligochaetes for two weeks, had reduced ability to capture live brine shrimp *Artemia salina* (Wallace *et al.* 2000).

The use of behavioral endpoints in ecotoxicology has advantages in hazard assessment. Behavioral studies can be very sensitive assays for determining the effects of sublethal concentrations of toxicants on populations of organisms as behavior integrates various biochemical, cellular and physiological processes (Kraus and Kraus 1986). Further, behavior is essential to the viability of organisms throughout biological

communities. Therefore, observation of behavior offers a toxicological perspective that links the cellular and community-level consequences of environmental contamination. In contrast to other sublethal behavioral criteria, such as reproduction, foraging and dominance are available for short-term testing (Vogl *et al.* 1999). For behavioral approaches to be utilized successfully in contaminant evaluations, the behavior being studied should be (1) easily observed in the laboratory or field, (2) sensitive to the pollutants of interest, and (3) ecologically relevant to the survival of the species (Cheung *et al.* 2002). The use of bioassays in the monitoring of environmental pollution is to establish a relationship between the toxicity and the concentration of a pollutant being studied. A rational sequence of toxicity tests starts by determining the lethal effects on a short-term basis and continues with more sophisticated tests designed to show more subtle physiotoxicological and ecological effects (Ramade 1987).

II. Foraging and Dominance Behavior

A. Foraging behavior:

1. Introduction:

Feeding is essential to the survival of organisms throughout their lifetime. It balances the energy budget and determines nutrient status, exercising a primary influence on growth, reproduction, dominance and other aspects of fitness (Hughes 1993, Weis *et al.* 2001). It is viewed as just one of many survival-related activities essential to reproductive fitness. As one activity demands greater investment of time and/or energy, less is available for the performance of other critical activities such as reproduction, parental care, defense of territory, etc. (Kamil and Sargent 1981). Thus, it is assumed that one dimension of the optimal solution to the general problem of time and/or energy

allocation resides in the maximization of benefits relative to the time and/or energy costs of any given activity.

2. Optimal foraging:

Changes in foraging behavior as a function of the spatial and temporal distributions of food affect foraging efficiency. The recognition of this aspect of foraging behavior has led to the development of optimal foraging theory (Kamil and Sargent 1981), which is based on the idea that an animal would promote its fitness by foraging in ways that maximize the net rate of energy gain, and that a forager can evaluate the profitability, in terms of yield per unit handling time, of each food type encountered (McArthur and Pianka 1966). Optimal foraging models predict that a forager should always accept the most profitable food type and that it should accept successively a less profitable type only when it becomes less energy efficient to control the most profitable food (McNamara and Houston 1990).

3. Competitive foraging:

Competition is a term used by ecologists to describe the active demand by two or more individuals for a resource that is potentially limiting. It is advantageous for an organism to compete aggressively for food, since it is energetically more efficient to fight few times for an area that contains food than to compete for each item of food (Archer 1988). Competitive foraging could lead (a) to direct conflict or aggressive interactions among foragers (contest competition) or (b) to the exploitation of resources by one forager (exploitive competition). Contest competition occurs when one individual obtains access to a food resource sufficient to maintain it, and it denies access to others through fighting (Archer 1988). Whereas, exploitive competition occurs principally when fighting would be inappropriate as a means of securing food resource because it is widely

distributed. In exploitive competition, the differential ability of competitors to harvest limiting resources leads to decreased resource availability, resulting in decreased growth and reproduction for those organisms with an inferior ability to harvest resources (Bertness 1999). In this case, dominant foragers deplete most of the food resources, and less successful competitors are likely to obtain some of the resource without being challenged directly (Krebs and Davis 1987, McArthur and Levins 1967).

The present understanding of the evolution of aggressive behavior in animals is primarily due to the application of game theory to animal conflicts (Enquist and Leimar 1983). Game theory largely dispenses with terms aggression and dominance (Francis 1988). It suggests that the time devoted to an aggressive interaction and the outcome of the interaction depends on the existence of asymmetries (i.e. differences) between competitors (Maynard Smith 1982, Parker 1984). If there are asymmetries, then game theory predicts that these asymmetries will often be used to settle disputes with little overt aggression. Asymmetries include differences in size and strength, ownership, and hunger. In asymmetric interactions with clear dominance-subordinate relations, the dominant individuals often force subordinates into sub-optimal patches (Schoener 1983). If, however, there are no clear asymmetries (e.g. same size contestants with no established ownership) then the result can be lengthy and costly fights, since contestants are willing to persist longer in fights for more valuable resources (Austad 1983, Giraldeau *et al.* 2000).

Aggressive interactions, however, take time away from foraging. This is generally true regardless of whether the focus is on dominant or subordinate individuals (Hughes 1993). Animal aggression is viewed as an optimum strategy for maximizing gain from resources present in the environment. In terms of guarding resources, animal aggression

is more obvious when there are fewer resources than competitors and when individuals can achieve an immediate gain in fitness by forcibly ousting others. It is more intense when the resource is limited, is easier to guard and provides higher yield (Parker 1974). Fighting outcomes are considered to be affected primarily by resource value and fighting ability (or resource holding potential). Size, strength, weaponry and prior experience are very important in determining the outcome of aggressive disputes (Keeley and Grant 1993, Smith *et al.* 1994). Even though fighting experience affects the outcome of subsequent contest by affecting how an individual assesses its fighting ability, these winning or losing experiences are extremely short-lived (Hsu and Wolf 1999, Whitehouse 1997).

4. Diet selection in estuarine deposit feeders:

Deposit feeders digest bacteria, protozoa, microalgae and detritus particulates attached to mud and sand. There are two categories of deposit feeders: surface deposit feeders that feed on at the sediment-water interface, while subsurface deposit feeders feed below it. An advantage to subsurface feeders in intertidal setting is that they can continue to feed even after the tide is out if capillary water remains. Surface deposit feeders can do so only if overlaying water remains (Hughes 1993).

Food quality for surface deposit feeders is determined by microbial density and organic content of surface sediments (DeFlaun and Mayer 1983, Weissburg 1993). Studies showed that there is a direct relationship between protein content of sediments and growth rate of deposit feeders (Taghon and Greene 1990). For intertidal communities, the tide constantly exchanges and delivers particles, providing a potential choice to surface deposit feeders. This is a reason, then, to expect diet selection by at least some deposit feeders.

5. Patch exploitation:

Feeding rate is in part determined by the ability of organisms to locate patches of high food quality. An explicit, experimental test of patch choice for deposit feeders was that of Robertson *et al.* (1980) with fiddler crabs *Uca pugnax*. It was found that these crabs spent more time foraging in patches that were amended with higher concentrations of benthic diatoms.

Patch models are among the most active areas of research within behavioral ecology. Patch exploitation models deal with optimal patch exploitation, that is, the time and extent to which patchy resources can be exploited. Hence, patch residence time can be incorporated into social patch models (Giraldeau and Caraco 2000). Also, individual payoff per patch is inversely related to the foraging group size. Nonetheless, increase in foraging group size improves patch-finding rate (Rita and Ranta 1998). Livoreil and Giraldeau (1997) tested the social patch model for depletable resources. It was concluded that finches that forage in groups interfered with one another, and that birds most affected by competition spent more time in the patch and collected more seeds. Social patch models are species-specific and different species behave differently. For instance, Smith *et al.* (2001) found that for dominant black birds *Turdus merula* foraging rate was greater with subordinate individuals present than when they were fed alone. As black bird group size increased, mean foraging rate also increased before it started dropping after group size reached a certain level.

B. Dominance behavior:

For animals that live in groups, individual differences in fighting ability determine who will have priority of access to food or mates (Krebs and Davies 1987). Agonistic behavior, which is a manifestation of dominance, is defined as social fighting including

threats, chasing, and physical combat but excluding predation (Earley *et al.* 2000, Francis 1988). Most agonistic behavior involves competition for resources (e.g. food, water, mates, or territories) that are in actual or potential short supply (Hyatt and Salmon 1979). Within a species, dominance is accomplished when an animal can control the behavior of another as a result of previous non-agonistic (e.g. hierarchy of food transfer from subordinate forager bees to dominant nurses) or agonistic interactions (Drickamer and Vessey 1982). However, in most cases, dominance is based on dyadic encounter (encounter between two individuals) (Earley *et al.* 2000).

Dominance is an important parameter in most behavioral studies. It is an attribute of repeated, agonistic interactions between two individuals, characterized by an overall fighting success of the same dyad member and an overall losing of the other. The status of the winner is dominant and that of the loser is subordinate (Francis 1988). The functional statement of dominance is that particular individuals in social groups have regular priority of access to resources in competitive situations; the dominant displaces the opponent in contests over resources (Drews 1993).

Agonistic behavior is viewed as a widespread solution by animals to the problems of self-preservation and resource competition. Examples of agonistic behavior can be found throughout the animal kingdom, from animals with very simple nervous systems such as planarian worms to those with the most complex neural mechanisms such as humans (Archer 1988, Bakker 1986). Dominance hierarchy occurs most often, however, in vertebrates and arthropods that live in social groups (Drickamer and Vessey 1982). For a dominance-subordinate relationship to exist, rivals must assess each other's fighting ability. The most common correlate of fighting ability is body size (Jennions and Backwell 1996). And the most obvious cues an animal could use to assess an opponent's

fighting ability would be physical attributes such as size or the possession of weapons. Where opponents are of different size, the larger is usually consistently victorious and therefore dominant over the other (Huntingford and Turner 1987, Lohrer *et al.* 2002). In symmetric contests, a consistency in the outcome of agonistic conflicts between two animals, following their first contest, indicates that a relationship between the subjects has been established, and the remaining period merely accentuates the differences in behavior of dominant and subordinate animals (Jakobsson and Kullberg 1995).

The increased risk of predation during escalated fights may be behind the evolution of ritualized fighting behavior (Jakobsson and Kullberg 1995, Palmer 1998). Grouping of animals into dyads maximizes the social interactions between contested animals (Sloman *et al.* 2002). The defeated member of the dyad submits in subsequent encounters with the other member without further escalation. On the basis of such a change from symmetric to asymmetric responses in subsequent agonistic interactions, dyads with a dominance relationship can be identified (Drews 1993). Animals, such as crabs that have won fights get fiercer and approach rivals more readily (Bakker and Sevenster 1983, Huntingford and Turner 1987). Because an animal reproductive success depends on its dominance status, and since dominant males sometimes acquire more mates and mate more frequently (Christy and Schober 1994), behavior is likely to be an important selective force in determining the genetic composition and the direction and speed of evolutionary change of a population (Krebs and Davis 1984).

Dominance behavior is a quality attributed to an individual, and it is based on the outcome of a dyadic contest and depends on asymmetry of an interaction between individuals (Earley *et al.* 2000), whereas, aggression is a quantifiable behavioral response. Some of the indices used to quantify aggressive behavior are frequency and

duration of threat display, frequency of attacks, latency of attacks, and number of attacks and time in aggression zone (Bakker 1986, Early *et al.* 2000, Hamilton and Poulin 1995).

III. Metal Pollution and its Implications

A. Introduction:

The discharge of metals into the environment, mainly through wastewater discharges, is a major environmental concern (Griscom *et al.* 2002, Seidemann 1991). Unlike organic pollutants, metals are nondegradable and cannot be destroyed by treatment technologies (Frazier 1979, Robson 2003). Metals in wastewaters normally represent materials utilized in casting, smelting and refining, coal mining etc. It is inherent, and often unavoidable for manufacturing industries to release metals as byproducts into the environment (Caussy *et al.* 2003, Patterson *et al.* 1998).

B. Fate of metals in the estuarine environment:

The sources of metals to estuaries have become more dominated by human activities (Laws 1993, Mance 1987, Mortimer and Rae 2000). Metals in their pure metallic state present little hazard; it is the soluble compounds of metals that are toxic to aquatic environments (Peakall and Burger 2003, Waldichuck 1974). Upon reaching the estuarine environment, most metals are converted from dissolved to particulate forms through the processes of precipitation, adsorption, or flocculation (Twiss *et al.* 1996). They settle out to the bottom of estuaries or in subtidal areas of salt marshes, hence, sediments are the principal sink for metals in aquatic environments. As a result, epibenthic organisms may be at higher risk of exposure to metals (Schlekat *et al.* 2001). As environmental conditions change, sediments can act as a source of contaminants that may bioaccumulate in the food chain (Feng *et al.* 1998, Izquierdo *et al.* 1997, Lee and

Cundy 2001). For instance, with increasing salinity, some weakly adsorbed metals can be desorbed through the action of ion exchange. The degradation of organic matter by bacteria in sediments can release metals, resulting in their dissolution and return to water column (Church and Scudlark 1998). Metals can form highly insoluble sulfide precipitates that substantially reduce their availability to organisms (Casas and Crecelius 1994). However, dredging, bioturbation and oxidation of salt marsh sediments by rooted vegetation can lead to oxidation of sulfides and release of metals into water column (Morse 1995).

C. Bioclassification of heavy metals:

Metals can be grouped according to their fundamental chemical properties into two major types. Type A include the oxygen seeking metals (e.g. Fe, Mn, Co, Cr etc). They tend to form cations, which react with negatively charged ions such as oxide and suspended particles and thus become enriched in sediments. Type B includes nitrogen/sulfur-seeking metals (e.g. Zn, Ni, Cu, Cd, Ag, Pb, Hg etc.), which tend to form stronger complexes with organic matter and thus are more stable for transport through aquatic environments. Many of these metals are essential (except for Cd, Hg, Pb, Ag and few more). However, above required concentrations, they can have adverse health effects (Church and Scudlark 1998, Laws 1993). In this context, metals exert their toxicity by (1) blocking of essential biological functional groups of biomolecules, (2) displacing essential metal ion in biomolecules and (3) modifying active conformation of biomolecules (Ochiai 1977, Sanders *et al.* 1996). Type B metal ions are more toxic than type A since the former can participate in all three general toxicity mechanisms. The mechanism of metal toxicity is frequently damage to an enzyme system. This occurs when metal ions bind to the enzyme and cause a change in its configuration that results in

the loss of its specific functions (Freedman 1989). For instance, the binding of Ag^{+}, Cd^{+2} or Hg^{+2} to the sulfhydral group of cysteine residues in the active site of enzymes inhibits enzymatic activities (Laws 1993, Nieboer and Richardson 1980, Robson 2003). Because many biological membranes are sulfhydral rich, membranes are considered to be important target sites of metal toxicity. Metals bound to membranes are likely to modify membrane integrity and function, inhibiting its various transport systems (Chang 1996). In intact cells, on the other hand, internalized metals readily react with cytoplasmic constituents and subcellular organelles (Foulkes 1996).

D. Metal bioaccumulation:

The tendency of metals to be bioaccumulated in aquatic organisms is perhaps one of the metals' most important biological properties (Waldichuk 1974). Metal concentrations in the tissues of organisms are widely used to evaluate the fate and distribution of biologically available contamination in aquatic ecosystems (Brown and Luoma 1995, Pyatt *et al.* 1997, Ritterhoff and Zaire 1997). The fact that metal concentrations in aquatic organisms are typically several orders of magnitude higher than concentrations in water means that metals may become progressively concentrated at higher trophic levels in aquatic food chains due to magnification (Bryan and Langston 1992, Griscom 2002, Laws 1993, Weis and Weis 1993). Exposure of estuarine organisms to metals through food or water can lead to the incorporation and transfer of metals into higher trophic levels in the aquatic food web, which may eventually lead to human consumption (Luoma 1989, Morrison *et al.* 1996). In the aquatic environment, organisms are exposed to sublethal concentrations of many contaminants simultaneously, and the concentrations of metals within the organisms result from the relative rate of metal accumulation and turnover (James and Kleinow 1992, Wolfe 1974). Hence, the

concentration of metals in aquatic organisms is a function of the balance between the rates of accumulation and depuration. This balance is determined by the physiological condition of the aquatic organisms as well as salinity and temperature of the exposure media, and metal-metal interactions (Brown and Luoma 1995, Bryan 1979, Lee and Luoma 1998, Khan *et al.* 1989, Mance 1987, Voyer *et al.* 1982).

E. Effect of salinity on metal uptake:

In general, metal uptake rates increase as salinity decreases (Mance 1987, Wildgust and Jones 1998). The most accepted paradigm explaining the negative relation of salinity on metal uptake assumes that, except for Hg, the most bioavailable metal form is the free metal ion; increasing complexation (mainly with chloride) at higher salinities reduces the free ionic concentration; the dominant species becomes chloro-complexes (Peakall and Burger 2003, Sung 1995, Wright 1995). Salinity in the estuarine environment is primarily controlled by freshwater discharge (Feng *et al.* 1998). The variations in freshwater flows expose estuarine biota to variations in salinity which will have a direct effect on metal uptake and therefore on tissue concentrations (Riedel *et al.* 1998, Vernberg *et al.* 1974).

F. Interactions of metals:

As for interaction of metals in relation to their toxicity to aquatic organisms, the toxicity of combinations of metals is of particular importance because aquatic ecosystems are often polluted with a mixture of metal pollutants rather than a single metal (Chu and Chow 2002, Preston *et al.* 2000). There is much less information available on the toxicity of mixtures of metals as compared to the toxicity of single metal (Preston *et al.* 2000, Schlekat *et al.* 2001). It is challenging to measure and evaluate the effects of metal mixtures because the combinations in which they occur in nature are endless, and because

physicochemical and physiological processes influence their effects (Peakall and Burger 2003). Predicting the response of aquatic organisms to exposed simultaneously to more than one potentially toxic chemical is one of the most difficult tasks in environmental toxicology (Norwood *et al.* 2003). Although it is possible to examine the effects of two metals in the laboratory, the combination of three or more becomes problematic, particularly given the possible variations in concentrations (Peakall and Burger 2003, Schlekat *et al.* 2001).

When organisms are exposed to mixtures of toxicants, the interactions can be described as additive, synergistic, or antagonistic. Additive effects arise when the toxicity of mixture is equal to the sum of toxicities of the individual chemicals. Synergistic or antagonistic interactions arise when the toxicity of the mixture is greater than or less than the sum of the toxicities of the individual chemicals (Laws 1993, Norwood *et al.* 2003, Preston *et al.* 2000, Ramade 1987). The toxicity of metal mixtures depends on their affinities to binding sites. For instance, if a more toxic metal in mixture has greater affinity for a key metallo-enzyme and causes greater conformational dysfunction than a competing metal, then additive or synergistic toxicity can result. But, if the more toxic metal in mixture has a strong binding affinity for metal storage proteins, this causes the less toxic metal to react in the cytosol, then antagonism can occur (Schlekat *et al.* 2001).

There are number of methods used to predict the impact of metal mixtures and metal interactions. All of these utilize water concentrations of metals of interest to generate dose-response curves for each individual metal, which are then used to generate specific critical concentrations for mixture models. These mixture models can broadly be classified into two basic types: Concentrations Addition and Response Addition. The most commonly used concentration Addition model is the Toxic Unit approach. In this

approach, the concentration of each metal in the mixture is divided by the toxic concentration for that metal and organism when present singly (e.g., lethal concentration killing 50% of organisms or LC_{50}, or lowest observed adverse effect concentration or LOAEC), to convert the concentration into toxic unit scale. The toxic units for all metals in the test mixture are then summed (Norwood *et al.* 2003).

The toxic interaction between metals is evident but inconsistent (Khan *et al.* 1989); it varies between study organisms (Lee and Luoma 1998, Mance 1987). For instance, the exposure of the sandy shore gastropod *Nassarius festivus* to sublethal concentrations of metals (specifically Cu, Cd, Zn and Cr) had a negative effect on its feeding behavior (Cheung *et al.* 2002). However, the curlew bird, *Numenius arquata*, with elevated body burden of these metals showed no difference in foraging and breeding success compared to birds from non-polluted areas (Currie and Valkama 1998). Hence, different species show different responses to same metal exposure; this highlights the need to consider species differences in ecotoxicological risk assessment (Sauvé *et al.* 2002).

Chapter Three:

FRAMEWORK OF THE RESEARCH

I. Introduction to Salt Marsh Ecology

Salt marshes are intertidal zones that act as links between land and water; they are transitional areas between dry terrestrial ecosystems, such as grasslands, and permanently wet aquatic ecosystems, such as bays and estuaries. Along the Northeastern Atlantic shorelines of the United States, salt marshes are closely linked to estuaries that are influenced by the twice-daily rise and fall of tides, and they are subject to rapid and extreme changes in salinity, as well in temperature and in water depth (U.S. EPA 2000). Salt marshes rank among the most productive ecosystems on earth (Bertness 1987, Nixon and Oviatt 1973), and they rival the tropical rainforests in the amount of plant material biomass produced each year. This high productivity is due to benthic algae, phytoplankton in the water, and salt marsh plants (Barnes and Hughes 1999). Only a small percent of marsh plant biomass is directly consumed by herbivores, most is decomposed by bacteria and fungi in the sediments. Scavengers such as worms, fishes, shrimps and crabs, feed on the decaying plants, along with the bacteria, fungi, and other attached organisms (Bertness 1992, Moy and Levin 1991). Long considered ecologically important habitats, salt marshes are nursery grounds for fishes, shrimps, and crabs, feeding and nesting areas for birds and mammals, and buffers that protect coastal environments from erosion and nutrients loading (Bertness 1999, Hinkle-Conn *et al.* 1998). Salt marshes help prevent flood by storing and slowing the flow of water, and act like filters by trapping sediments and nutrients suspended in water (U.S. EPA 2000).

As salt marshes trap nutrients and provide rich productive nurseries and feeding areas for numerous organisms, they also trap pollutants that can harm estuarine organisms (Burke *et al.* 2000). Estuaries, which have historically acted as centers for industrial and urban development, are deemed to receive urban and industrial

anthropogenic wastes. Hence, estuarine sediments are an important sink for a wide range of contaminants (Mortimer and Rae 2000). According to the U.S. Environmental Protection Agency, the leading causes of pollution in estuaries are municipal and industrial discharges, urban runoff and atmospheric deposition. Almost one-half of estuarine areas in the U.S. are affected by pollution. Metals are one of leading pollutants in affected estuaries (U.S. EPA 1998) since they show high affinities for fine-grained estuarine sediments (Croudace and Cundy 1995). Many metal compounds are stable, which explains their pervasiveness in the environment (Caussy *et al.* 2003, Robson 2003). Metals are released into the environment by natural processes, such as volcanic activity and erosion; and anthropogenic processes, such as mining, smelting, industrial uses and fossil fuel combustion (Caussy *et al.* 2003). Once metals have been released into the aquatic environment they may interact with fine-grained suspended sediments, and may subsequently be removed from the water column by deposition in mud flats and salt marsh environments (Izquierdo *et al.* 1997, Schlekat *et al.* 2001). The removal may be only temporary since sediments can also act as a source of metals. Following deposition and burial, metals can be subject to a variety of physical, chemical and biological processes that may remobilize and rework metals into the water column. These processes may be natural (e.g. erosion, bioturbation) and/or anthropogenic (e.g. dredging, pH changes, land reclamation) (Lee and Cundy 2001).

II. Fiddler Crabs of the Genus *Uca*

A. Ecology of fiddler crabs:

Fiddler crabs (kingdom: Animalia, phylum: Arthropoda, class: Crustacea, order: Decapoda, family: Ocypodidae and genus *Uca*) are semi-terrestrial and highly social

crustaceans inhabiting intertidal zones of sheltered bays and estuaries. Fiddler crabs are ubiquitous in tropical and temperate estuaries around the world (Doherty 1982). Only three species of fiddler crabs occur in the temperate zone of northeastern United States. They are well adapted to fluctuating water levels, salinities and temperatures of brackish tidal marshes (Grimes *et al.* 1989, Vernberg 1984). Fiddler crabs are highly tolerant to salinity since in estuaries salinity changes from near 30ppt in dry season to around 5ppt during the heights of the rains (Crane 1975). To a certain extent, the crabs can compensate for changes in temperature; they can keep almost the same level of metabolic activity in a temperature range between 18° to 26°C. Above that, their metabolism speeds up rapidly. Below 16°C, their metabolism slows down very rapidly, as crabs quickly go into torpor. In cold seasons, fiddler crabs retreat into their burrows; they have developed the ability to withstand extended periods without oxygen (Teal and Teal 1969). The three species differ slightly. *Uca pugilator* (Bosc) is restricted to a sandy substratum habitat; it is able to sort food from sand and is least tolerant to reduced salinity (Vernberg 1984). *U. pugnax* (Smith) ranges from Massachusetts to northeast Florida; it is found in muddy areas of cordgrass *Spartina* marsh and has feather-like hairs on the mouthparts to handle the mud more efficiently (Teal *et al.* 1969, Vernberg *et al.* 1972). The fiddler crabs *U. pugnax*, like other fiddler crabs, is active at low tide, but live in burrows during high tide; this crab can survive anoxia for 24 hours at 21°C (Vernberg and Vernberg 1972). *U. minax* (LeConte) is the largest of the local fiddler crabs, it exhibits the greatest resistance to reduced salinity, and it penetrates greatest distance up the estuary with some populations living in freshwater (Gray 1942, Miller and Maurer 1973, Thurman 1982).

Fiddler crabs account for the greatest macrofaunal biomass in the intertidal salt marshes. They are not uniformly distributed within their respective salt marshes. Fiddler

crabs are frequent burrowers in temperate western Atlantic marshes. Burrows of adult *U. pugnax* contain standing water, are typically 15-25 cm in depth, and lack chimneys (Grimes *et al.* 1989, Montague 1980). The burrow is the center of a territory defended both for the protection offered and courtship (Oliveira *et al.* 1998). When walking or fleeing back into their burrows, fiddler crabs display a unique style of locomotion; they run sideways (Full and Herreid 1984). Root mat density in salt marsh soils greatly influences the frequency of burrowing by *U. pugnax* (Bertness 1985). In general *Uca* densities decrease from the low to the high marsh areas (Murai *et al.* 1987). Estimates for *U. pugnax* density range from 27 to 152 fiddler crabs per square meter (Montague 1980, Weissburg 1992).

B. Predation on fiddler crabs:

Fiddler crabs are an important intermediate step in the food web of estuaries. Fiddler crabs, which feed on organic detritus of salt marshes, are consumed by a large number of avian predators including egrets, herons and other shore birds (Grimes *et al.* 1989). Blue crabs and channel bass are also common predators of fiddler crabs. In addition, terrestrial mammals, such as raccoons, feed on fiddler crabs (Klein and Lincer 1974). Only few fiddler crab consumers are permanent marsh inhabitants. Thus fiddler crab predation may represent an energy export from salt marshes (Bildstein *et al.* 1989, Montague 1980).

C. Dimorphism in fiddler crabs:

Conspicuous sexual dimorphism is a major feature of fiddler crabs. Males possess one small claw that they use for feeding and grooming and an enlarged claw used for waving (Aicher and Tautz 1990, Salmon 1965), which may be as much as a third of their body weight (Bildstein *et al.* 1989). The claws are symmetrical in young crabs and only

during subsequent development become asymmetric in a random manner i.e. the major cheliped can occur either on the right or left side (Govind *et al.* 1986). Female *Uca* lack the large claw and use both chelae during feeding (Weissburg 1993). Sexual dimorphism may result from natural and/or sexual selection. Darwin (1874) reasoned that intrasexual contests for mates exert, usually on males, producing sexual dimorphism, which is maladaptive with respect to natural selection. This sexually selected male trait enhances male fighting ability and attractiveness to females, but often at the expense of reduced survival. In some species, the enlarged male claw may inhibit rapid escape from predators (Montague 1980) and the added nutritional content of the claw muscle may make males more profitable and hence preferred prey (Koga *et al.* 2001, Rhodes 1986). If a larger than average size of a claw confers a mating advantage on male crabs, the mean expression of the trait will increase until the directional force of sexual selection is balanced by an opposite force of equal magnitude from natural selection (Christy 1991, Lande 1980). Hence, to restore the loss of foraging ability caused by sexual selection for increased major claw size, male fiddler crabs feed longer and can move their single claw faster than the individual claw of a female (Caravello and Cameron 1987, Valiela *et al.* 1974). While feeding, *Uca* crabs extract nutrients attached to sediments. However, because of their more rapid feeding, female *U. pugnax* are extraction-time limited at any food concentration, whereas males are scoop rate limited at low food levels, and extraction-time limited at high food levels (Weissburg 1992). The claw is also used to signal to females, and to threaten or to actually fight with other males over food, mates, and borrow possession (Takeda and Murai 1993). Male-male combat is especially prevalent during the breeding season, and probably used to space out males and

determine which individuals will possess the best territories from which to court females (Christy and Schober 1994, Grimes *et al.* 1989).

D. Foraging behavior of fiddler crabs:

Fiddler crabs of the genus *Uca* live in burrows in the intertidal zone of sandflats and salt marshes. At low tide, crabs leave their burrows to forage on exposed sediments (Grimes *et al.* 1989). Studies showed that these semiterrestrial deposit feeder crabs prefer substrates with high organic content of high-energy value (Whiting and Moshiri 1974), but they must determine when initiation and continuation of feeding optimize energy intake (Robertson *et al.* 1981). Fiddler crabs are capable of detecting organic content of sediment by means of chemoreceptors located on their minor chelae (Robertson *et al.* 1981). The chemoreceptor neurons in the feeding legs of female *U. pugnax* have lower threshold and greater average sensitivity than in males. This explains the observed sexual differences to feeding stimulants, and suggests that females can orient to more dilute stimuli than males (Weissburg *et al.* 1996, Weissburg and Derby 1995, Weissburg and Zimmer-Faust 1991). Another important factor that determines where fiddler crabs forage is sediment water content. The presence of water in the sediment is important for initial stimulation of these chemoreceptors. Optimal conditions for foraging are found in sediment with high organic content that is fully saturated with water. Salinity, the most variable of the environmental factors, has no effect upon foraging behavior (Reinsel and Rittschof 1995). Consequently, spatial patchiness of the foraging environment of *Uca* crabs is mainly due to sediment organic and water contents.

Fiddler crabs feed by scraping the surface of the substratum with the minor chelae. Among the particles being scooped up are detritus, algae and bacteria. The marsh cordgrass *Spartina* is an important sort of detritus; it is the main primary producer in

Atlantic salt marshes (Weis and Weis 2000). Miller (1961) extensively studied the feeding mechanism of *Uca* crabs. Sorting of food material from the coarse mineral fraction first occurs in the mouthparts as the material is moistened by respiratory water. After flooding the buccal cavity with respiratory water, some water is returned to the gill cavities, some is lost as food is ingested, and some is lost by evaporation while the mouthparts are exposed to the air. Thus, to continue feeding, the crab must have access to an external supply of water in order to replenish its respiratory water. This factor is important in limiting the areas where crabs can feed, and is reflected by the moist conditions of the material from which they prefer to feed. The need of water as an integral part of the feeding mechanism is one major factor preventing *Uca pugnax* crabs from living in a terrestrial habitat. At low tide, a marsh-inhabiting fiddler crab can readily replenish its respiratory water from small pools of water in the intertidal areas by lowering the thorax into the water. For *U. pugnax*, however, it was observed these pools are insufficient to replenish its respiratory water and additional burrow water is needed. This relationship between the burrow and a source of respiratory water may be a key factor contributing to the burrow-centered territoriality observed in *U. pugnax* (Miller 1961).

E. Factors affecting fight outcome between crabs:

In species living in social groups, aggression among individuals to gain access to limiting resources, such as food, can lead to the formation of stable social hierarchies (Whiteman and Cöte 2004). Dominant individuals monopolize a disproportionate share of the available resources (Lahti and Lower 2000). This can have significant consequences on foraging rate (Lahti and Lower 2000, Whiteman and Cöte 2004), individual growth (Forrester 1990), risk of predation (Grand and Dill 1999) and mating

success (Kroon *et al.* 2000) of individuals. Two hypotheses are commonly proposed to explain the establishment of dominance hierarchies. The first suggests that dominance is determined by the attributes (e.g. body size, physical strength and level of aggression) of individuals (Drews 1993, Lohrer and Whitlatch 2002). Dominance can, alternatively, result from previous social interactions (Hsu and Wolf 1999). For instance, Huntingford *et al.* (1995) studied the dyadic antagonistic interactions in two species of swimming crabs (*Liocarcinus depurator* and *Necora puber*). In both species, most fights were won by the larger crabs, but the outcome of previous fight had no significant effect on the result of a second fight staged immediately afterwards.

In fiddler crabs *Uca pugnax*, Hyatt and Salmon (1978) found that size often determines the outcome of dyadic fights and that combat duration is not related to environmental factors such as temperature, time of the day or tide cycle. Field observations of *Uca* crabs showed that the smaller the size difference between males, the longer the encounter (or interlocking of claws) lasted (Jennions and Backwell 1996). The length of the major cheliped (weapon size) was significantly correlated with carapace width (the use of major claw length or carapace width as a measure of size produced similar results), while handedness of opponents has no effect on fight results (no difference in the outcome of encounters between opposite-handed males and same-handed males) (Jennions and Backwell 1996).

F. Osmoregulation in fiddler crabs:

Since changing salinities are characteristics of estuaries, it is not surprising that estuarine animals exhibit a wide diversity of metabolic responses to variable salinities. Exposure to low salinity waters generally causes increased oxygen consumption in intertidal animals originally maintained at higher salinities. This type of response has

been reported in crabs of genus *Uca* (Vernberg and Vernberg 1972). Observed changes in metabolic rate may reflect behavioral changes since a change in salinity may alter the locomotor activity of an organism. It was also suggested that change in oxygen consumption with salinity might reflect the influence of hydration on the hormonal and/or enzymatic activities within the cells (Henry *et al.* 2002, Vernberg and Vernberg 1972).

The fiddler crabs have developed water salt control systems so that they not only can live in diluted seawater and still maintain their internal environment constant, but they can also regulate their body fluids when in contact with concentrated seawater (Teal and Teal 1969). They are excellent hyperosmotic regulators in dilute media (salinity of 1-5ppt) and can hypoosmoregulate well in concentrated media (salinity of 40-60ppt). Sodium and chloride ions are the major osmotic effectors in crab hemolymph, which are regulated by Na^+/K^+ -ATPase enzyme (Holliday 1990). These ions must be actively absorbed into the hemolymph of *Uca* in dilute media and actively excreted in concentrated media; the gills are presumably the sites of transport in both cases (Baldwin and Kirschner 1976). Fiddler crabs also drink at a much greater rate in concentrated media than in dilute media. Studies showed a rapid (<3h) increase in the gill enzymatic activity in the osmoregulating fiddler crabs when abruptly transferred to dilute media (Holliday 1985).

Changes in salinity can affect the bioavailability and uptake of metals by euryhaline invertebrates (Caruso *et al.* 2003, Peakall and Burger 2003). In many cases, salinity-related effects on metal uptake can be explained by changes in chemical speciation, but salinity may also influence uptake indirectly through its action on osmoregulatory mechanisms (Lawson *et al.* 1995, Lucu 1993, Roast *et al.* 2002, Towle 1993). Although most studies demonstrate the effect of a single environmental parameter

(e.g. salinity) on organisms, one should keep in mind that the environment is the sum total of interactions of many separate factors (e.g. pH, temperature, salinity, metal interactions, complexation and chelation). These factors may act synergistically on organisms to produce an effect that is more extreme than that which results when studying each factor independently (Baker *et al.* 2003, Peakall and Burger 2003, Schlekat *et al.* 2001, Vernberg 1975).

G. Fiddler crabs as bioindicators:

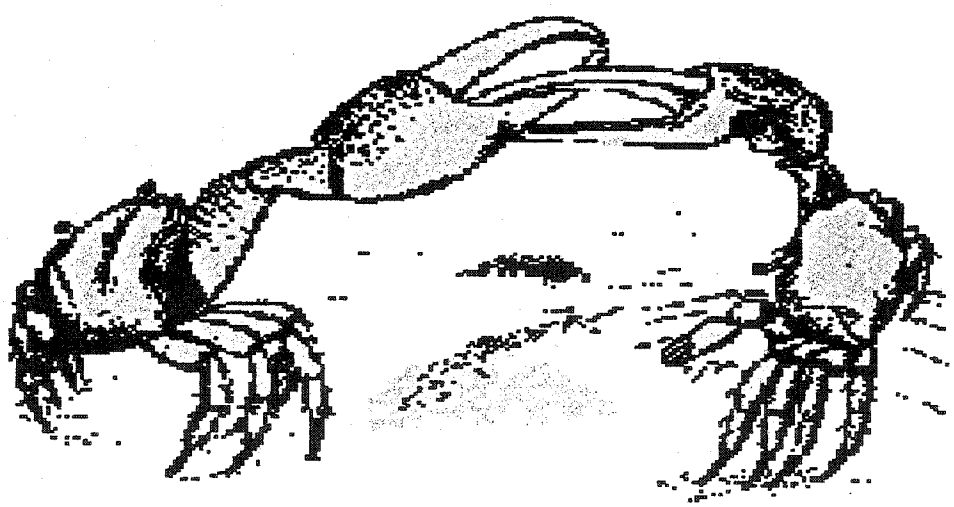
Indicator organisms are used to provide early warning signals of environmental hazards, including the presence of pollutants like heavy metals and organic contaminants (Chu and Chow 2002, Peakall and Burger 2003). These organisms are used as biomonitoring tools to assess the health of ecosystems (Burger *et al.* 2003). Features that are usually considered in selecting a bioindicator species include the species' widespread persistence in the system being examined, its sensitivity to change, acceptable methods for its assessment, characteristics of the ecosystem, and economic feasibility (Burger 1994, Ramade 1987). Within the same species, however, individual organisms show variations depending on breeding cycle, age, sex, size, weight and nutritional status. These variations must be considered in selecting test organisms (Peakall and Burger 2003).

Animals selected for this research are the Western Atlantic fiddler crabs *U. pugnax*. The specific name of this species refers to the fighting nature of the males, which are territorial especially during breeding season (Teal and Teal 1969). These animals were selected because (1) they are abundant intertidal zone species and are easily collected, (2) their systematics are well understood, (3) they are hardy animals and

survive well in the laboratory with minimal care (Hyatt and Salmon 1979), and (4) they readily show aggression (see Figure 1 below).

The extensive literature describing normal behavior in this species provides a basis for identifying abnormal behavior (Klein and Lincer 1974). These crabs can be used as reference species in the monitoring of biological water quality. They easily accumulate pollutants, playing the role of 'sentinel' organisms (Ramade 1987). In field combat studies, crabs can visually assess the fighting ability of opponents and are free to select opponents before engaging in potentially costly fights (Jennions and Backwell 1996). The advantage of laboratory behavioral study is that it eliminates the advantage of allowing males to choose opponents by visual assessment.

Figure 1: An illustration depicting two male fiddler crabs with interlocking major claws. These crabs were chosen in this study because they easily show aggression in the laboratory



III. Metal Uptake and Detoxification in Decapod Crustaceans

A. Metal uptake in decapods:

In aquatic organisms, uptake of metals from water can take place either by diffusion through the general body surface or more particularly through the gills (Roesijadi and Robinson 1992). Studies on the kinetics of metal uptake by aquatic organisms indicate that this is a two-phase process composed of a rapid adsorption to the surface and a slow penetration into the organism (Siriwardena *et al.* 1995). Transport of metals to the interior of cells may occur either by active transport or by diffusion of ions across epithelial membranes. Active transport of metals is carried through ion pumps that have overlapping specificities and most do not transport a single metal species but rather are able to transport several metals with differing affinities (Baker *et al.* 2003). In contrast, passive diffusion is not a major route of entry for most metals (Baker *et al.* 2003).

To limit uptake, some organisms use extracellular barriers that limit binding of the metal to transport sites. In decapod crustaceans, for instance, carapaces help to limit uptake by either adsorbing the metals to their chitin-based exoskeleton or by reducing the surface area of exposed epithelium available for metal transport. Chitin, a natural polymer present in crab exoskeleton, is an important storage site for metals in addition to hepatopancreas and gills. It is an effective ion-exchanger with high adsorption capacity for metals such as Cu, Cd, Zn, As, Pb, Fe, and Ag. It is used commercially as a metal adsorbent in the treatment of industrial wastewaters (Benguella and Benaissa 2002, Evans *et al.* 2002). While the adsorption of metals to the chitinous exoskeleton of crustaceans may contribute to the overall metal body burden, it has no toxic effect on these organisms (Hook and Fisher 2001). The exoskeleton is essentially impermeable, and appears to be

used as an adaptive strategy to confer metal tolerance. Since the majority of ion exchange with the medium is limited to specialized areas, such as the gills, the control of ion fluxes can be highly regulated (Ray and McLeese 1987). This mechanism is particularly important in estuarine and brackish water crustaceans that have adapted to fluctuating salinities.

B. Intracellular handling of metals:

1. Intracellular homeostasis:

The occurrence of adverse toxic effects from metal exposure is dependent not only on the concentration of bioavailable metal in the environment but also on the organism's ability to handle and maintain intracellular homeostasis (Cosson 1994, Luk *et al.* 2003). Since many metals are essential for all organisms in trace amounts but toxic in excess, cells have developed homeostatic mechanisms that reduce or prevent a metal-induced toxic effect by blocking metals from binding with essential biomolecules (Baker *et al.* 2003, Peakall and Burger 2003); metals can be sequestered by cellular ligands that act as important 'sinks' for both essential and nonessential metals. Two important cellular ligands have been identified; they include high affinity metal-binding proteins (e.g. metallothioneins) and mineralized organic-based granules. Without showing any direct physiological effect, these intracellular ligands can bind metals and effectively immobilize them before they can disrupt cellular activities (Roesijadi and Robinson 1992).

2. Metallothioneins (MT):

Metallothioneins (MT) are a class of soluble cytoplasmic sulfhydral-rich, heat stable, low molecular weight proteins. MT plays a central role in the regulation and homeostasis of the essential metals such as Cu and Zn, and in the detoxification of

nonessential metals such as Cd and Ag (Baker *et al.* 2003, Hogstrand *et al.* 1996, Park *et al.* 2001, Ratte 1999, Russell and Kramer 1999, Temara *et al.* 1997). MT was originally isolated from equine kidney cortex of horses (Kägi and Vallee 1960), and chemically similar proteins have been identified in a variety of aquatic species such as mammals, amphibians, fish and many invertebrates including crabs (Roesijadi 1992, Roesijadi 1994). Typically, these proteins lack aromatic amino acids and approximately thirty percent of the residues is sulfur-rich cysteine, which accounts for the high capacity to bind a variety of metals including Cd, Zn, Cu, Ag and Hg (Cosson 1994, Sanders *et al.* 1996). MT has two cluster domains (see Figure 2), although, the exact coordination and the number of metal complexed varies with metals and tissue of origin (Hamer 1986, Roesijadi *et al.* 1989). The order of binding affinity between the cysteine of MT and various metals (high-to-low: Hg, Ag, Cu, Cd and Zn) reflects the relative strengths of the metal-thiolate bond (Cosson 1994, High *et al.* 1997, Sanders *et al.* 1996).

The role of MT proteins in sequestering metals is well established, and their induction by metal exposure is associated with conferring protection against metal toxicity (Roesijadi and Fellingham 1987). This prior induction may result in the accumulation of an increased amount of preexisting MT proteins, which is then available for binding potentially toxic metals in subsequent exposure (Park *et al.* 2001). Studies on the regulation of MT gene expression have established that induction by metals is a direct response to increases in the intracellular metal concentration (Brouwer *et al.* 1993, High *et al.* 1997, McNamara and Buckley 1994, Serafim and Bebianno 2001). Research shows that exposure of aquatic organisms to some metals enhances tolerance to metal toxicity through increased copies of the MT gene (Roesijadi 1994, Sanders *et al.* 1996). This amplification of the MT gene has evolutionary significance since amplified genes can be

stably transmitted over generations. The capacity of MT induction is greater in tissues that are active in metal uptake, storage and excretion (Domouhtsidou *et al.* 2004, Roesijadi 1992). Induction can shift the kinetics of metal binding to favor binding to MT and reduce, but not eliminate, the rates of binding to other cellular structures (Roesijadi 1992).

MT and Metallothionein-like proteins (MTLP) have been isolated from numerous tissues including liver, kidney, lung, spleen and intestine of vertebrates and digestive gland, gill and kidney in mollusks and crustaceans (Barka *et al.* 2001, Gillis *et al.* 2004, Roesijadi 1981, Roesijadi and Robinson 1992). MTLP are heat stable, low molecular weight proteins that binds metals (Hamza-Chaffai *et al.* 1995, High *et al.* 1997). Their molecular weights, metal contents, and induction by metals such as Cd, Zn, Cu, and Hg suggest close similarities to MT (Roesijadi 1981). MT isolated by ultracentrifugation of heat-treated cytosol (Hamza-Chaffai 1995, Wallace and Lopez 1997). Purification and sequencing of isolated MT showed that the isolated heat-treated cytosol also contained proteins with MT properties i.e. MTLP (Legras *et al.* 2000).

Increased levels of MT appear to be characteristic of exposure to Hg, Ag, Cd, Cu, and Zn in aquatic animals (Cosson 1994). Levels of MT and metals bound to MT can be used as an indicator of metal exposure (Domouhtsidou *et al.* 2004, Hogstrand *et al.* 1996, Roesijadi and Robinson 1992). Therefore, these proteins have been proposed as biomarkers to assess metal contamination in the environment (Depledge *et al.* 1995, High *et al.* 1997, Oost *et al.* 2003, Serafim and Bebianno 2001). This induction confers enhanced metal tolerance to both cells and individuals, and, coupled with the relatively long turnover time for MT-bound metals, higher burden of metals can be accumulated and tolerated by aquatic animals. A possible consequence of this increased capacity for

sequestration via MT is an increase in the potential for trophic transfer of metals (Wallace and Lopez 1997, Wallace *et al.* 1998).

Studies show that MT protein plays two interrelated roles. First, it plays an essential role in the compartmentalization and in sequestering of essential metals such as Zn and Cu when their concentrations exceed cellular requirements (Laws 1993). In doing so, it limits the nonspecific binding of these metals to metabolically important proteins (Laws 1993). Once sequestered, these metals can be released as required by metallo-enzymes. Second, MT is responsible of the detoxification of nonessential metals, which otherwise can bind nonspecifically to various proteins within the cell (Sanders *et al.* 1996, Serafim and Bebianno 2001). However, excessive exposure of MT to metals can compromise the ability of MT to sequester additional amounts of a toxic metals, and a "spillover" of metals from MT to high molecular weight cystolic protein and various organelles can occur, thereby causing toxic effects (High *et al.* 1997, Roesijadi 1992, Wallace and Lopez 1996). Hence, the rates of MT synthesis and metal binding to MT can become limiting as exposure concentrations increase and result in increased binding of metals to other subcellular structures (Laws 1993, Luoma *et al.* 1995).

3. Intracellular granules:

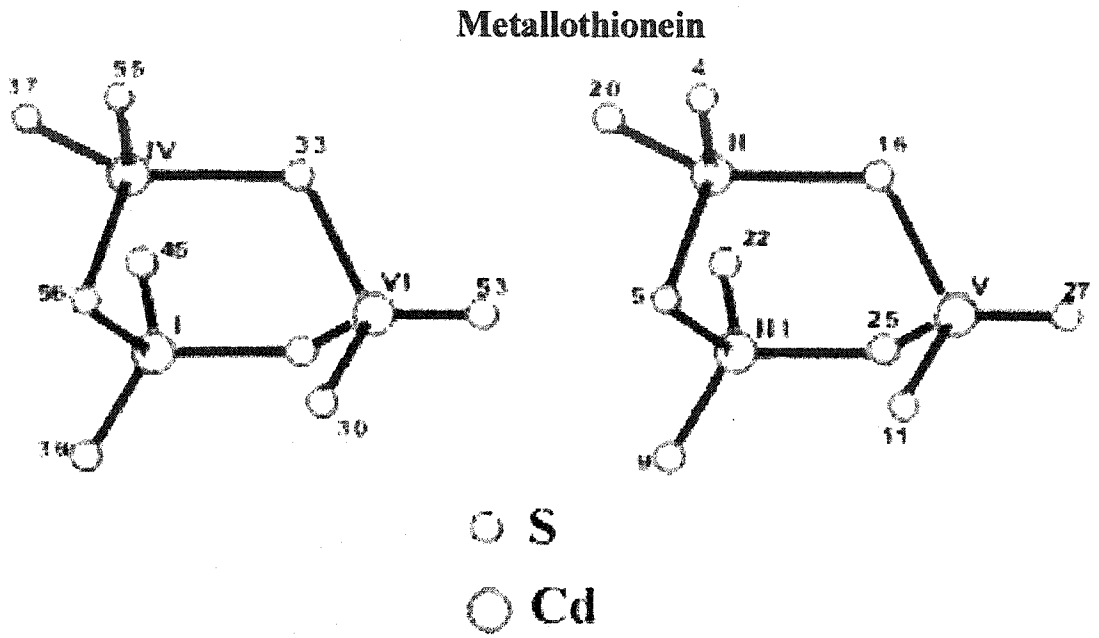
All aquatic organisms contain a wide variety of membrane-bound intracellular 'granules', many of which bind metals. A number of studies have shown that metals such as Ag, Al, B, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, and etc. can be sequestered by these subcellular particles (Roesijadi 1994). The deposition of metals in these sulfur-rich granules, which are probably breakdown products of metallothioneins, has been used as evidence for their function in metal detoxification (Peakall and Burger 2003). Although these granules show a high degree of variation in form and composition, they are all

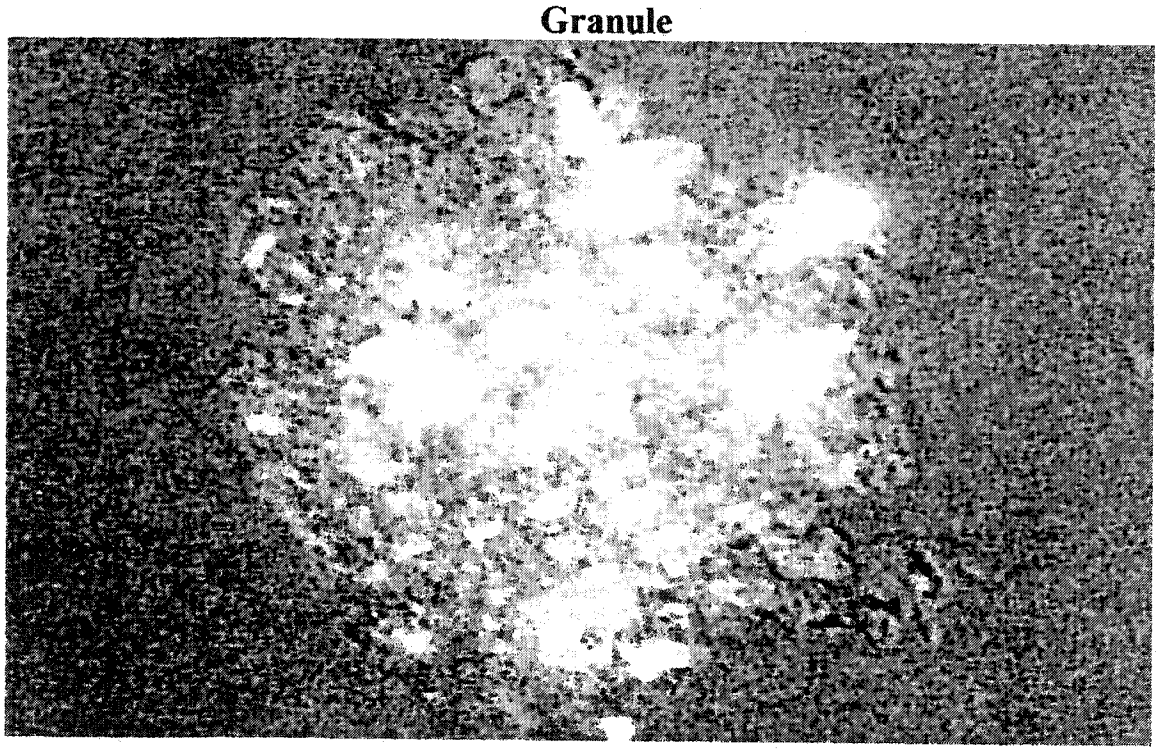
metal-rich inclusions containing a high inorganic mineral component (see Figure 2). Most of these particles also contain organic components although, in general, the organic content of these granules is relatively low (George 1982). They are highly insoluble, they irreversibly sequester a number of metals (Peakall and Burger 2003), and they tend to be of greater density and hardness than the surrounding cellular structures and can be isolated by centrifugation (Klerks and Bartholomew 1991, Wallace *et al.* 1998).

Granules are particularly prevalent in invertebrates. They occur in a variety of cellular locations that are species-specific. These subcellular particles are generally associated with the digestive and excretory tissues of invertebrates, but they can also be found within almost every tissue type (Roesijadi and Robinson 1992, Simkiss and Taylor 1994). Based on their inorganic composition, granules can be classified as either Cu-, Fe- or Ca-containing granules. Nonessential metals, such Cd and Ag, have been found in these granules, the sequestration of nonessential metals indicates that these subcellular metal-containing particles are not simply a depository of essential metals. In fact, the ability of granules to bind a variety of metals suggests that they play a role in the accumulation, storage and detoxification of metals (Luoma *et al.* 1995, Pyatt *et al.* 1997).

It is clear that a variety of strategies are available to aquatic organisms to limit the impact of metals and to maintain intracellular homeostasis. These include strategies that limit uptake by binding metals to their exoskeletons, and sequester or compartmentalize metals in MT proteins and intracellular granules. The mechanism and occurrence of these strategies is species-specific (Roesijadi and Robinson 1992). Although it may be difficult to extrapolate these strategies from one species to another, an understanding of these strategies can provide a basis for establishing testable hypotheses as to the specific mechanisms of metal detoxification in a given aquatic species.

Figure 2: Metallothionein structure (top) showing its two cluster domains (Otvos *et al.* 1982), and granule (bottom) from debris fraction of *Limnodrilus hoffmeisteri* (Wallace *et al.* 1998)





IV. Objective

A. Rationale:

This is a multi-faceted research project aimed at examining the behavioral and subcellular toxicities of two toxicologically important waterborne metals, Cd and Ag, on the Atlantic salt marsh crab *U. pugnax*. To account for the extreme salinity change in estuarine environment, *Uca* crabs were exposed to dissolved metal at two different salinity regimes. Estuarine organisms are rarely exposed to one metal at a time in their natural environment; the effect of exposure to mixtures of these two metals on the behavior of *Uca* crabs was also investigated.

The toxicity of exposure in water to a range of concentrations of each metal on foraging and dominance behaviors of *Uca* crabs was examined under two different salinity regimes (5ppt and 30ppt). To better understand the toxic mechanism of each metal, subcellular fractions of *Uca* crabs were isolated, and then analyzed for metal content. Specifically, the following fractions were isolated: enzymes, organelles, MT proteins and insoluble fraction (intracellular granules and adsorbed metal). Total body metal content was also determined. The enzymes and organelles are considered the metal-sensitive fractions, whereas MT proteins and insoluble fractions contain the biologically detoxified metal (Wallace *et al.* 2003). It is suspected that there may be a relationship between behavioral toxicity in *Uca* crabs and binding of metal to the subcellular metal-sensitive fractions, as well as between metal bioaccumulation in various fractions and salinity of the exposure media.

The interaction of Cd and Ag in *Uca* crabs was also investigated. The effect of exposure to mixtures of these metals on foraging and dominance behaviors was investigated, and their bioaccumulation in various subcellular fractions was determined.

Literature review showed limited work on the interaction of Cd and Ag. For instance, Voyer *et al.* (1982) studied the influence of dissolved Ag on Cd toxicity. When tested in combination with Cd, Ag reduced the toxicity of Cd on the embryos of winter flounder. It is known, however, that Both Cd and Ag are nonessential to animal metabolism, they have similar atomic size and they are more toxic in their ionic forms. Although intracellular MT proteins have higher affinity for Ag (Sanders *et al.* 1996), and aquatic organisms tend to accumulate Ag more efficiently (Berry *et al.* 1999, Wang *et al.* 1996), it is not expected that Ag will reduce the toxicity of Cd in adult *Uca* crabs.

A field study was carried to verify if *Uca* crabs from a polluted site behave differently than those collected from a cleaner site. First, foraging and dominance behavior of crabs from both sites was investigated. Then, metal contents of various subcellular fractions of 'impacted' and 'reference' crabs were compared. The polluted site chosen for *Uca* crabs collection is Meredith Creek marsh (Figure 3). This creek is connected to the Arthur Kill that is a part of New York-New Jersey Harbor estuary. The Harbor is one of the most polluted estuaries in the world; it is surrounded by a population of more than 20 million people and concentrated refining industries and wastewater treatment facilities (N.Y.C. DEP 1996, 1997). The Arthur Kill itself is surrounded by a dense urban population, heavy industries and major shipping lanes. Its sediments are contaminated with an array of metals and organic pollutants, which moderately-to-highly impact benthic organisms (U.S. EPA 1995, 1998). The reference site is Rutgers' Marine Research station in Tuckerton located in a natural reserve in southern New Jersey.

B. Hypotheses:

Studies have shown that the exposure of estuarine organisms to metals can negatively impact their behavior (Cheung *et al.* 2002, Scherer *et al.* 1997, Sloman *et al.*

2003, Smith and Weis 1997, Wallace *et al.* 2000). In this study, it is hypothesized that the exposure of dominant *Uca* crabs to Cd and Ag (individually or in mixture) will affect their foraging behavior and dominance status. Since both Cd and Ag are non-essential metals with high affinities for -S, it is unlikely that their effects on the behavior of *Uca* crabs will be antagonistic. It is hypothesized that the effect of exposure to a mixture of Cd and Ag on *Uca* crab behavior may be either additive or synergistic.

In crustaceans, metal uptake from water occurs mainly through the gills (Silvestre *et al.* 2004). As salinity increases, metal uptake decreases due to the formation of less available chloro complexes (De Wolf *et al.* 2004, Peakall and Burger 2003, Roast *et al.* 2001). Hence, in this study, it is hypothesized that accumulation of Cd and Ag in fiddler crabs may decrease as salinity increases from 5ppt to 30ppt.

Metal accumulation by estuarine invertebrates results in their accumulation and distribution in various subcellular components (e.g. organelles, enzymes, MT and insoluble fractions) of exposed animals (Selck and Forbes 2004, Wallace *et al.* 2003). Enzymes are very sensitive to metals; both Cd and Ag ions showed inhibitory action against enzymes in euryhaline crabs (Skaggs and Henry 2002). It is hypothesized therefore that there may be a relationship between behavioral changes and metal (Cd and Ag) accumulation in enzymes and/or organelles of exposed *Uca* crabs (Wallace *et al.* 2000).

A field investigation was conducted to determine if exposure to environmental contaminants under natural setting alters the behavior of *Uca* crabs. It is hypothesized that *Uca* crabs collected from a relatively polluted site may forage less than those collected from a relatively pristine site. For instance, Perez and Wallace (2004) showed that grass shrimps, *Palaemonetes pugio*, collected from a creek adjacent to landfills

exhibited a prey capture rate that was about two times lower than that of shrimps collected from a creek located farther away from the landfills. Weis *et al.* (2001) showed that killifish, *Fundulus heteroclitus*, originating from a contaminated site are slow to capture prey and to escape predators. Study conducted by Wallace *et al.* 2000 revealed that grass shrimps, *Palaemonetes pugio*, fed Cd-exposed *Artemia salina* exhibited significant reductions in their ability to capture live prey. Most importantly, the study showed that successful prey capture by grass shrimps decreased with increased body burdens and increased Cd concentration bound to enzymes. Smith and Weis (1997) showed that mummichogs, *Fundulus heteroclitus*, collected from an uncontaminated environment captured prey at a faster rate and had lower level of mercury in their tissues than those collected from a mercury-polluted creek.

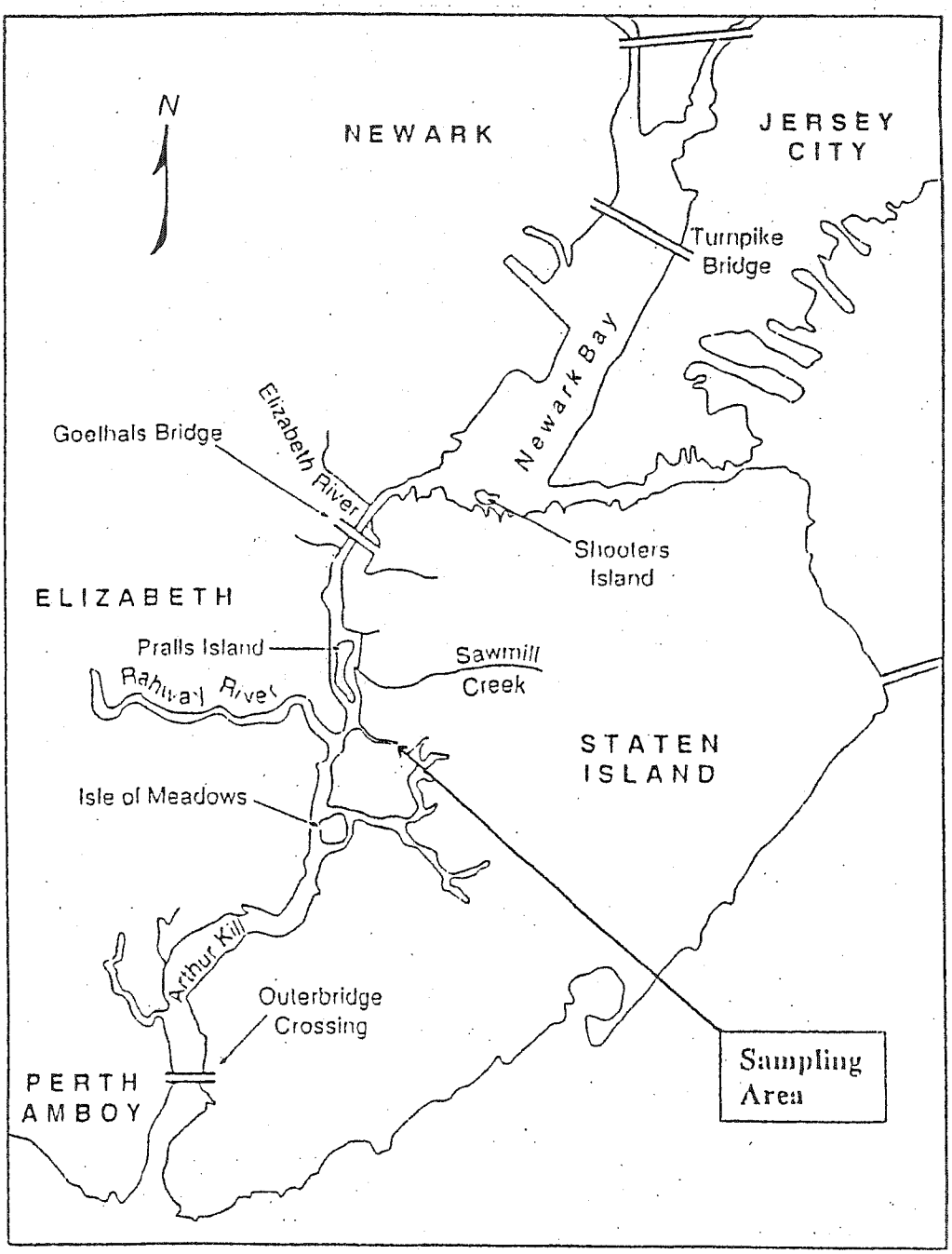
V. Methodology

A. Field collection and laboratory holding of *Uca* crabs:

Male fiddler crabs *U. pugnax* 18-20 mm in carapace width (Figure 4) were collected in the summer (July-August) of 2001 from Meredith Creek marsh on Staten Island, NY (Figure 3). Once in the lab, crabs were housed in (40 + 90 + 30 : w + l + h) cm tanks at a density of ~100/m^2, density equivalent to that observed in the field (Montague 1980). Tanks were maintained at 24°C with a light: dark cycle of 12:12 hours.

Half the bottom of each tank was filled with 15-20 cm of mud collected from the banks of Meredith Creek marsh; the rest of the tank was filled with Meredith Creek water (salinity ~15ppt). Water level was maintained at a level below the surface of the mud. Water within this terrarium was filtered with an aquarium pump positioned on the water end of each tank. This setting allowed the crabs an access to both aquatic and terrestrial

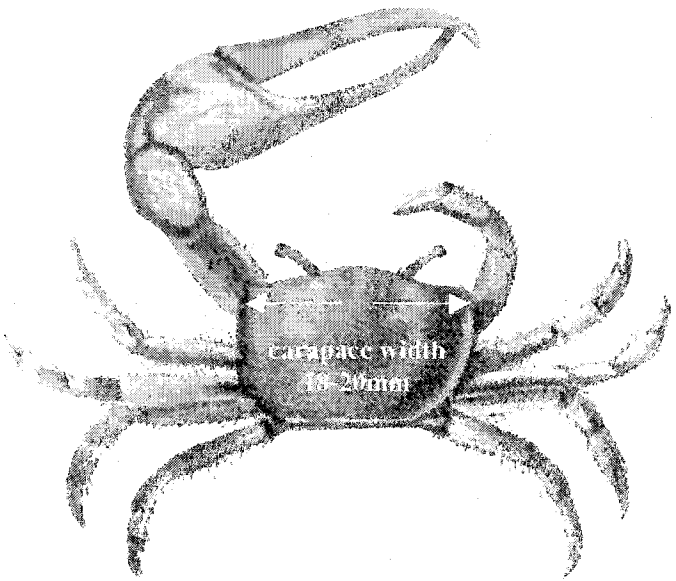
Figure 3: Map showing the location of Meredith marsh sampling site



Adapted from "Before and After an Oil Spill: The Arthur Kill", 1994

environments. Fiddler crabs readily constructed burrows and fed on the mud surface that was artificially flooded on a weekly basis. Crabs were held in the laboratory awaiting use in behavioral studies.

Figure 4: A top view of male fiddler crab showing the carapace width. It is generally accepted that carapace width in fiddler crabs correlates with the size of major claw



B. Behavioral studies:

Fiddler crabs were used in various treatments to assess the individual and combined effects of Cd and Ag with changing salinity. A total of 24 treatments were conducted, and each included assessment of various behavioral parameters prior to metal exposure (Trial 1 - no metal exposure), followed by exposure to metals and subsequent re-assessment of behavior (Trial 2 - after exposure to metal). The exposure conditions of each treatment are described in Table 2. All behavioral studies were conducted in a temperature-controlled room at 24°C.

For each treatment, crabs (n=16) of similar carapace width were removed from tanks, and were placed individually in labeled plastic cups with mesh bottoms that were placed in a fish tank filled with filtered water (salinity ~ 15ppt). Crabs were allowed to depurate for 48 hours. At the end of depuration period, each crab was placed in a 250 ml acid-washed Erlenmeyer flask containing 100 ml of Nano® pure water of corresponding salinity for a 72-hour period (Instant Ocean™ salt was used to prepare solutions). This holding was included in Trial 1 to mimic the conditions during exposure to metals in Trial 2. Crabs were then paired at random and were placed in a 15 x 20 cm opaque plastic container containing a ~1 cm layer of Meredith marsh mud (a shallow layer of mud was used to prevent crabs from burrowing). In the center of mud, a 1.5 cm diameter protein-rich patch (of crushed Freeze Dried Plankton™ containing Euphausiids with a minimum of 69% crude protein and 13% crude fiber) was placed on mud surface for the crabs to compete for. While in the container, each pair of crabs was videotaped by a JVC Digital Signal Processing camcorder for one hour (Figure 5). Tapes were immediately reviewed and data on competitive foraging behavior (total number of scoops and number of scoops on protein-rich patch), and on competitive dominance behavior (time in control of the patch and aggressiveness/ number of successful attacks) of each crab were collected. When viewing video footage, it became obvious that one crab from each pair was dominant and the other was submissive. Dominance was assigned to the most aggressive crabs (i.e. the crabs that had most successful attacks are here referred to as dominant, those crabs with less or no successful attacks are referred to as submissive). At the end of the videotaping session, each crab was allowed to feed individually on fresh Meredith marsh mud in a labeled plastic container of 20 cm in diameter for 48 hours. This feeding is referred to de-acclimation.

Table 2: List of treatments investigating the individual and combined effects of Cd, Ag and salinity on behavior of fiddler crabs

Treatment #	Metal(s)	Concentration (mgl^{-1})	Molarity ($\mu molel^{-1}$)	Salinity (ppt)
1	Blank	-	-	5
2	Blank	-	-	30
3	Cd	0.5	4.45	5
4	Cd	0.5	4.45	30
5	Cd	2.5	22.3	5
6	Cd	2.5	22.3	30
7	Cd	5.0	44.5	5
8	Cd	5.0	44.5	30
9	Cd	10.0	88.9	5
10	Cd	10.0	88.9	30
11	Ag	0.10	0.93	5
12	Ag	0.48	4.45	5
13	Ag	0.48	4.45	30
14	Ag	2.4	22.3	5
15	Ag	2.4	22.3	30
16	Ag	4.8	44.5	5
17	Ag	4.8	44.5	30
18	Ag	9.6	88.9	30
19	Ag	16.0	148.0	30
20	Cd, Ag	0.5, 0.48	4.45	5
21	Cd, Ag	0.5, 0.48	4.45	30
22	Cd, Ag	2.4, 2.5	22.3	5
23	Cd, Ag	2.4, 2.5	22.3	30
24	Tk vs. Mer	-	-	-

In Trial 2, crabs were transferred to labeled depuration chambers as before to allow the evacuation of gut contents. This second depuration period also lasted 48 hours. Dominant crabs were exposed in flasks containing 100ml solution at the corresponding metal(s) concentration –if any- and salinity (see Table 2). Metal solutions were prepared using Perkin Elmer ultra pure metal solution. Submissive crabs were placed in similar flasks, each containing 100 ml of metal-free solution to mimic exposure. Exposure lasted 72 hours. Subsequent to exposure, crabs were then re-paired as in Trial 1 and were videotaped for a second one-hour period (see Table 3). Tapes were reviewed and behavioral data were again collected. At the end of Trial 2, crabs were depurated for 48 hours. Subsequently, crabs were stored individually in zip lock bags at -80°C.

Before starting metal exposure treatments, two blank treatments (Treatment #1 and #2) were conducted in order to verify that salinity and experiment duration (Trial 1 vs. Trial 2) do not affect the behavior of fiddler crabs. These were blank treatments conducted at 5ppt and 30ppt salinity. Blank treatments followed the same protocol as the rest of the treatments with one exception: dominant *Uca* crabs were not exposed to any metal in Trial 2, i.e., in the two blank treatments, Trial 2 was conducted exactly the same way as Trial 1.

Another blank treatment was conducted to verify whether the presence of the protein-rich patch affects the foraging behavior and aggressiveness of *Uca* crabs. The treatment also had Trial 1 and Trial 2; crabs were not exposed to metal in either trial. This blank treatment was conducted in a way as to correct for any change in crabs behavior that may arise as switching from Trial 1 to Trial 2. For instance, in Trial 1, eight pairs of crabs with similar carapace widths were allowed to depurate for 72 hours. At the end of depuration period, pairs 1 to 4 were allowed to feed, one pair at a time, for one

Figure 5: A drawing depicting the container used for video surveillance. Two crabs were placed covered with mud with a protein-rich patch in the center

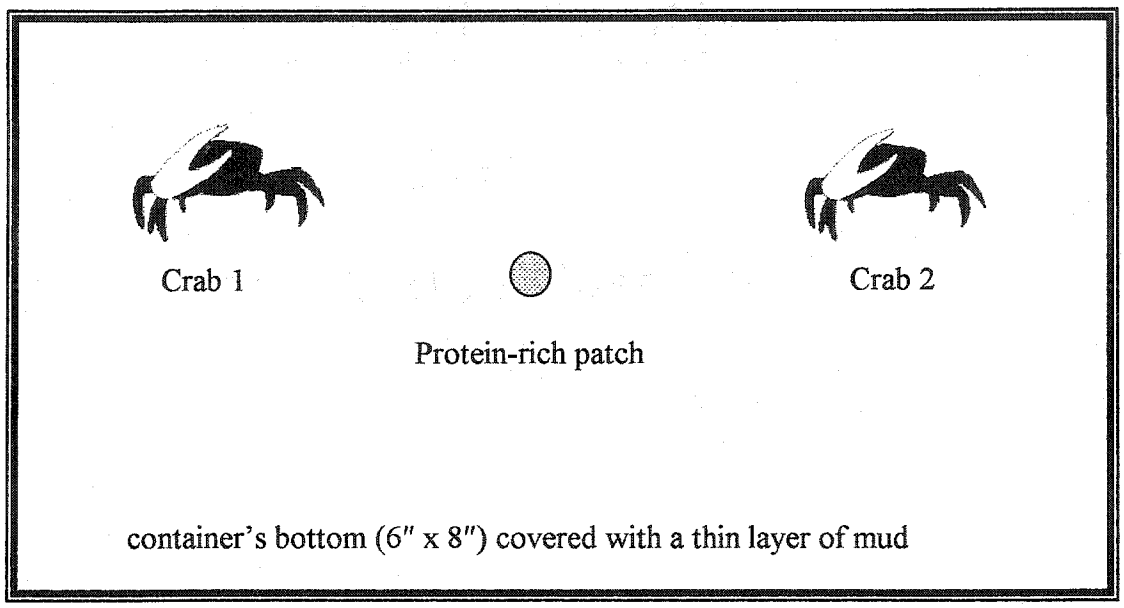


Table 3: A flow chart showing the major steps in Trial 1 and Trial 2 in behavioral studies

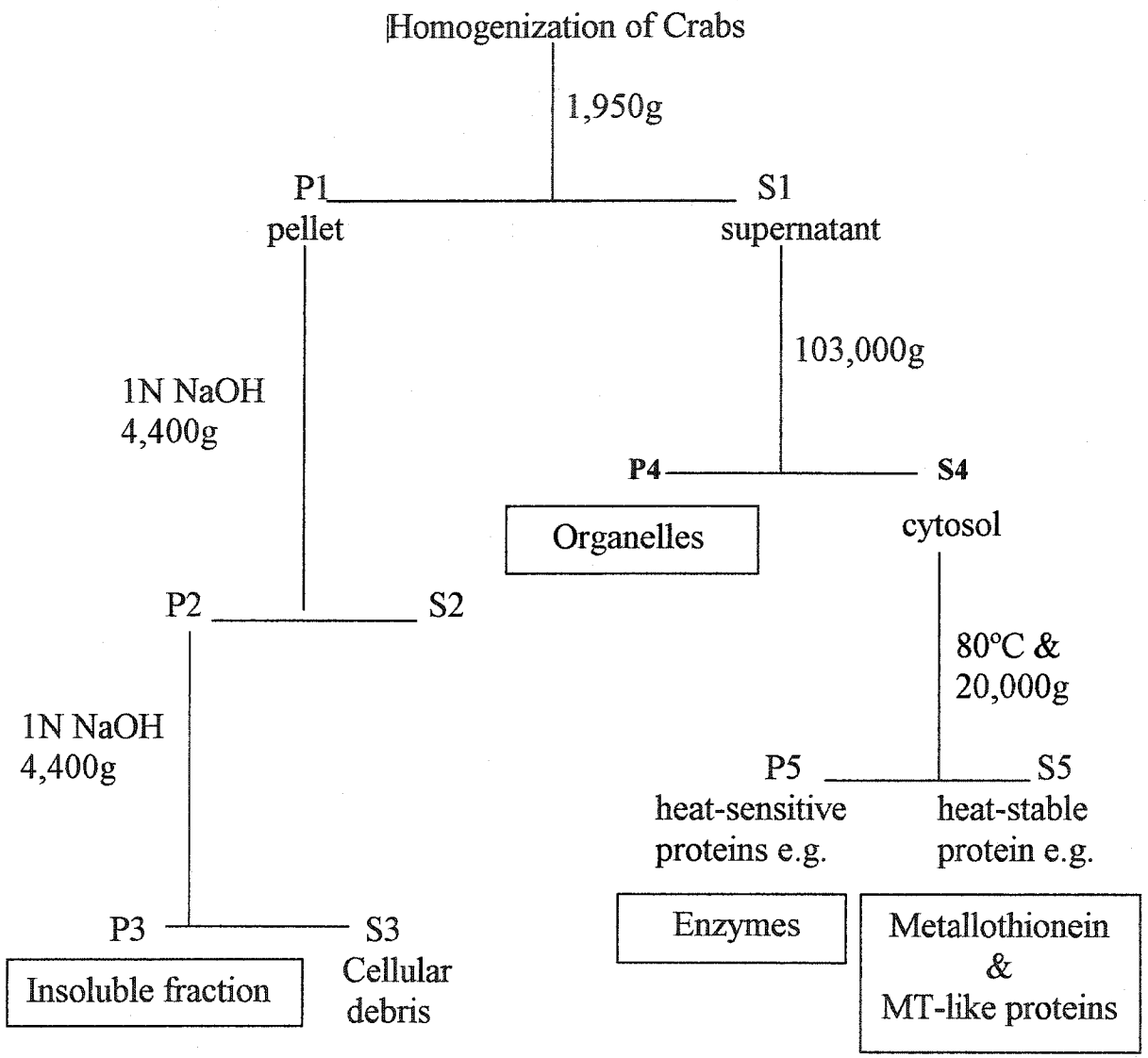
Trial 1	Duration	Trial 2	Duration
Depuration	48 hours	Depuration	48 hours
↓		↓	
Pre-exposure	72 hours	Exposure	72 hours
↓		↓	
Videotaping	1 hour	Videotaping	1 hour
↓		↓	
De-acclimation	48 hours	Depuration	48 hours

hour in a mud container; no protein-rich patch was used, whereas, pairs 5 to 8 were allowed to feed on mud, one pair at a time, for one hour in a container with protein-rich patch placed at the center. Crabs were videotaped while feeding, tapes were reviewed and behavioral data were collected. In Trial 2, however, pairs 1 to 4 were allowed to feed on mud containing protein-rich patch at the center while pairs 5 to 8 were fed on mud with no protein patch. Crabs were videotaped, and all tapes were reviewed for data collection.

C. Fractionation procedure and sample preparation:

Metal-exposed and control crabs were subjected to subcellular fractionation as described by Wallace *et al.* 2003 (Figure 6). Briefly, crabs (four dominant and four submissive from each treatment) were thawed on ice. Then, each entire crab was placed in a 50 ml Nalgene® centrifuge tube containing 5.8 ml of 20 mM Tris buffer solution (prepared by adding 3.152g of J.T. Baker Ultrapure Grade TRIS Hydrochloride to 1 liter of Nano® pure water and adjusted to pH of 7.6). Crabs were then homogenized using Polytron® PT 2100 homogenizer. Subsequent to homogenization, a one-milliliter sample was removed from the centrifuge tube and was transferred to a pre-weighed vial for future analysis of total body burden. Homogenized crabs were first centrifuged in a Sorval® (RC 5C Plus model) at 1,950g for 15 minutes. Supernatants (S1) were transferred to a set of centrifuge tubes, which were further centrifuged in a Beckman centrifuge (L8-M Ultracentrifuge model) at 103,000g for one hour. After adding 5.0 ml of Tris buffer to pellets (P1), they were placed in water bath at 100°C for 2 minutes; 5.0 ml of 1N NaOH was added to each tube before being vortexed and placed again in a water bath at 65°C. After one hour, pellets (P1) were removed from water bath; they were vortexed and spun in Sorvall centrifuge at 4,400g for 15 minutes. Supernatants (S2) were

Figure 6: Fractionation Procedure of Fiddler Crabs



Adapted from Wallace *et al.* 1996, 1997.

transferred to pre-weighed vials; these are the cellular debris. Five ml of 1N NaOH was added to pellets (P2), pellets were then vortexed and centrifuged in the Sorvall centrifuge at 4,400g for 15 minutes. Supernatants (S3) were transferred to the same vials containing the cellular debris. Pellets (P3) were transferred to a set of pre-weighed vials after being resuspended in 4 ml of Nano® pure water; this constitutes the insoluble fraction (intracellular granules plus carapace). After centrifuging supernatants (S1), supernatants (S4) were transferred to another set of Beckman centrifuge tubes that were placed in a water bath at 80°C for 10 minutes, then on ice for 45 minutes. Pellets (P4) contain the organelles; they were transferred to a set of pre-weighed vials after being resuspended in 2 ml of Nano® pure water. After supernatants (S4) were cooled on ice, they were centrifuged in the Beckman centrifuge at 20,000g for 30 minutes. Supernatants (S5), which contain MT proteins, were transferred to a set of pre-weighed vials. Pellets (P5) contain enzymes, were transferred to pre-weighed vials after being resuspended in 2 ml of Nano® pure water.

Crab tissues and subcellular fractions were digested for metals following standard protocol. Vials containing subcellular fractions were dried in a drying oven at 65°C, and then they were weighed. Concentrated HNO_3 (5 ml) was then added to each vial. Samples, covered with reflux bulbs, were refluxed until tissues dissolved. Samples were then evaporated to dryness, resuspended in 10 ml of 2% Ultragrade HNO_3 and filtered through .45 μm Millipore filters. Filtered samples were then analyzed for Cd and Ag using Perkin Elmer model 3100 atomic absorption spectrophotometer. Quality Control and Quality Assurance samples included (1) standard tissue samples, (2) fractionation procedural blanks, (3) digestion blanks and (4) filter blanks. During analysis, background

and standards were periodically run to verify the accuracy of the instrument. If background or standard samples did not meet QA/QC the instrument was recalibrated.

D. Statistical analysis:

Behavioral data (comparison of total number of scoops, number of scoops on patch, time in control of protein-rich patch and percent successful attacks) of both dominant and submissive crabs at both salinities were compared using non-parametric test (Mann-Whitney *U*-test, $p<0.05$). This statistical test is used to compare two groups of data. Changes in behavioral parameters were analyzed using another non-parametric test (Kruskal-Wallis ANOVA, $p<0.05$). These two non-parametric tests make no assumptions about the distribution of data. And like many other non-parametric tests, these tests use the median of the data rather than means to calculate the statistic (Bart *et al.* 1998).

Data on bioaccumulation of metals in various subcellular fractions were tested using t-test (unpaired, $p<0.05$). The t-test requires either that data are normally distributed (which is rare in practice) or that the sample size is large enough (which varies according to the distribution of the variable measured). In many cases investigated by behavioral ecologists, sample sizes of 10-15 are sufficiently large enough for the t-test to be valid (Bart *et al.* 1998). All statistical analyses were performed using Statistica 5.0 software.

VI. Results

A. Effects of salinity and holding time on the behavior of fiddler crabs:

The effects of salinity and experiment duration (Trial 1 vs. Trial 2) on foraging behavior (total number of scoops as well as number of scoops on protein-rich patch), and on dominance behavior (ability to control a protein-rich patch as well as aggressiveness/ fighting ability) of dominant and submissive *Uca* crabs, were examined.

To study the effect of salinity and experiment duration on the competitive behaviors of fiddler crabs, two blank treatments (Treatment #1 and #2 at 5ppt and 30ppt, respectively) were conducted with no exposure to metal. Results of blank treatments (Figs. 7a,c,e,g vs. Figs. 7b,d,f,h respectively) showed that, at both salinities, dominant and submissive crabs had a similar (Mann Whitney U-test, $p>0.1$, see Appendix A) total number of scoops (~70 vs. ~36 scoops respectively, Figs. 7a-b), number of scoops on patch (~35 vs. ~10 scoops, Figs. 7c-d), time in control of the patch (~12 vs. ~2.5 minutes, Figs. 7e-f), and aggressiveness (~95% vs. ~5% of successful attacks, Figs. 7g-h). This is an important finding since it shows that salinity does not affect the competitive behaviors of fiddler crabs.

Results of the blank treatments also showed that the competitive behaviors of fiddler crabs were similar in both Trial 1 and Trial 2. Irrespective of the trial, the foraging and dominance behaviors of both submissive and dominant crabs did not change ($p>0.1$) (Figs. 7a-d and Figs. 7e-h). This means that holding time did not influence the behavior of *Uca* crabs, and that the behavior of fiddler crabs is independent of the trial underway.

B. Effect of protein-rich patch on the behavior of fiddler crabs:

In a separate treatment, the effect of the protein-rich patch presence on the behavior of fiddler crabs was examined. Competitive behaviors of fiddler crabs were observed in the presence as well as in the absence of a protein-rich patch. Results showed that foraging behavior parameters of both submissive and dominant crabs were unaltered (Mann Whitney U-test, $p>0.1$) by the presence of the protein patch, and that dominant crabs consistently fed more ($p<0.05$) than the submissive ones irrespective of the patch presence (Fig. 8a). It was observed that although dominant crabs were able to monopolize and, consequently, forage more on the protein-rich patch, submissive crabs occupied the

broader area of the mud surface. As for aggressiveness, interestingly, the presence of high food density patch did not alter number of successful attacks launched by both dominant and submissive crabs ($p>0.1$), nor did it affect the already established dominance hierarchy (Fig. 8b).

C. Background metal concentrations in dominant and submissive crabs:

Environmental contaminants may affect exposed aquatic organisms (Cheung *et al.* 2002, Smith and Weis 1997, Weis *et al.* 2001). In an effort to determine the influence of accumulated Cd and Ag on dominance behavior of fiddler crabs, subcellular fractions of both dominant and submissive crabs subsequent to Trial 2 were analyzed for their metal contents. The analysis showed that at any given salinity, there was no difference in Cd and Ag concentrations (unpaired t-test, $p>0.05$) between dominant and submissive crabs (Figs. 9a-d).

VII. Discussion

Previous studies have shown that salinity can alter the physiology and behavior of decapod crustaceans. For instance, Grimes *et al.* (1989) reported that the Atlantic mud fiddler crab, *Uca pugnax* prefers seawater as it lacks tolerance to freshwater. These crabs also osmoregulate at a much greater rate at low salinity (Holliday 1985). McGaw *et al.* (1999) reported changes in the locomotor activities of four different osmoregulating crabs with change in salinity. Also, Delorenzi *et al.* (2000) showed that an increase in salinity enhances the memory of *Chasmagnathus* brackish-water crab. Hence, It was critical for the purpose of this study to show that salinity had no impact on foraging and dominance behaviors of *Uca pugnax*. This is consistent with the findings of Reinsel and Rittschof (1995) that salinity, the most variable of all environmental factors, had no effect on

feeding of the sand fiddler crab *Uca pugilator*. This suggests that any change in the foraging and/or dominance behavior of *Uca* crabs subsequent to metal exposure may not be attributed to salinity.

The diet of fiddler crab *Uca pugnax* consists mainly of diatoms, fungi and vascular plant tissues (Grimes *et al.* 1989). Hoffman *et al.* (1984) reported that these crabs significantly reduce the abundance of salt marsh meiofauna (crustaceans, nematodes, and segmented worms), probably by feeding on them. Foraging response of Atlantic sand crab *Uca pugilator* on food patches was directly correlated with food density (Robertson *et al.* 1980), as well as with the presence of proteins, sugars and living organisms (Robertson *et al.* 1981). Also, freshwater fiddler crab *Uca minax* prefer substrates with high organic content of high-energy value (Whiting and Moshiri 1974). Accordingly, this recent study showed that marsh mud crabs *Uca pugnax* also compete for protein-rich diet.

Additionally in this study, dominant crabs were found to be able to control, and as a result, to forage longer on the protein-rich patch. This is an important finding as it shows that dominant individuals in a species have priority access to high quality food. Hence, there is a relationship between dominance and foraging. This is consistent with results of previous studies that showed that in a group of gobies, dominant individuals were able to monopolize areas with high food density and achieve higher foraging rates (Whiteman and Cöte 2004). Also, Lahti and Lower (2000) showed a relation between aggression and feeding in fish, where the most aggressive fish obtained the greater portion of food.

In this study, metal analysis showed that Cd and Ag levels in total body, organelles, heat-sensitive enzymes, heat-stable MT and insoluble fractions were similar

in both dominant and submissive crabs. This shows that in blank treatments, background Cd and Ag body burden does not affect the foraging and dominance of *Uca* crabs.

Sloman *et al.* (2003) showed that the waterborne exposure of a group of rainbow trout to Cd affected the social status and tissue accumulation of Cd, with dominant fish accumulating more Cd at the gill. The exposure of rainbow trout, however, to sublethal levels of Cu had no effect on the social hierarchies with subordinate fish accumulating more metal in their gills (Sloman *et al.* 2002). It is interesting, therefore, to study the effect of exposure to various metals on the behavior of both dominant and submissive *Uca* crabs, as well as the difference in metal accumulation in various subcellular fractions in these crabs.

In this study, the effect of social ranking on the foraging behavior of fiddler crabs was examined utilizing pairs of crabs held under conditions designed to maximize social interactions. Various studies have shown that foraging is affected by group size (Cezilly *et al.* 1990). For instance, in salmon, as group size increased from one to four, fish captured more prey (Grand and Dill 1999). As group size increases, however, competition for resources may also increase, and individuals may be expected to increase their foraging effort in an attempt to increase their share (Grand and Dill 1999). The extent of feeding benefits from increasing group size is likely to vary from species to species. It would be interesting to study the effect of increase in the group size on competitive foraging and dominance behaviors of *Uca* crabs.

Fig. 7: Quantification of competitive foraging behavior (a, b, c and d) and competitive dominance behavior (e, f, g, and h) of *Uca* crabs at 5ppt (left) and 30ppt (right)

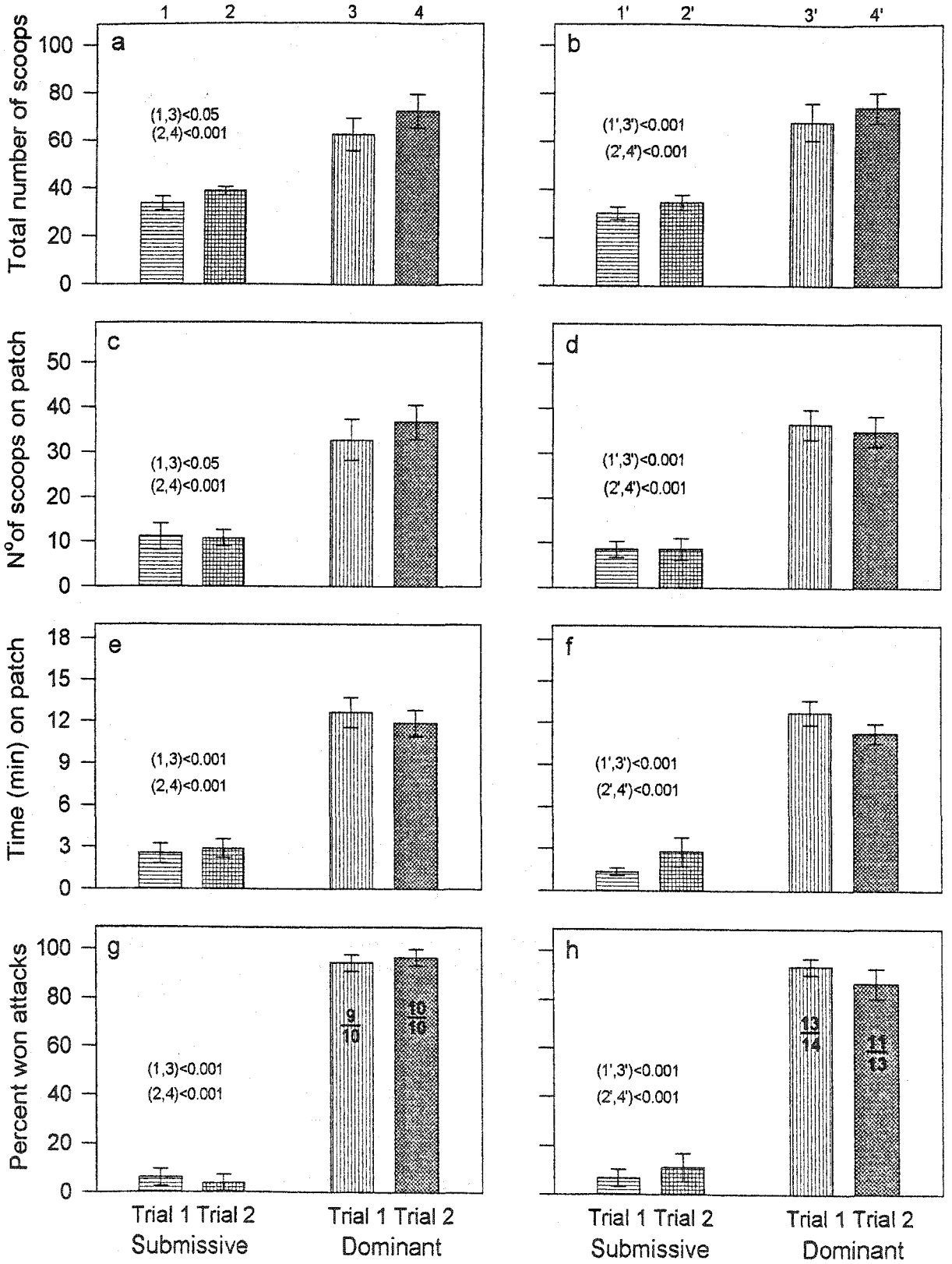
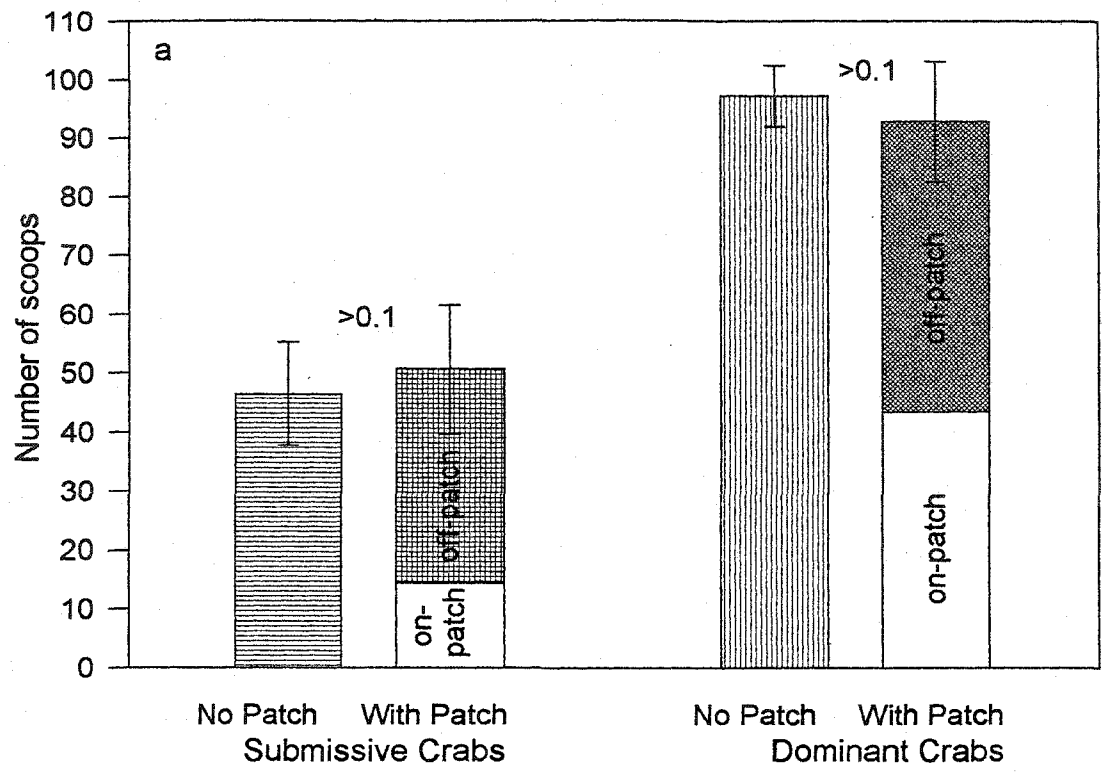


Fig. 8: Effect of the presence of protein-rich patch on foraging behavior (top) and dominance (bottom) of the fiddler crab *U. pugnax*



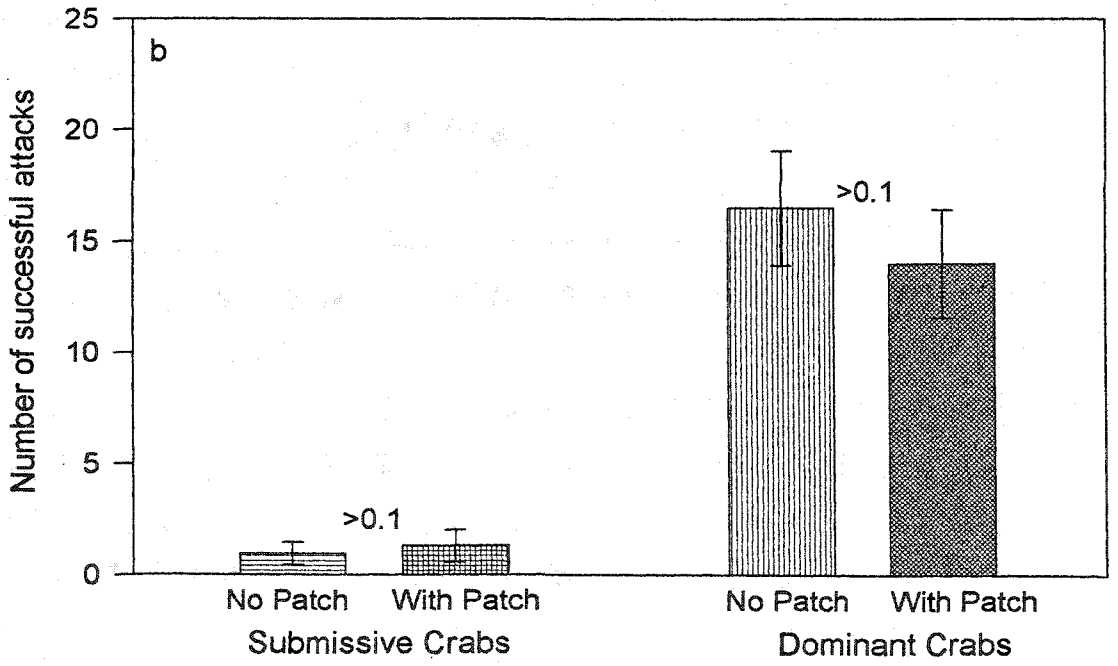
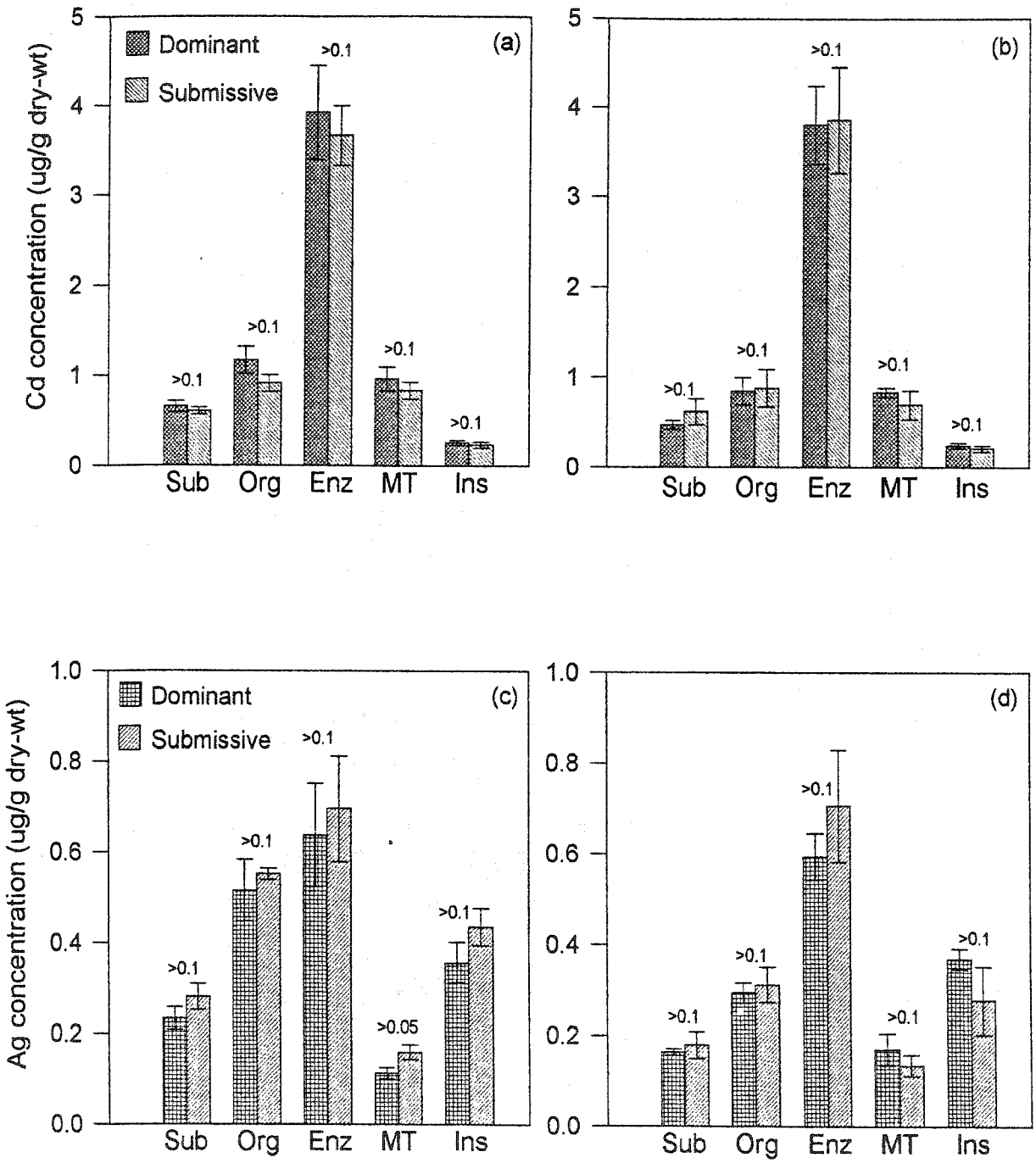


Fig. 9: Metal concentrations in various fractions of dominant and submissive crabs: (a, b) Cd concentrations at 5ppt and 30ppt, (c, d) Ag concentrations at 5ppt and 30ppt respectively



§ Chapter Four §

BEHAVIORAL TOXICITY & BIOACCUMULATION OF CADMIUM IN *UCA* CRABS

I. Toxicity of Cadmium

A. Cadmium and the environment:

In 1817, Cadmium (Cd) was first discovered as a distinct element in Germany by Stromeyer (Browning 1969). It is a highly toxic metal with no known biological function (Berman 1980, Szebedinszky *et al.* 2001). Cd occurs naturally in trace amounts (from 1 to 250 μg/kg) in the surface rocks of the earth's crust, it is present in amounts a thousand times higher in many plants and animal tissues (Browning 1969, Kalisińska *et al.* 2004, Laws 1993). As Cd and Zn are similar in atomic structure and chemical behavior, they often occur together in nature (Berman 1980). There are no ore deposits sufficiently rich in Cd to warrant its extraction. Instead, Cd is obtained as a by-product of mining of other metals, primarily ores of zinc, and to a much lesser extent of lead and copper (Friberg *et al.* 1971, Laws 1993).

In the last few decades, the introduction of Cd into aquatic ecosystems has increased drastically (Laws 1993). Research shows that ingestion of even trace quantities of Cd can influence not only the physiology and health of individual organisms, but also the demographics and distribution of species (Domouhtsidou *et al.* 2004, Larison *et al.* 2000). Although Cd is a relatively rare element, it is a common pollutant because of its wide use in industrial processes. The main uses of Cd are in electricity (storage batteries), in electronics (photovoltaic cells) and in metallurgy (surface-coating by Cd electroplating). The plastics industry also employs large quantities of Cd stearate as a stabilizer in certain polymers (Duffus 1980, Patnaik 2002). Increased industrial usage of Cd has therefore caused an increase in Cd production. Since less than 5% of Cd used in the U.S. is recycled, its use over the last few decades has resulted in an increase in soil, air and water contamination (Waalkes and Misra 1996). Extraction of Cd from Zn ore,

fossil fuel consumption and burning of plastics stabilized by Cd stearate are important sources of air pollution by Cd. Electroplating is considered a major cause of the Cd contamination of waters when it is discharged into the aquatic system (Ramade 1987).

Aquatic ecosystems are particularly sensitive to Cd pollution due to the strong tendency of this element to bioaccumulate in the food web; many plants and animals absorb it efficiently and concentrate it within their tissues (Hopkins *et al.* 2004, Prankel *et al.* 2004, Ray and McLeese 1987). In 1955 a well-documented Cd poisoning incident took place in Japan. This occurred when wastewaters from Zn mines was used to irrigate rice fields which led to the accumulation of Cd in rice. This was called Itai-itai (ouch-ouch) disease because of the severe bone pain associated with it (Duffus 1980). Cd therefore is widely regarded as a priority contaminant, and, historically, guidelines by regulatory agencies have been established for its levels in public water supplies. In considering the effects of Cd on human health, the U.S. EPA (1985) established drinking water standard of 10 parts per billion (ppb).

B. Mechanisms of Cd toxicity to aquatic organisms:

Cd has only one oxidation state (Cd^{2+}), which is believed to be the most toxic form of Cd (Bjerregaard and Depledge 1994, Laws 1993, Patnaik 1992). It readily forms complexes, reacts with sulphur and other non-metals, and binds to many biologically important groups, including phosphates, cysteine, histidine, purines and porphyrins. Thus, Cd can inhibit a range of enzymes that possess active thiol groups. It can also disrupt pathways of oxidative phosphorylation (Berman 1980). Cd ion was shown to interact with phospholipid monolayers of cell membranes and to be very effective in altering their structure even at very low concentrations (Domouhtsidou *et al.* 2004, Ochiai 1977). This implies that Cd ions may have a toxic effect on biomembranes. The

Cd ion, like other metal ions, affects the conformation of polynucleotides and the physical properties of DNA (Ochiai 1977). Cd and Zn are chemically similar; their similar chemistry, combined with the greater affinity of Cd for various bioligands, allows Cd to displace Zn in many biological processes. For instance, Cd can substitute for Zn ions at the active site of some enzymes, rendering them active but with possibly altered substrate specificity (Waalkes and Misra 1996).

The toxicity of Cd in crustaceans has been well studied. The free Cd ion is believed to be the most bioavailable and toxic form of Cd to crustaceans (Wildgust and Jones 1998). Among crustaceans, crabs are the most tolerant to Cd with acute LC_{50} ranges from 0.5 to 50 mg/l (McLeese *et al.* 1987, Zanders and Rojas 1986). Crustaceans exposed to low concentrations of Cd have shown different behavioral and physiological responses, e.g. reduction in feeding rate, change in burrowing activity, disruption of swimming behavior, reduction in growth rate, inhibition of reproduction, change in respiration rate, change in ATPase activities, and so on (McGeer *et al.* 2000, McLeese *et al.* 1987, O'Hara 1973, Roast *et al.* 2000, Rodriguez *et al.* 2000, U.S. EPA 1977, Voyer *et al.* 1982).

C. Effect of salinity on Cd toxicity:

The toxicity of Cd to aquatic organisms is influenced by various environmental factors. For estuarine organisms, salinity is the most important factor. In most studies, accumulation of Cd increased with decrease in salinity (Chan *et al.* 1992, De Wolf *et al.* 2004, Feng *et al.* 1998, Mance 1987, Voyer *et al.* 1982). For instance, Lee and Cundy (1998) showed that uptake of Cd by the bivalves, *Macoma balthica*, increased as salinity decreased. However this is not consistent with Blust *et al.* (1992) finding that the uptake of Cd by the brine shrimp *Artemia franciscana* did not change over a wide range of

salinity. Part of the explanation for the increased toxicity of Cd at low salinity levels may be the result of increased stress on animals that are not adapted to low-salinity regimes (McLeese *et al.* 1987). However, there is clearly another reason for increased Cd toxicity at low salinity. The decreased toxicity of Cd at high salinities may be related to the greater complexation of the free Cd ion with the chloride ion. As salinity decreases, the chloride ion concentration decreases, resulting in an increase in the availability of the free Cd ions (Bjerregaard and Depledge 1994, Waeles *et al.* 2004, Wildgust and Jones 1998). As salinity increases, the dominant species becomes chloro-complexes, with $CdCl^+$ dominant in estuarine waters and $CdCl_2$ in oceanic water. It is generally accepted that only free Cd ions can be accumulated by organisms, whereas chloro-complexes are not taken up (Peakall and Burger 2003). Sunda *et al.* (1978) showed that Cd bound to chloride ion is nontoxic or less toxic than the free Cd ion, and that there is more free Cd ion present at low salinity. For instance, at 5 ppt salinity, about 23% of Cd is present in its free form. As salinity increases to about 30 ppt, only 4% of Cd is present in its free form, and Cd toxicity dropped five-fold (Engel and Fowler 1979).

II. Methods

A. Behavioral studies:

Male fiddler crabs *Uca pugnax* were collected from Meredith Creek marsh on Staten Island, NY (Figure 3). In the lab, crabs were housed in tanks that were maintained at 24°C with a light:dark cycle of 12:12 hours. Half the bottom of each tank was filled with Meredith Creek marsh mud; the rest of the tank was filled with Meredith Creek water (~15ppt in salinity). The level of water was maintained below the surface of the

mud. This setting allowed the crabs an access to both aquatic and terrestrial environments.

Fiddler crabs were used in various treatments to assess the interactions of Cd with changing salinity. A total of 8 treatments were conducted, and each included assessment of various behavioral parameters prior to metal exposure (Trial 1 - no metal exposure), followed by exposure to metals and subsequent re-assessment of behavior (Trial 2 - after exposure to metal). The exposure conditions of each treatment are described in Table 4. All behavioral studies were conducted in a temperature-controlled room at 24°C.

Table 4: List of treatments investigating the effects of Cd exposure at 5ppt and 30ppt on the behavior of fiddler crabs

Treatment #	Metal(s)	Concentration (mgl^{-1})		Molarity ($\mu molel^{-1}$)	Salinity (ppt)
		Nominal	Observed		
1	Cd	0.5	0.45	4.45	5
2	Cd	0.5	0.47	4.45	30
3	Cd	2.5	2.46	22.3	5
4	Cd	2.5	2.45	22.3	30
5	Cd	5.0	4.49	44.5	5
6	Cd	5.0	4.78	44.5	30
7	Cd	10.0	9.38	88.9	5
8	Cd	10.0	9.41	88.9	30

Trial 1 for Cd treatments was conducted the same as that of control treatments. Briefly, for each treatment, crabs (n=16) of similar carapace width (18-20 mm) were removed from tanks, and were placed individually in labeled plastic cups with mesh bottoms that were placed in an aquarium filled with filtered water (salinity ~ 15ppt). Crabs were allowed to depurate for 48 hours. At the end of depuration period, each crab was placed in a 250 ml acid-washed Erlenmeyer flask containing 100 ml of Nano® pure

water of corresponding salinity for a 72-hour period (Instant Ocean™ salt was used to prepare solutions). This holding was included in Trial 1 to mimic the conditions during exposure to metals in Trial 2. Crabs were then paired at random and were placed in a 15 x 20 cm opaque plastic container containing a ~1 cm layer of Meredith marsh mud. In the center of mud, a 1.5 cm diameter protein-rich patch (of crushed Freeze Dried Plankton™ with a minimum of 69% crude protein and 13% crude fiber) was placed on mud surface for the crabs to compete for. While in the container, each pair of crabs was videotaped by a JVC Digital Signal Processing camcorder for one hour. Tapes were immediately reviewed and data on competitive foraging behavior (total number of scoops and number of scoops on protein-rich patch), and on competitive dominance behavior (time in control of the patch and aggressiveness/ number of successful attacks) of each crab were collected. When viewing video footage, it became obvious that one crab from each pair was dominant and the other was submissive. Dominance was assigned to the most aggressive crabs (i.e. the crabs that had most successful attacks are here referred to as dominant, those crabs with less or no successful attacks are referred to as submissive). At the end of the videotaping session, crabs were de-acclimated by feeding individually on Meredith marsh mud in a labeled plastic container for 48 hours.

In Trial 2, crabs were transferred to depuration chambers as before to allow the evacuation of gut contents. This second depuration period also lasted 48 hours. Dominant crabs were exposed in flasks containing 100-ml Cd solution at the corresponding concentration and salinity (see Table 4). Cd solutions were prepared using Perkin Elmer ultra pure metal solution. Submissive crabs were placed in similar flasks, each containing 100 ml of metal-free solution to mimic exposure. Exposure lasted 72 hours. Crabs were then re-paired as in Trial 1 and were videotaped for a second one-hour period. Tapes

were reviewed and behavioral data were collected. At the end of Trial 2, crabs were depurated for 48 hours. Afterward, crabs were stored in zip lock bags at -80°C.

B. Fractionation procedure and sample preparation:

Metal-exposed and control crabs were subjected to subcellular fractionation as described in Chapter 3 (Figure 6). Briefly, crabs (four dominant and four submissive from each treatment) were thawed on ice. Then, each crab was placed in a 50 ml Nalgene® centrifuge tube containing 5.8 ml of 20 mM Tris buffer solution. Crabs were then homogenized using Polytron® PT 2100 homogenizer. Subsequent to homogenization, a one-milliliter sample was removed from the centrifuge tube and was transferred to a pre-weighed vial for future analysis of total body burden. Homogenized crabs were first centrifuged in a Sorval® (RC 5C Plus model) at 1,950g for 15 minutes. Supernatants (S1) were transferred to a set of centrifuge tubes, which were further centrifuged in Beckman centrifuge (L8-M Ultracentrifuge model) at 103,000g for one hour. After centrifuging supernatants (S1), the resulting pellets (P4) contain the organelles; supernatants (S4) were transferred to another set of Beckman centrifuge tubes that were placed in water bath at 80°C for 10 minutes, then on ice for 45 minutes. After supernatants (S4) cooled on ice, they were centrifuged in Beckman centrifuge at 20,000g for 30 minutes. The resulting supernatants (S5) and pellets (P5) constitute MT proteins and enzymes, respectively.

After adding 5.0 ml of Tris buffer to pellets (P1), they were placed in water bath at 100°C for 2 minutes; 5.0 ml of 1N NaOH was added to each tube before being vortexed and placed again in a water bath at 65°C. After one hour, pellets (P1) were removed from water bath; they were vortexed and spun in Sorvall centrifuge at 4,400g for 15 minutes. Supernatants (S2) were transferred to pre-weighed vials; these are the

cellular debris. A 5 ml of 1N NaOH was added to pellets (P2), pellets were then vortexed and centrifuged in Sorvall centrifuge at 4,400g for 15 minutes. Supernatants (S3) were transferred to the same vials containing the cellular debris. Pellets (P3) constitute the insoluble fraction (intracellular granules plus carapace).

Crab tissues and subcellular fractions were digested for metals following standard protocol. Vials containing subcellular fractions were dried in a drying oven at 65°C, and then they were weighed. Concentrated HNO_3 (5 ml) was then added to each vial. Samples, covered with reflux bulbs, were refluxed until tissues dissolved. Samples were then evaporated to dryness, resuspended in 10 ml of 2% Ultragrade HNO_3 and filtered through .45 μm Millipore filters. Filtered samples were then analyzed for Cd using Perkin Elmer model 3100 atomic absorption spectrophotometer. Quality Control and Quality Assurance samples included (1) standard tissue samples, (2) fractionation procedural blanks, (3) digestion blanks and (4) filter blanks. During analysis, background and standards were periodically run to verify the accuracy of the instrument.

III. Results

A. Effects of exposure to Cd on competitive behaviors of *Uca* crabs:

The effects of exposure to different Cd concentrations on competitive foraging behavior (total number of scoops as well as number of scoops on a protein-rich patch), and on competitive dominance behavior (ability to control a protein-rich patch as well as aggressiveness/fighting ability) of *Uca* crabs, were examined under 5ppt and 30ppt salinity.

In Trial 1 (prior to exposure), and as compared with submissive fiddler crabs, dominant crabs consistently exhibited a higher number of total scoops (~75 vs. ~40

scoops, Mann Whitney U-test, $p<0.05$) (Figs. 10-13, a-b), and a higher number of scoops on patch (~36 vs. ~10 scoops, $p<0.01$) (Figs. 10-13, c-d). These unexposed dominant crabs also spent more time feeding on the patch than submissive crabs (~11 vs. ~2 min./ hour, $p<0.05$) (Figs. 10-13, e-f), and, by definition, had more successful attacks than submissive crabs (~97% vs. ~3%, $p<0.001$) (Figs. 10-13, g-h). Additionally in Trial 1, and except for Figs. 12e-f, there was no influence of salinity ($P>0.1$, see Appendix A) on the behavior of either dominance or submissive fiddler crabs (Figs. 10-13, a-h).

In Trial 2, following a 3-day exposure of dominant crabs to 0.5 and 2.5mg/l Cd (nominal concentrations), foraging and dominance behaviors in these crabs, as well as that of submissive crabs, were comparable ($p>0.1$) to those exhibited in Trial 1 (Figs. 10-11, a-h). These concentrations are considered as the 'no observed adverse effects level' (NOAEL) for Cd in fiddler crabs *Uca pugnax*.

However, following an exposure to 5.0mg/l Cd (nominal concentration), the foraging behavior parameters (total scoops and scoops on patch) of dominant crabs (Trial 2) decreased significantly ($p<0.01$) at both 5ppt and 30ppt (Figs. 12a-d). Specifically, the foraging behavior of dominant crabs decreased 50-70% at 5ppt (Figs. 12a,c), and 40-45% at 30ppt following exposure to 5.0mg/l Cd (Figs. 12b,d) (Trial 2 vs. Trial 1). Interestingly, at 5ppt where the decrease in foraging of dominant crabs was greater after exposure, submissive crabs showed a 100-200% increase ($p<0.05$) in foraging parameters (Figs. 12a,c) (Trial 2 vs. Trial 1). At 30ppt, where the decrease in foraging of the dominant crabs was less drastic, there was no change in foraging behavior of submissive crabs (Figs. 12b,d) ($p>0.1$).

In Trial 1, dominant crabs controlled the protein-rich patch (Figs. 12e,h), and won more attacks than the submissive crabs (Figs. 12g,h) ($p<0.001$) at both 5ppt and 30ppt. It

should be noted that these were the criteria for determining dominance behavior. After exposure to 5.0mg/l Cd at 5ppt, dominant crabs spent ~65% less time controlling the patch (Fig. 12e) and won ~50% fewer attacks (Fig. 12g) ($p<0.01$); correspondingly, submissive crabs were able to attain 10 times greater access to patch (Fig. 12e) and to win 50% more attacks (Fig. 12g) ($p<0.05$) in Trial 2 than in Trial 1. After exposure at 30ppt, dominant crabs spent 65% less time on the patch (Fig. 12f) ($p<0.01$), but won just as many attacks as they did before exposure ($p>0.1$). The dominance behavior of submissive crabs was unaffected ($p>0.1$) by the change in the behavior exhibited by dominant crabs.

Following the exposure of dominant crabs to 10.0mg/l Cd (nominal concentration) at 5ppt and 30ppt salinity, dominant crabs showed a decrease in their foraging behavior parameters ($p<0.01$) and submissive crabs showed an increase ($p<0.05$) (Figs. 13a-d). Specifically, as compared to these behaviors in Trial 1, the total number of scoops and number of scoops on patch of the exposed dominant crabs (Trial 2) decreased 80-85% at 5ppt (Figs. 13a,c), and 60-75% at 30ppt (Figs. 13b,d). As compared to that in Trial 1, the foraging behavior parameters of submissive crabs in Trial 2 increased dramatically by 75-350% at both 5ppt and 30ppt (Figs. 13a-c). As for dominance behavior (Figs. 13e-h), after exposure to 10.0mg/l Cd at 5ppt and 30ppt, dominant crabs spent 95% ($p<0.01$) and 60% ($p<0.05$) less time on the patch ($p<0.01$) respectively (Figs. 13e,f). These crabs also won 95% less attacks at 5ppt (Fig. 13g) ($p<0.01$), and 70% less attacks at 30ppt (Fig. 13h) ($p<0.01$). Submissive crabs, however, were able to attain greater access to patch by spending 4-6 times more time ($p<0.05$) on the patch at both 5 and 30ppt (Figs. 13e,f), and winning 95% and 70% more attacks ($p<0.01$) at 5ppt and 30ppt respectively (Figs. 13g,h).

The overall post-exposure changes in competitive behavior of submissive and dominant fiddler crabs are summarized in Figs. 14-15. As Cd exposure concentrations for dominant crabs were increased from 0-10mg/l at 5ppt and 30ppt, foraging behavior of these exposed crabs decreased (Kruskal-Wallis ANOVA, $p<0.05$), and foraging behavior of submissive crabs was increased ($p<0.05$) (Figs. 14a-d). As for dominance behavior, the increase in Cd exposure concentrations led to a decrease in the number of successful attacks by dominant crabs, and to an increase in the of successful attacks by submissive crabs ($p<0.05$) (Figs. 15c,d).

B. Bioaccumulation of Cd in fiddler crabs

Fiddler crabs were exposed to a series of Cd solutions (0.0, 0.5, 2.5, 5.0 and 10.0mg/l) at both 5 and 30ppt salinity. For each exposure concentration, the bioaccumulation of Cd in whole body (Fig. 16a), metal-sensitive fractions e.g. organelles and enzymes (Figs. 16b,c), and metal-detoxifying fractions e.g. Metallothioneins and insolubles (granules and carapace) (Figs. 16d,e) was determined. Results are summarized in Figs. 16a-e.

The background Cd concentrations in total body burden, organelles, enzymes, metallothioneins (MT) and insoluble fractions were 0.6, 1.0, 3.9, 1.1 and 0.2 μg/g dry-weight respectively (Figs. 16a-e). After exposure to 0.5mg/l, Cd total body burden of crabs increased by 32-fold at 5ppt (t-test, $p<0.01$), and 7.5-fold at 30ppt ($p<0.01$) (Fig. 16a). The level of Cd in enzymes and organelles (metal-sensitive fractions) increased by ~30-fold at 5ppt ($p<0.001$), and by ~9-fold at 30ppt ($p<0.05$) (Figs. 16b-c). In MT metal-detoxifying fraction), Cd concentrations increased by 15-fold at 5ppt ($p<0.001$), but by only 5 folds at 30ppt ($p<0.01$) (Fig. 16d). The concentration of Cd in the 'insoluble'

(metal-detoxifying fraction) remarkably increased by 95-fold at 5ppt ($p<0.001$), but by only 23-fold at 30ppt ($p<0.01$) (Fig. 16e).

As Cd exposure concentration increased by 4-fold to 2.5mg/l, the levels of Cd in whole body, organelles, enzymes, MT and insoluble fractions increased by 1- to 2-fold ($p<0.001$) at 5ppt (Figs. 16a-e). At 30ppt, the levels of Cd increased by ~2-fold ($p<0.05$) in whole body, MT and insoluble fractions (Figs. 16a,d,e); there was no significant increase in Cd levels in organelles and enzymes fractions at this exposure concentration (Figs. 16b,c) ($p>0.05$).

A two-fold increase in Cd exposure concentration to 5.0mg/l at 5ppt resulted in a significant increase ($p<0.05$) of Cd in the enzyme fraction only (Fig. 16c). At 30ppt, this increase in Cd exposure level resulted in an almost 2-fold increase ($p<0.05$) in Cd whole body burden (Fig. 16a), a 2.3-fold increase ($p<0.01$) of Cd in the enzyme fraction (Fig. 16c), almost 2-fold increase in MT ($p=0.06$) and insoluble ($p<0.01$) fractions (Figs. 16d,e).

Another two-fold increase in Cd exposure concentration to 10.0mg/l at 5ppt did not lead to any change ($p>0.1$) in the levels of Cd in all fractions (Figs. 16a-e). At 30ppt, this one-fold increase in Cd exposure concentration led to a 0.5- to 2-fold increase ($p<0.05$) in Cd levels in all fractions (Figs. 16a-e).

In conclusion, background Cd concentrations were similar in all fractions at both 5ppt and 30ppt ($p>0.05$, see Appendix A). At the no observed adverse effect levels (NOAEL) e.g. 0.5 and 2.5mg/l Cd, and at the lowest observable adverse effect level (LOAEL) e.g. 5.0mg/l Cd, crabs accumulated more Cd in all fractions at 5ppt than at 30ppt ($p<0.005$, see Appendix A).

IV. Discussion

A. Introduction:

In general, the sublethal effects of exposure to environmental pollutants are assessed by measuring one, or few, responses (e.g. behavioral, physiological and cellular) of studied organisms (Hebel *et al.* 1997). Behavioral responses may include changes in feeding and mating behavior (Currie and Valkama 1998, Perez and Wallace 2004), physiological responses may manifest as alteration in osmoregulation and ion uptake (McGeer and Wood 2000), and cellular responses are reflected in alterations in enzyme activities and metallothionein induction (Domouhtsidou *et al.* 2004, Skaggs and Henry 2002). It is established that variations in these behavioral, physiological and cellular responses can be used as biomarkers in assessing the biological impact of pollutants on organisms (Depledge *et al.* 1995). Most studies, however, demonstrate the effects of pollutants, including metals, on a particular response, but few studies examine the relationship between various responses (Burger *et al.* 2003). In metal toxicity studies, the occurrence of adverse toxic effects of metals depends not only on their exposure levels but also on the organism's ability to handle metals and maintain intracellular homeostasis (Baker *et al.* 2003, Caussy *et al.* 2003). Hence, in toxicity studies, it is important to relate pollutant exposure level to the various responses of organisms. This provides insights into the use of NOAEL (no observed adverse effect levels) and LOAEL (lowest observable adverse effect level) in risk assessment (Burger *et al.* 2003).

Competitive foraging and dominance behaviors in fiddler crabs *Uca pugnax* were used as the endpoints to determine the toxic effects of Cd. A behavioral toxicological response is believed to occur when the exposure to a pollutant leads to a significant deviation in an organism's normal behavior (Depledge *et al.* 1995). This current research

showed that the exposure of fiddler crabs to aqueous Cd at nominal concentrations ≤ 22.3μM had no short-term effects on fiddler crabs' competitive behavior. In addition, salinity of exposure did not affect the toxicity of Cd at or below 22.3μM. Also, dominant crabs exposed to Cd at or below the no-observed-adverse effect nominal concentration of 22.3μM consistently forage at a higher rate than submissive crabs at both salinities. With the exception of the time spent on patch before exposure to 44.5μM Cd (nominal concentration)(Figs. 12e-f), it was demonstrated that, prior to exposure to Cd, salinity had no effect on competitive foraging as well as dominance behaviors of both dominant and submissive fiddler crabs.

B. Effects of Cd on the behavior of fiddler crabs: Protective effect of salinity

Previous studies showed that exposure of trout to low levels of Cd affected their behavior. For instance, Scherer *et al.* (1997) showed that Cd decreased the foraging rates of exposed lake trout. Sloman *et al.* (2003), in examining the effect of exposure to waterborne Cd on trout dominance, demonstrated that exposure of rainbow trout to low levels of Cd decreased their abilities to compete with non-exposed fish. Behavioral changes in guppies exposed to Cd included swimming in an imbalanced manner, capsizing and difficulty breathing (Yilmaz *et al.* 2004).

In this study, the exposure of dominant fiddler crabs to 44.5μM Cd (LOAEL) resulted in a significant drop in their foraging behavior parameters i.e. total number of scoops as well as number of scoops on protein patch. Specifically, Cd exposure resulted in 50-75% drop in foraging behavior parameters of the exposed dominant crabs at 5ppt salinity, and 35-40% at 30ppt. When compared to submissive crabs, foraging behavior of exposed dominant crabs is 55-75% less at 5ppt, but no such change was exhibited at 30ppt. The lower toxicity of Cd at higher salinity to fiddler crabs may be due to the

increase in complexation of Cd by chloride, which has seen to decrease the bioavailability of the more toxic free Cd ions (Bjerregaard and Depledge 1994, Engel and Fowler 1979, Peakall and Burger 2003, Roast *et al.* 2001, Sunda *et al.* 1978, Wildgust and Jones 1998).

Interestingly, the drop in foraging behavior parameters exhibited by exposed dominant crabs was accompanied by an increase in the foraging of submissive crabs at 5ppt but not at 30ppt. Specifically, after exposure of dominant crabs to 44.5μM Cd, the foraging parameters of submissive crabs at 5ppt were 1.5-3 times greater than at 30ppt. As a result, by limiting Cd toxicity due to complexation with chloride, salinity appeared to play a role in determining the foraging behavior of submissive crabs. This data sheds additional light on the importance of interactive nature of the dyadic relationship among fiddler crabs.

Impacts of Cd on competitive dominance were also observed. The exposure of dominant crabs to 44.5μM Cd solution at 5ppt resulted in ~50% drop in their successful attacks which was counterbalanced by a 50% increase in the number of successful attacks of their submissive counterparts. The exposure of dominant crabs to the same Cd solution at 30ppt, however, did not result in a change in their aggressive behavior. Once again, the reduction in toxicity of Cd at higher salinity is reemphasized.

What should be noted however is that the dominance behavior in fiddler crabs was not as sensitive to Cd exposure at 44.5μM as foraging behavior. In fact, after the exposure of dominant crabs to Cd at LOAEL, submissive crabs became as aggressive as the dominant crabs at 5ppt but remained less aggressive at 30ppt. It is believed that the ability of dominant individuals in a population to maintain their dominant status after

exposure suggests that these 'most fit' individuals are still capable of protecting their resources, and of mating, more than their submissive counterparts.

Unlike exposure to 44.5μM Cd, exposure of fiddler crabs to 88.9μM Cd (nominal concentration) at both salinities had devastating effects on both foraging and dominance behaviors. These toxic responses exhibited by dominant crabs were mirrored by improvements in these behavioral parameters of the submissive crabs. Ultimately, the protective effect that salinity had on crab behaviors at 44.5μM Cd was reduced, though not eliminated, by the increase in presence of the toxic free Cd ions in the 88.9μM Cd exposure solution. In particular, fiddler crabs exposed to 88.9μM Cd at 30ppt salinity managed to feed on and control the protein-rich patch 3 times more than those exposed at 5ppt. To fiddler crabs, Cd exposure concentration of 88.9μM is ranked at the upper sublethal Cd concentration limit; beyond it, not all exposed crabs would survive. Zanders *et al.* (1996) reported that the 96-h LC_{50} value for Cd in *Uca* crabs is two to six times higher than the values reported for other euryhaline crustaceans. Interestingly, even at this unreasonably high Cd exposure concentration salinity still reduced Cd toxicity to fiddler crabs. This is also demonstrated by 90% reduction in the aggressiveness of dominant crabs at 5ppt salinity, as compared to 50% reduction at 30ppt.

C. Relationship between Cd bioaccumulation and behavioral changes in *Uca* crabs:

1. Implications of osmoregulation in metal uptake in fiddler crabs:

The mud fiddler crabs *Uca pugnax* are one of the most euryhaline of all crustaceans (Holiday 1985), these crabs however are more frequently exposed to low-salinity seawater in their salt marsh habitats (Montague 1980). The salinity of estuarine waters changes continuously, and fiddler crabs can control, to a certain extent, the salt concentration of their body fluid (or hemolymph); they are referred to as osmoregulators

(Baldwin and Kirschner 1976, Zanders and Rojas 1986). At low salinity, the body fluid of these estuarine crabs is hypertonic to the medium, which promotes the osmotic uptake of water. Crabs respond to the increased uptake of water by increasing urine production; the urine is isotonic to the blood and its increased production results in the loss of major osmotic ions (Holiday 1985, Rainbow 1995 and 1997, Rainbow and Black 2002). In order to keep their hemolymph osmotic concentration constant, *Uca pugnax* must hyperosmoregulate by actively transporting major ions through their gills into the hemolymph in dilute media (Rainbow and Black 2002). It is believed that Na^+ and Cl^- are the two major ions that make up more than 90% of the total hemolymph osmotic concentration in euryhaline crabs (Henry *et al.* 2002), but other ions such as Ca^{+2} and Mg^{+2} are also involved (Skaggs and Henry 2002).

Among decapod crabs, *Uca* possess remarkably well-developed control of ionic and osmotic regulation (Zanders and Rojas 1996). The gills of these euryhaline crabs possess mainly two enzymes, which appear to have a universal distribution in all osmoregulating crabs (Henry *et al.* 2002). The enzymes allow crabs to maintain fairly constant levels of Na^+ and Cl^- in their hemolymph. These are the Na/K-ATPase and carbonic anhydrase, and it is believed that they are critical to the osmoregulatory functions of the gill major ion pumps of all aquatic invertebrates during acclimation to dilute media (Henry *et al.* 2002, Skaggs and Henry 2002). The gill Na/K-ATPase provides the electrochemical force for Na^+ and Cl^- uptake. Carbonic anhydrase catalyzes the reversible hydration of CO_2 and water to H^+ and HCO_3^-, the counterions for Na^+ and Cl^- respectively, in hyperosmoregulating crabs (Henry *et al.* 2002, Lucu 1993, Skaggs and Henry 2002, Towle 1993, Vitale *et al.* 1999). This means that carbonic anhydrase is involved not only in CO_2 excretion but also in osmoregulation. Impairment of the

activities of these enzymes can compromise osmoregulation as well as acid-base balance in animals (Lionetto *et al.* 1998). Holiday (1985) showed that the gill Na/K-ATPase enzyme activity of male mud fiddler crabs *Uca pugnax* immersed in 5ppt saltwater is significantly higher than that of crabs in 30ppt. Moreover, Henry *et al.* (2002) showed the induction of activity of both enzymes in euryhaline crabs upon transfer from 32ppt to 10ppt salinity.

During metal exposure, particular attention is focused on the gills as they are responsible for about 80-90% of the water taken up by hyperosmoregulating crabs (Rasmussen *et al.* 1995). The surface of these crustaceans is covered by an impermeable cuticle that acts as a site for the passive adsorption of dissolved metals, but such metals will not be transported into the body of the crustacean (Rainbow 1997). Uptake of trace metal ions into the decapod gill cells and hemolymph occurs either passively with the aid of carrier proteins, and/or through the action of active transport pumps (Hebel *et al.* 1997, Phillips 1995, Rainbow and Black 2002). The increase in the rate of uptake of major ions via ion pumps at low salinity can have implications for increased bioaccumulation of metals in osmoregulating *Uca* crabs. Rainbow (1995) proposed that since the rate of uptake of major ions via ion pumps in decapod crustaceans is increased at low salinity, therefore, it is possible that any observed increased rate of uptake of metal in *Uca* decapod crabs may simply be a reflection of the enhanced activity of ion pumps at low salinity.

2. Cd uptake in exposed fiddler crabs: osmoregulation vs. salinity effects

In this study, the exposure of *Uca pugnax* to 0.93-88.9μM Cd at 5ppt and 30ppt salinity resulted in an increased Cd body burden, as expected, as compared to controls (Burke *et al.* 2003, Campbell *et al.* 2002, Pedersen and Bjerregaard 1995, Lionetto *et al.*

1998, O'Hara 1973, Rainbow 1995, Soegianto *et al.* 1999, Vitale *et al.* 1999, Wallace *et al.* 2003, Zanders and Rojas 1996). In accordance with the findings of Selck and Forbes (2004) findings, this increase in Cd uptake by *U. pugnax* crabs is translated into an increase in Cd accumulation in all subcellular fractions (enzymes, organelles, MT protein, and the insoluble chitin and granules). At these Cd exposure concentrations, *Uca* crabs accumulated more Cd in their tissues and cellular parts at 5ppt salinity than at 30ppt. This may be interpreted by the physiological response of osmoregulating crabs to low salinity, and by the physicochemical effect of increased free Cd ions. Firstly, since the rate of uptake of major ions via ion pumps in decapod crustaceans is increased at low salinity, it is possible that the observed increased in the accumulation of Cd in *Uca* crabs is simply the result of the enhanced uptake of Cd ions by energy-dependent ion pumps at low salinity (Rainbow 1995). Also, because cadmium ions (Cd^{+2}) have similar ionic radii and charge as Ca^{+2}, it is inevitable that some Cd will enter the organism by Ca^{+2} energy-dependent pump (Burke *et al.* 2003, Pedersen and Bjerregaard 1995, Phillips 1995, Rainbow 1997, Silvestre *et al.* 2004, Zanders and Rojas 1986). However, the increase in Cd accumulation at low salinity does not depend only on the physiological status of osmoregulating crabs, i.e. the increased activity of major ion pumps, but also on physicochemical changes in Cd speciation at these low salinities. For instance, at low salinity, the chloride concentration of the medium is lower with concomitant reduction in the formation of chloro complexes. This results in an increase in the availability of free Cd ions (Peakall and Burger 2003, Rainbow 1997, Wright 1995). Rainbow (1995) showed that approximately 20% of Cd at 5ppt is present in its available hydrophilic ionic form, but at 30ppt only 3% is present as free Cd ions. The remaining Cd^{+2} forms chloride complexes ($CdCl^{+}$, $CdCl_2$, $CdCl_3^{-}$ and $CdCl_4^{-2}$) that dominate at high salinities (Burke *et*

al. 2003, Rainbow 1997). These complexes, however, are not biologically available (Burke *et al.* 2003, Luoma *et al.* 1995).

Fiddler crabs exposed to 88.9μM Cd accumulated as much Cd at 5ppt as they did at 30ppt. It is suspected that at this elevated exposure concentration, free Cd ions are abundant at both salinities, and speciation plays less of a role in Cd bioaccumulation. At this elevated exposure concentration, the uptake of Cd through gill ion pumps may become the limiting factor in Cd accumulation. On the other hand, exposure of *Uca* crabs to high Cd concentration at both salinities resulted in the deaths of significant number of *Uca* crabs. This may be due to the inhibition of Na/K- ATPase and carbonic anhydrase activity that leads to ionoregulation and osmoregulation dysfunction (Hansen *et al.* 1992, Lionetto *et al.* 1998, Morgan *et al.* 1997, Skaggs and Henry 2002, Vitale *et al.* 1999).

3. Relationship between intracellular distribution of Cd and behavioral toxicity:

Internalization of Cd occurs mostly through the gills either passively with carrier protein, and/or actively through major ion pumps (Hebel *et al.* 1997). Inevitably, some Cd ions are bioadsorbed to the *Uca* chitinous exoskeleton that has a high affinity for Cd (Benguella 2002, Evans *et al.* 2002). Once inside the body, Cd ions, which have high affinity for S^- and N^- sites, bind non-specifically intracellularly to proteins, enzymes and organelles (Lionetto *et al.* 1998, Rainbow 1997). Simultaneously, at least three detoxification mechanisms are activated; at the organismal level, there is an increase in the excretion rate (or efflux) of Cd through the gills (Phillips 1995); at the cellular level, there is an increase in the formation of granules that sequester Cd in relatively non-toxic forms (Peakall and Burger 2003, Phillips 1995, Simkiss and Taylor 1994); and at the molecular level, there is an increase in the binding of Cd to MT and MT-like proteins,

along with an increase in MT synthesis (Baker *et al.* 2003, Hebel *et al.* 1997, Roesijadi 1992).

Previous studies showed that Cd accumulation in the tissues of fiddler crabs is enhanced at low salinity, and that at high salinities the toxicity and uptake of Cd is diminished (Zanders and Rojas 1996). In this study, at 5ppt salinity, the binding of Cd to MT proteins and insolubles (granules and crab exoskeleton) reached its maximum capacity at Cd exposure concentration of 22.3μM, whereas at 30ppt, the maximum binding capacity of these metal-detoxifying fractions was reached at 88.9μM. Interestingly, the onset of behavioral toxicity of Cd on fiddler crabs at both salinities appeared at 44.5μM Cd (LOAEL), i.e. a concentration that was higher than needed for metal-detoxifying fractions to reach its capacity at 5ppt, and below that needed at 30ppt. This suggests that 'spill-over effect', or the saturation of metal-detoxifying fraction, cannot explain the initial onset of Cd behavioral toxicity at either exposure salinity. Moreover, at the onset of Cd behavioral toxicity, Cd concentrations in the metal-sensitive fractions (i.e. organelles and enzymes) were on average 3-fold less at 30ppt than at 5ppt. It can be inferred from these findings that there is no direct relationship between Cd behavioral toxicity and enzymatic bioaccumulation of Cd. This means physiochemical events alone do not explain salinity-dependent behavioral toxicity. Alternatively, physiological conditions of crabs at the time of exposure may affect their tolerance to Cd, hence, physiological status of organisms may be important in predicting Cd behavioral toxicity (Burke *et al.* 2003, Rainbow and Black. 2002, Zanders and Rojas 1996).

For instance, Harris and Santos (2000) showed that various euryhaline decapods have greater capacity for regulating blood osmotic concentrations at low salinity. This capacity is significantly reduced at high salinity. The exposure of the Mediterranean

decapod shrimp *Penaeus japonicus* to Cd at high salinity resulted in a decrease in basal infoldings and mitochondria of gills (Soegianto *et al.* 1999). Also, Lawson *et al.* (1995) showed that exposure of euryhaline decapod *Carcinus maenas* to a sublethal Cu level caused greater damage to the gill epithelial cells (including decrease in the number of plasma membrane infoldings, fewer mitochondria, and extensive vacuolation) at a high salinity of 35ppt than at a low salinity of 10ppt. These plasma membrane infoldings provide the surface area for exchange of ions and respiratory gases; the structural integrity of the plasma membranes is essential for the functioning of ionic exchange and osmoregulation. The disruption of plasma membrane infoldings by metal exposure decreases the surface area available for Na/K-ATPase and carbonic anhydrase pumps that are responsible for ionoregulation and respiratory gas exchange respectively which could affect metal uptake. Mitochondria supply energy for active transport pumps; any decrease in the number of mitochondria would result in a reduction of available energy for ionoregulation and respiratory gas exchange (Lawson *et al.* 1995). Finally, vacuolation of gill epithelial cells potentially affects the physiological efficiency of gills by increasing the diffusion distance for respiratory gases and ions. Vacuolation after exposure to metals at high salinity may indicate a failure to regulate water content (Lawson *et al.* 1995). This is supported by the findings of Rasmussen *et al.* (1995) that showed water permeability of several euryhaline decapods decreased with increasing Cd concentrations.

Metal exposure causes greater ultrastructural alterations to the epithelial cells of euryhaline crabs at high salinity. It is not unlikely to observe these alterations in *Uca* crab gill cells at high salinity since they are physiologically more adapted to survive at low salinity (Montague 1980, Zanders and Rojas 1986). This may explain the increase in behavioral toxicity of intracellular Cd in *Uca* crabs at high salinity.

Fig.10: Effect of exposure to 0.5mg/l Cd on competitive foraging (a,b,c,d), and on competitive dominance (e, f, g, h) of *Uca* crabs at 5ppt (left) and 30ppt (right)

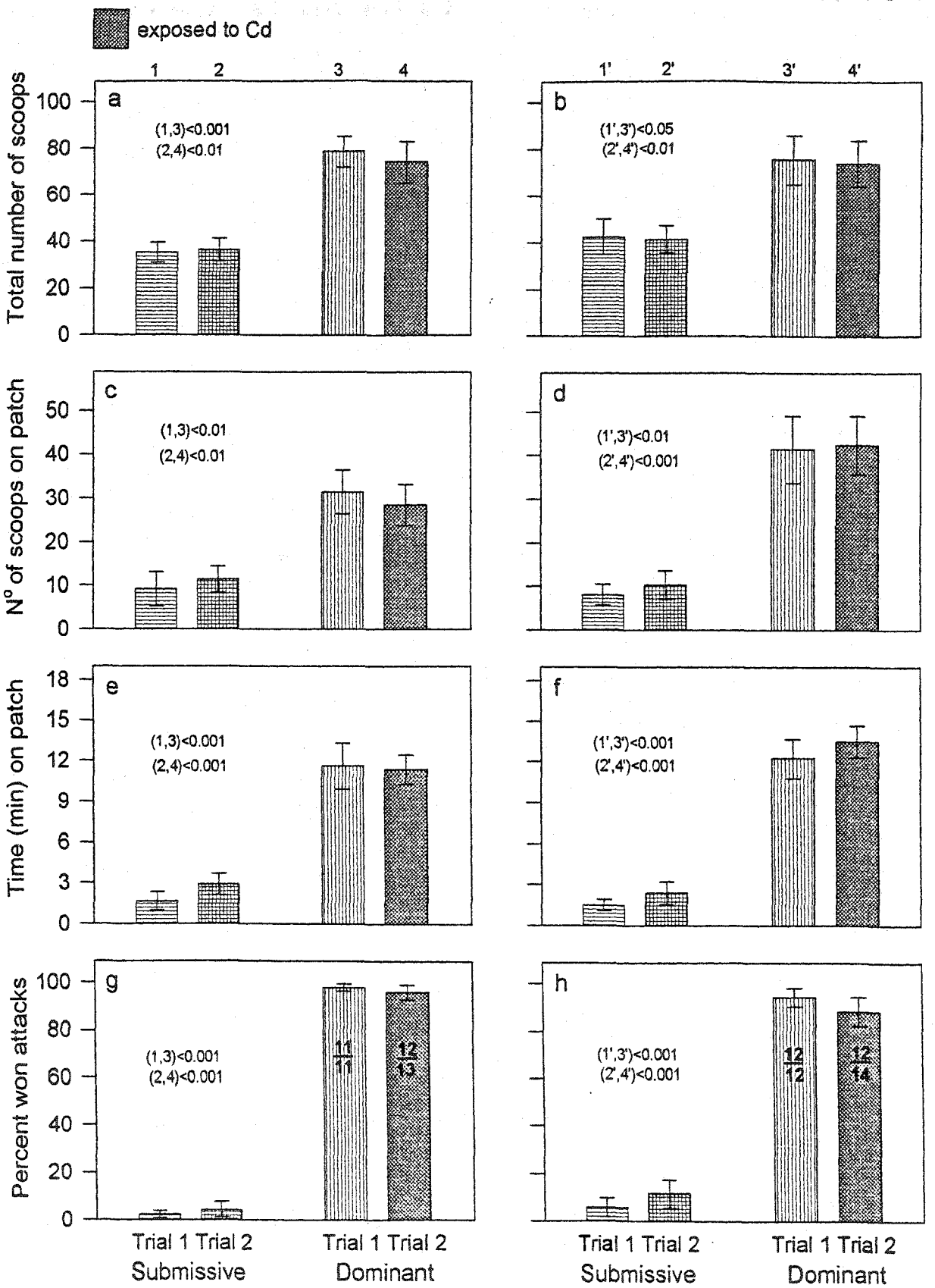


Fig.11: Effect of exposure to 2.5mg/l Cd on competitive foraging (a, b, c, d), and on competitive dominance (e, f, g, h) of *Uca* crabs at 5ppt (left) and 30ppt (right)

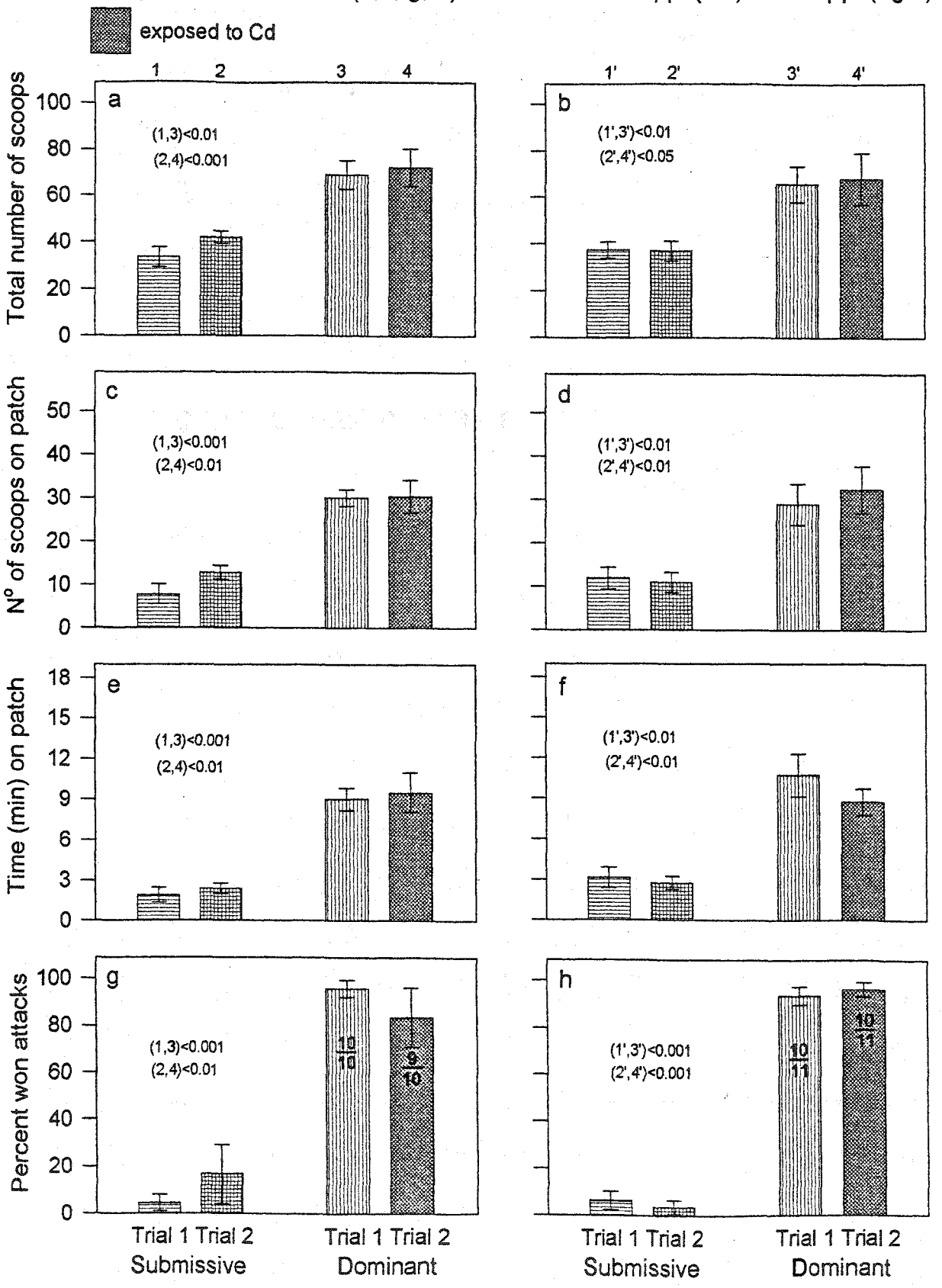


Fig.12: Effect of exposure to 5.0mg/l Cd on competitive foraging (a, b, c, d), and on competitive dominance (e, f, g, h) of *Uca* crabs at 5ppt (left) and 30ppt (right)

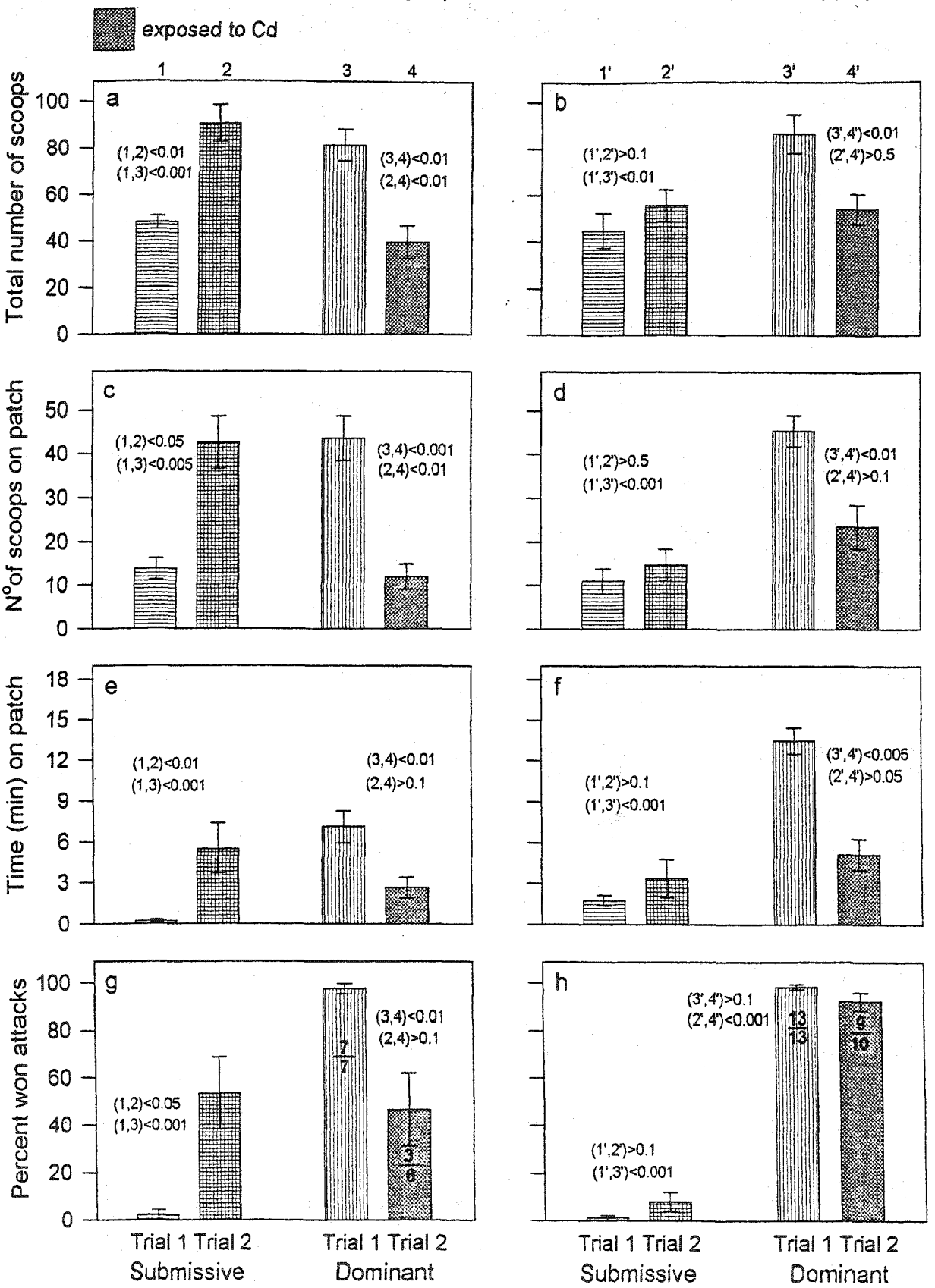


Fig.13: Effect of exposure to 10mg/l Cd on competitive foraging (a, b, c, d), and on competitive dominance (e, f, g, h) of *Uca* crabs at 5ppt (left) and 30ppt (right)

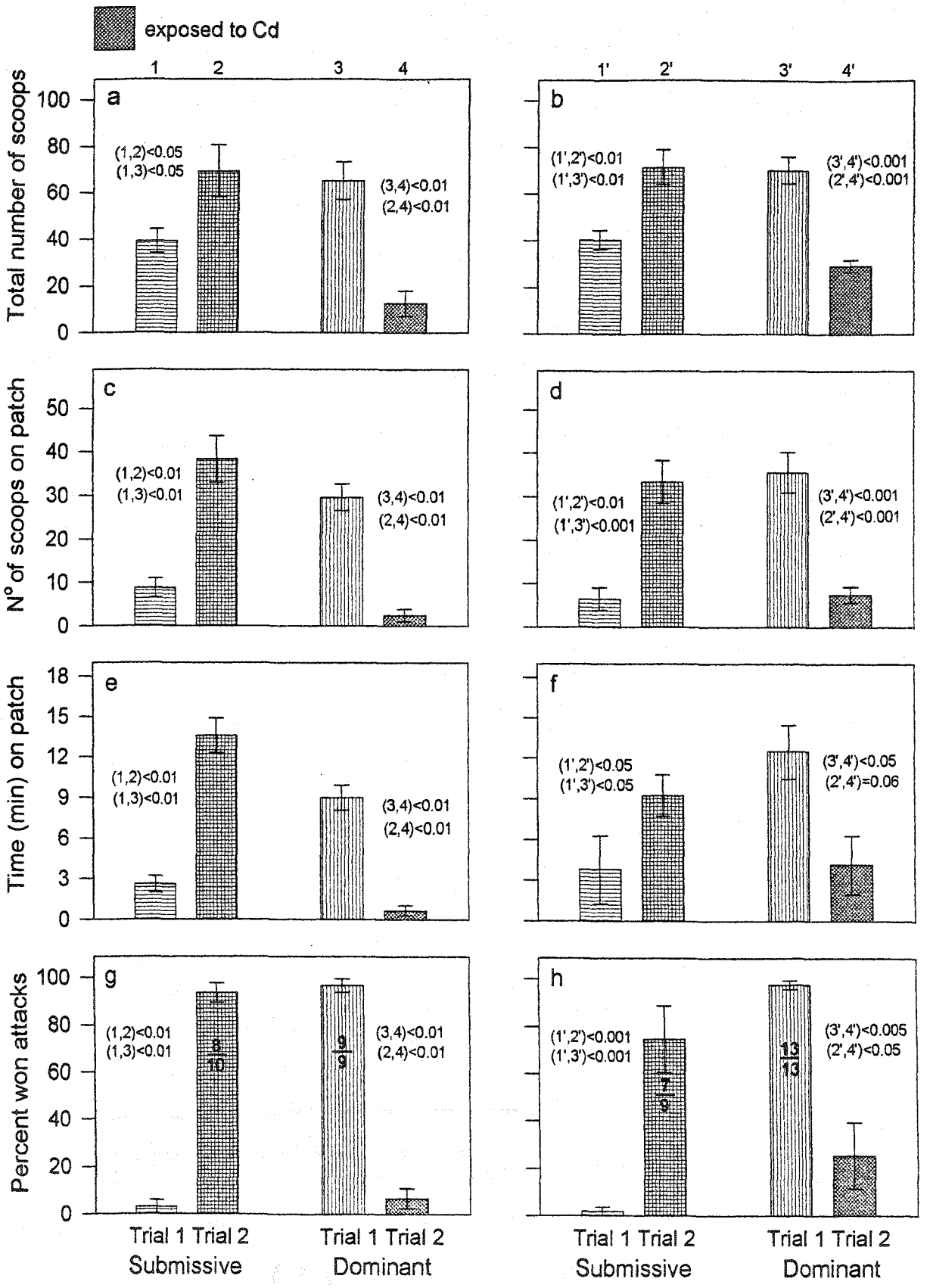


Fig. 14: Change in foraging behavior in terms of total scoops (a,b) and scoops on protein-rich patch (c,d) of submissive (gray bars) and Cd exposed dominant (black bars) *Uca* crabs, at both 5ppt (left) and 30ppt (right)

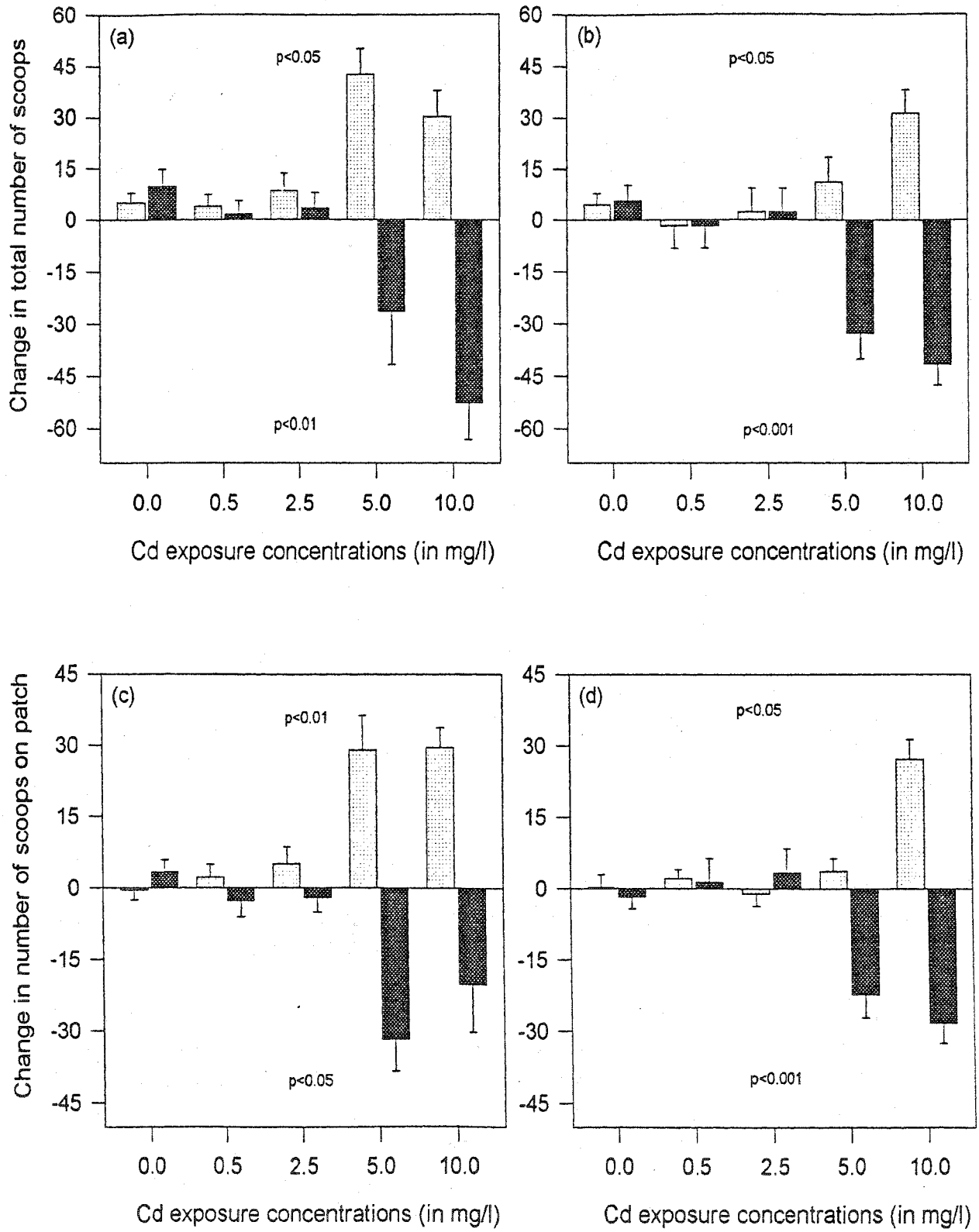


Fig. 15: Change in dominance behavior in terms of ability to control patch (a,b) and aggressiveness (c,d) of submissive (gray symbols) and Cd exposed dominant (black symbols) *Uca* crabs, at both 5ppt (left) and 30ppt (right)

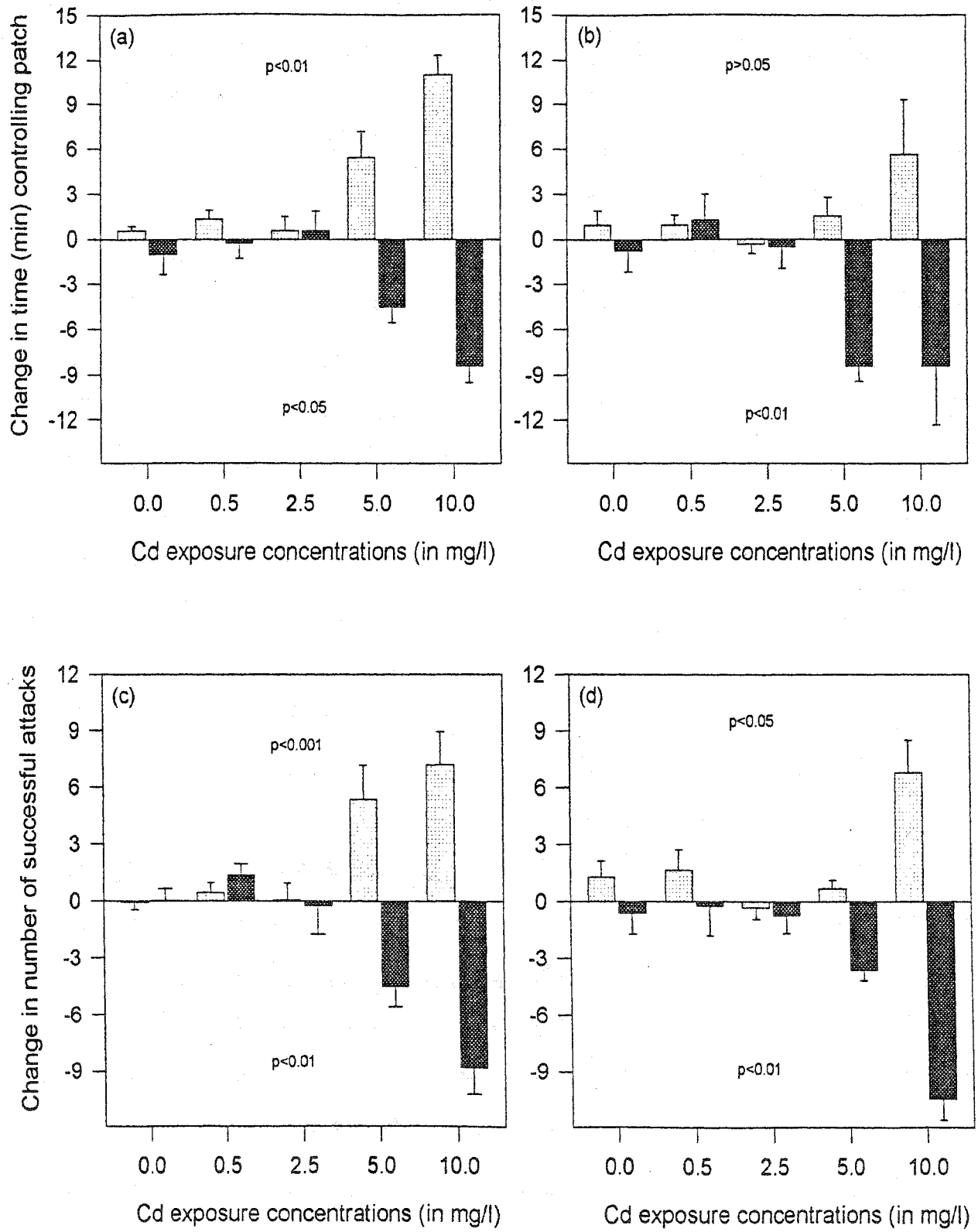
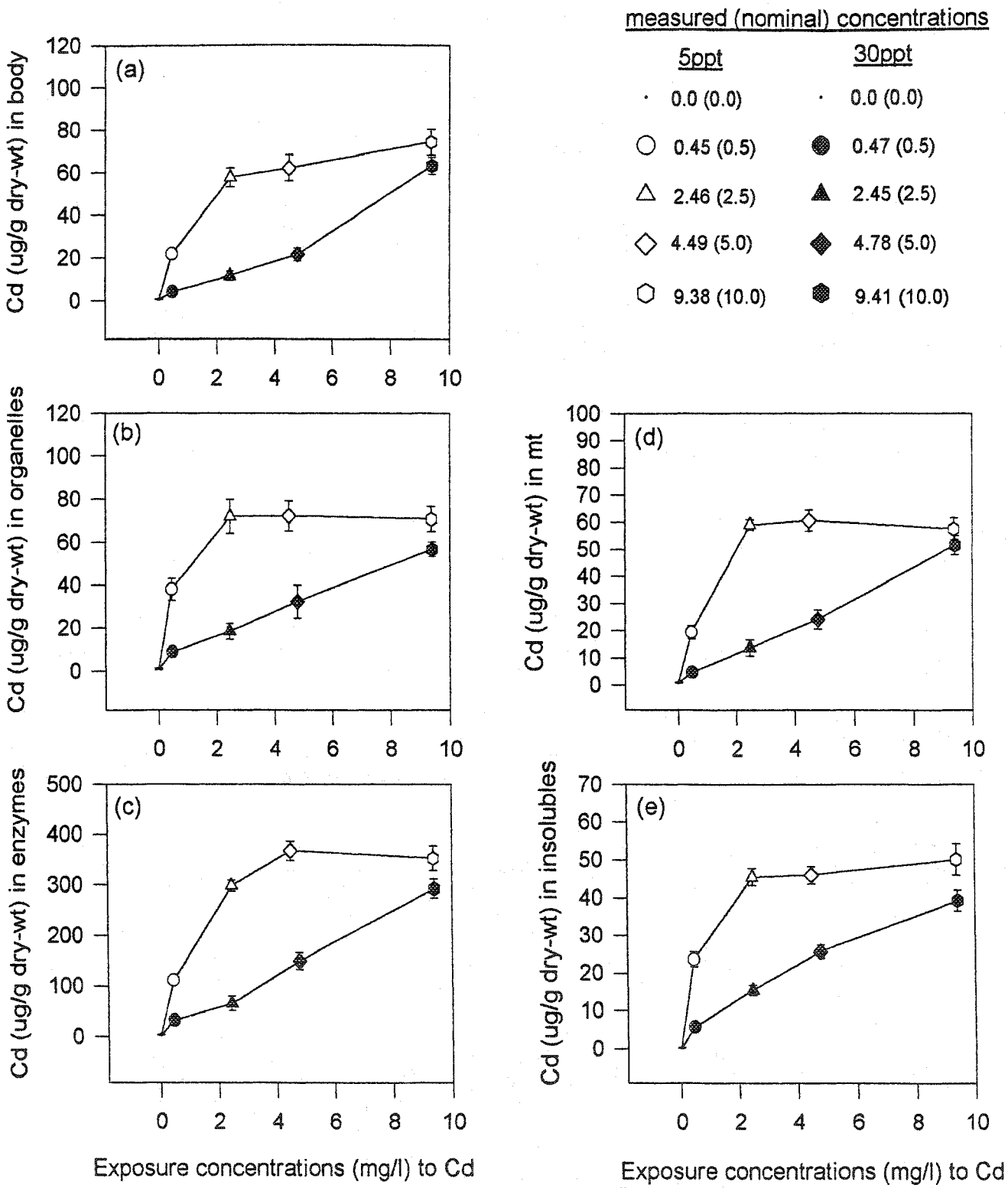


Fig. 16: Bioaccumulation of Cd at different concentrations and salinities in (a) total body, (b) organelles, (c) enzymes, (d) metallothioneins, and (e) insolubles in fiddler crabs *Uca pugnax*



§ Chapter Five §

BEHAVIORAL TOXICITY & BIOACCUMULATION OF SILVER IN *UCA* CRABS

I. Toxicity of Silver

A. Background:

Silver (Ag) is a naturally occurring precious metal known to ancient people in the Mediterranean region as early as 4000 B.C. Ag is a geologically rare element. It is found in the native state or associated with copper, gold and lead (Berman 1980). It is a trace metal with no known biological function (Bell and Kramer 1999, Fisher *et al.* 1996, Fisher and Wang 1998).

The Ag ion is one of the most toxic metals, surpassed only by mercury and thus has been assigned, together with Cd, to the highest toxicity class (Ratte 1999). Aquatic organisms readily accumulate Ag ions, which have a particularly high affinity for sulfhydryl groups, and thus bind predominantly to proteins (Bell and Kramer 1999 and Connell *et al.* 1991). As a type B metal, Ag exhibits a very high affinity for sulfur (Berman 1980). This affinity for sulfur leads to its strong association with proteins and its potential interference with protein metabolism. Consequently, the metal inactivates sulfhydral enzymes and also combines with amino, imidazole, carboxyl and phosphate groups (Bell and Kramer 1999, Fisher and Wang 1998). Ag is also likely to inhibit important enzymes. It exerts its toxicity by inhibiting the Na^+/ K^+ -ATPase activity, which leads to the blocking of active Na^+ and Cl^- uptake at the gills and therefore inhibits osmoregulation by aquatic organisms (Bury *et al.* 1999a, Guadagnolo *et al.* 2001, Hogstrand and Wood 1998, Morgan *et al.* 1997, Wood *et al.* 1999).

Silver is used in the manufacture of electronics, silverware, jewelry and plating (Berman 1980, Browning 1969). Many silver compounds, such as silver nitrate, silver chloride and silver oxides, have wide commercial applications. The most extensive uses are in batteries and photographic industry; the latter is considered the major source of Ag

to the environment (Hogstrand *et al.* 1996, Patnaik 2002, Purcell and Peters 1998). Wastes arising from such commercial uses of Ag are often discharged to wastewater treatment facilities, and potentially high concentrations of Ag (three orders of magnitude higher than the receiving waters) may be discharged to receiving estuaries (Shafer *et al.* 1998).

B. Toxicity of Ag in aquatic environment:

Since Ag is an element, it does not break down once it is released into the aquatic environment, but it can change its form by combining with other substances (U.S. DOH 1990). Depending on Ag species, the toxicity of Ag ranges through several orders of magnitude. Ag is found in the environment in four oxidation states: 0, 1+, 2+, and 3+, with the first two being the most common (Ferguson and Hogstrand 1998, Purcell and Peters 1998). The free Ag ion in the form of silver nitrate is the most toxic form of Ag (Hogstrand *et al.* 1996). It is known to be highly toxic to aquatic animals, and it is extremely reactive to dissolved ligands, such as, thiosulfate, sulfide, chloride and dissolved organic carbon (Hogstrand and Wood 1998). The U.S. Environmental Protection Agency has therefore imposed strict water criteria for Ag; the Maximum Permissible Concentration of Ag in natural water is only 13.4 parts per billion (U.S. EPA 1987). Silver thiosulfate, a highly soluble compound and a main component of wastewaters of photoprocessors, is less toxic than silver nitrate (Gorsuch and Klaine 1998, Morgan *et al.* 1997, Ratte 1999). Silver sulfide is the least toxic of all tested silver compounds because of its low solubility and bioavailability; it is however the most predominant form of Ag under reducing conditions in soil, sludge and sediments (Andren and Armstrong 1999, Nicholas *et al.* 1998, U.S. DOH 1990). Silver in sediments however can be remobilized and resuspended in water through bioturbation and changes in

chemistry of pore and overlaying water (Call *et al.* 1999, Rouleau *et al.* 2000). The toxicity of Ag in estuarine environments, where there is potential of silver chloride complexation, is and will be an important issue for investigation (Ratte 1999).

C. Effect of salinity on Ag toxicity:

The behavior of Ag in the environment makes it an element of concern as a contaminant in estuarine ecosystems (Luoma *et al.* 1995). It is well documented that the toxicity of Ag in the aqueous environment depends on the concentration of active free Ag. Accordingly, many water characteristics reduce Ag toxicity by binding free Ag ions with colloids and particles in wastewaters, and with chlorides in receiving estuarine waters (Andren and Armstrong 1999, Gorsuch and Klaine 1998, Peng *et al.* 2002). The salinity of brackish water greatly affects the chemical behavior of Ag. As the chloride concentration is increased, the activity of Ag ions decreases, and the anionic chloro complex ($AgCl_n^{n-1}$) becomes the predominant dissolved Ag species (Erickson *et al.* 1998, Ferguson and Hogstrand 1998, Hogstrand and Wood 1998, Voyer *et al.* 1982, Wood *et al.* 1999). Thus, instead of precipitating in the estuarine environment, chloro-complexation favors the retention of at least some Ag in the dissolved form that can be dispersed from anthropogenic input sources (Luoma *et al.* 1995). In the grass shrimp *Palaemonetes pugio*, the effect of salinity on the uptake of Ag was examined. Ag uptake decreased with increasing salinity. By binding to Ag ions, chloride ions reduce the concentration of free Ag ions (Ratte 1999). In rainbow trout *Oncorhynchus mykiss*, increasing the concentration of chloride ions in water protected against the inhibition of Na^+/K^+ -ATPase activity (Ferguson and Hogstrand 1998, McGeer and Wood 1998, Webb and Wood 1998). However, the effect of salinity on Ag toxicity depends on the study organism. For instance, in acute toxicity tests, chloride was protective against Ag toxicity

in rainbow trout, but it had no effect in fathead minnows, *Pimephales promelas* (Bury *et al.* 1999b, Galvez and Wood 1997, Karen *et al.* 1999). There is still a demand for further toxicity studies in salinity ranges typical to estuarine environments where Ag concentrations might be high and the influence of salinity may be considerable (Hogstrand and Wood 1998).

D. Bioaccumulation of Ag in aquatic organisms:

Impact of Ag on aquatic ecosystems is an area of research that investigators have been relatively late in exploring. While the toxicities of other metals, such as Cd, have been studied extensively, the literature on the effects of Ag on aquatic organisms is limited (Hogstrand and Wood 1998). The strong bioaccumulation of Ag in marine benthic organisms is of major concern since there may be potential for long-term sublethal effects in these organisms (Ratte 1999). Several studies show that Ag is the most strongly bioaccumulated metal by at least some marine and estuarine invertebrates, and it is one of most readily accumulated metal by phytoplankton (Berry *et al.* 1999, Fisher *et al.* 1996, Luoma *et al.* 1995, Wang *et al.* 1996). The bioavailability of Ag disproportionately increases as compared to the increase in its contamination level (Bryan and Langston 1992). The strong bioaccumulation of Ag in estuarine environment may partly result because the neutral chloro complex ($AgCl^{\circ}$) is highly available. It is suggested that the low polarity of this complex facilitates its diffusion across biological membranes (Bryan and Langston 1992, Engel *et al.* 1981, Hogstrand and Wood 1998). Moreover, the gill epithelium of aquatic animals contains negatively charged binding sites, which are due to phosphate, carboxyl, amino, sulfate groups and others. Ag ions are attracted to these negative binding sites (Janes and Kleinow 1995). In estuaries, the

bioaccumulation of Ag increases so steeply with contamination, thus, the range of tolerance to Ag in estuaries could be narrower than for many metals (Luoma *et al.* 1995).

II. Methods

A. Behavioral studies:

Male fiddler crabs *U. pugnax* 18-20 mm in carapace width were collected from Meredith Creek marsh on Staten Island, NY. They were used in various treatments to assess the effects Ag with changing salinity. A total of 9 treatments were conducted, and each included assessment of various behavioral parameters prior to metal exposure (Trial), followed by exposure to metals and subsequent re-assessment of behavior (Trial 2). The exposure conditions of each treatment are described in Table 5. All behavioral studies were conducted in a temperature-controlled room at 24°C.

Table 5: List of treatments investigating the individual effects of Ag and salinity on behavior of fiddler crabs

Treatment #	Metal(s)	Concentration (mgl^{-1}) Nominal	Observed	Molarity ($\mu molel^{-1}$)	Salinity (ppt)
1	Ag	0.10	0.03	0.93	5
2	Ag	0.48	0.10	4.45	5
3	Ag	0.48	0.10	4.45	30
4	Ag	2.4	0.33	22.3	5
5	Ag	2.4	0.39	22.3	30
6	Ag	4.8	0.73	44.5	5
7	Ag	4.8	0.83	44.5	30
8	Ag	9.6	1.40	88.9	30
9	Ag	16.0	1.45	148.0	30

For each treatment, crabs (n=16) of similar carapace width were removed from tanks, and were placed individually in labeled plastic cups with mesh bottoms that were placed in a fish tank filled with filtered water (salinity ~ 15ppt). Crabs were allowed to depurate for 48 hours. At the end of depuration period, each crab was placed in a 250 ml acid-washed Erlenmeyer flask containing 100 ml of Nano® pure water of corresponding salinity for a 72-hour period (Instant Ocean™ salt was used to prepare solutions). This holding was included in Trial 1 to mimic the conditions during exposure to metals in Trial 2. Crabs were then paired at random and were placed in a 15 x 20 cm opaque plastic container containing a ~1 cm layer of Meredith marsh mud (a shallow layer of mud was used to prevent crabs from burrowing). In the center of mud, a 1.5 cm diameter protein-rich patch (of crushed Freeze Dried Plankton™ containing a minimum of 69% crude protein) was placed on mud surface for the crabs to compete for. While in the container, each pair of crabs was videotaped by a JVC Digital Signal Processing camcorder for one hour. Tapes were immediately reviewed and data on competitive foraging behavior (total number of scoops and number of scoops on protein-rich patch), and on competitive dominance behavior (time in control of the patch and aggressiveness/ number of successful attacks) of each crab were collected. When viewing video footage, it became obvious that one crab from each pair was dominant and the other was submissive. Dominance was assigned to the most aggressive crabs (i.e. the crabs that had most successful attacks are here referred to as dominant, those crabs with less or no successful attacks are referred to as submissive). At the end of the videotaping session, each crab was allowed to feed individually on fresh Meredith marsh mud in a labeled plastic container of 20 cm in diameter for 48 hours. This feeding is referred to de-acclimation.

In Trial 2, crabs were transferred to labeled depuration chambers as before to allow the evacuation of gut contents. This second depuration period also lasted 48 hours. Dominant crabs were exposed in flasks containing 100-ml metal(s) solution at the corresponding Ag concentration and salinity (see Table 5). Metal solutions were prepared using Perkin Elmer ultra pure metal solution. Submissive crabs were placed in similar flasks, each containing 100 ml of metal-free solution to mimic exposure. Exposure lasted 72 hours. Subsequent to exposure, crabs were then re-paired as in Trial 1 and were videotaped for a second one-hour period. Tapes were reviewed and behavioral data were again collected. At the end of Trial 2, crabs were depurated for 48 hours. Subsequently, crabs were stored individually in zip lock bags at -80°C.

B. Fractionation procedure and sample preparation:

Metal-exposed and control crabs were subjected to subcellular fractionation as described by Wallace *et al.* 2003 (Figure 6). Crabs (four dominant and four submissive from each treatment) were thawed on ice. Then, each crab was placed in a 50 ml Nalgene® centrifuge tube containing 5.8 ml of 20 mM Tris buffer solution (prepared by adding 3.152g of J.T. Baker Ultrapure Grade TRIS Hydrochloride to 1 liter of Nano® pure water and adjusted to pH of 7.6). Crabs were then homogenized using Polytron® PT 2100 homogenizer. Subsequent to homogenization, a one-milliliter sample was removed from the centrifuge tube and was transferred to a pre-weighed vial for future analysis of total body burden. Four subcellular fractions were prepared from the homogenate by differential centrifugation. The resulting pellets (P3) were defined as the insoluble fraction (1,950g for 15 minutes, twice at 4,400g for 15 minutes), organelles were defined as pellets (p4) (1,950g for 15 minutes, 103,000g for 1 hour), pellets (P5) and supernatant

(S5) were defined as the enzyme- and Metallothionein-containing fractions respectively (S4 centrifuged at 20,000g for 30 minutes) (Figure 6).

Crab tissues and subcellular fractions were digested for metals following standard protocol. Vials containing subcellular fractions were dried in a drying oven at 65°C, and then they were weighed. Concentrated HNO_3 (5 ml) was then added to each vial. Samples were refluxed until tissues dissolved. Samples were then evaporated to dryness, resuspended in 10 ml of 2% Ultragrade HNO_3 and filtered through .45 μm Millipore filters. Filtered samples were then analyzed for Ag using Perkin Elmer model 3100 atomic absorption spectrophotometer. Quality Control and Quality Assurance samples included (1) standard tissue samples, (2) fractionation procedural blanks, (3) digestion blanks and (4) filter blanks. During analysis, background and standards were periodically run to verify the accuracy of the instrument. If background or standard samples did not meet QA/QC the instrument was recalibrated.

III. Results

A. Effects of exposure to Ag on competitive behaviors of *Uca* crabs:

In Trial 1, dominant crabs consistently had more total scoops than submissive crabs (~74 vs. ~33 scoops, Mann Whitney U-test, $p<0.05$) (Figs. 17-20, a-b), and had more scoops on protein-rich patch (~39 vs. ~8 scoops, $p<0.01$) (Figs. 17-20, c-d). They also spent more time in control of the patch (~13 vs. ~2 minutes, $p<0.01$) (Figs. 17-20, e-h), and had a higher percentage of successful attacks (~98 vs. ~8 percent of attacks on average, $p<0.01$) (Figs. 17-20, g-h); again, this was the criterion used to determine "dominance". Additionally, there was no influence of salinity ($P>0.05$) on the behavior of either dominance or submissive fiddler crabs (Figs. 17-20, a-h) (see Appendix A).

After exposure of dominant crabs to 0.48 and 2.4mg/l Ag (nominal concentrations) at both salinities, foraging and dominance behavior parameters in dominant and submissive crabs was unaltered as compared with Trial 1 ($p>0.1$) (Fig. 17-17a-h). These exposure concentrations represent the no observed adverse effect levels (NOAEL) for Ag.

Subsequent to an exposure of 4.8mg/l Ag (nominal concentration) (Trial 2), dominant crabs had ~50% less total number of scoops ($p<0.05$) at 5ppt and 30ppt than prior to exposure (Trial 1) (Figs. 19a,b). Total number of scoops by submissive crabs however were ~100% greater ($p<0.05$) at 5ppt and 70% greater ($p<0.01$) at 30ppt (Figs. 19a-b) as compared to Trial 1. Also after exposure, number of scoops on patch by dominant crabs decreased by 80% ($p<0.001$) at 5ppt and by 40% ($p<0.05$) at 30ppt (Figs. 19c,d). That of submissive crabs increased by 650% ($p<0.05$) at 5ppt and by 210% ($p<0.01$) at 30ppt (Figs. 19c-d). Also, after their exposure, dominant crabs spent ~70% less time ($p<0.01$) at 5ppt and 30ppt in control of the patch (Figs. 19e,f). Time spent by submissive crabs on patch increased 500% ($p<0.01$) at 5ppt but remained unchanged ($p>0.1$) at 30ppt (Figs. 19e,f). After exposure to 4.8mg/l Ag, the percentage of successful attacks by dominant crabs dropped by 50% ($p<0.01$) at 5ppt but was unchanged ($p>0.1$) at 30ppt. The percentage of successful attacks by submissive crabs increased by 50% ($p<0.01$) at 5ppt but was unchanged at ($p>0.1$) at 30ppt (Figs. 19g-h).

Fig. 16 shows the effects of exposure to Ag concentrations of 9.6 and 16.0mg/l (nominal concentrations) at 30ppt. After exposure (Trial 2) of dominant crabs to Ag, total number of scoops declined by 35% ($p<0.05$) at 9.6mg/l Ag, and by 70% ($p<0.001$) at 16.0mg/l Ag (Figs. 20a,b). Total number of scoops by submissive crabs increased by 100% at 9.6mg/l (marginally significant, $p=0.07$), and by 160% ($p<0.01$) at 16.0mg/l

(Figs. 20a,b). Also after exposure, number of scoops on patch of dominant crabs was unchanged ($p>0.1$) at 9.6mg/l, but decreased by 85% ($p<0.001$) at 16.0mg/l (Figs. 20c,d). Number of scoops on patch of submissive crabs in Trial 2 increased by 175% ($p=0.05$) at 9.6mg/l, and by 400% ($p<0.001$) at 16.0mg/l (Fig. 20c,d). After exposure of dominant crabs, time spent in dominating the patch dropped by 40% ($p<0.05$) at 9.6mg/l, and by 90% ($p<0.001$) at 16.0mg/l (Figs. 20e,f). Time spent by submissive crabs in dominating the patch was unchanged ($p>0.1$) at 9.6mg/l, but increased by almost 700% ($p<0.001$) at 16.0mg/l (Figs. 20e,f). The percentage of successful attacks by the dominant crabs was unchanged ($p>0.1$) at 9.6mg/l Ag, and decreased 80% ($p<0.001$) at 16.0mg/l (Figs. 20g,h). Whereas, the percentage of successful attacks by submissive crabs in Trial 2 was unaltered ($p>0.1$) at 9.6mg/l, submissive crabs were more successful by 80% ($p<0.001$) at 16.0mg/l (Figs. 20g,h).

Post-exposure changes in competitive behavior of submissive and dominant fiddler crabs are summarized in Figs. 21-22. As Ag exposure concentration was increased from 0-9.6mg/l at 5ppt; the foraging and dominance behavior of dominant crabs became impaired (Kruskal-Wallis ANOVA, $p<0.05$), while that of submissive crabs improved ($p<0.05$) (Figs. 21a,c and Figs. 22a,c). At 30ppt, foraging behavior of dominant crabs decreased ($p<0.05$) with the increase in Ag exposure levels (Figs. 21b,d), dominance behavior was unaltered (Figs. 22b,d). Foraging and dominance behaviors of submissive crabs were unchanged ($p>0.05$) with the increase in exposure concentrations (Figs. 21b,d and Figs. 22b,d).

B. Bioaccumulation of Ag in fiddler crabs:

Fiddler crabs were exposed to a series of Ag solutions (0.10, 0.48, 2.4 and 4.8mg/l) at 5ppt, and to Ag solutions (0.48, 2.4, 4.8, 9.6, and 16.0mg/l) at 30ppt. For each

exposure concentration, the bioaccumulation of Ag in various fractions was determined. Results are summarized in Figs. 23a-e.

The background concentrations of Ag in total body burden, organelles, enzymes, metallothioneins (MT) and insoluble fractions were 0.18, 0.35, 0.61, 0.23 and 0.31 μg/g dry-weight respectively. After the exposure of crabs to 0.10mg/l Ag at 5ppt, the Ag concentrations increased significantly (t-test, $p<0.01$) in all fractions. Specifically, Ag concentrations increased by 22-fold in total body fraction (Fig. 23a), by ~23-fold in the organelles and enzymes (Figs. 23b-c), and by 13-fold in the MT and insoluble fractions (Figs. 23d-e). As Ag exposure concentration was increased to 0.48, 2.4 and 4.8mg/l at 5ppt; the only fraction that showed a significant increase ($p<0.05$) in Ag level was the metal-sensitive enzymes (Fig. 23c).

At 30ppt, the exposure of crabs to 0.48mg/l Ag led to a significant increase ($p<0.01$) in Ag concentrations in all fractions as compared to background levels (Figs. 23a-e). Specifically, there was a 30-fold increase ($p<0.01$) of Ag in whole body (Fig. 23a), a 40-fold increase ($p<0.001$) in organelles (Fig. 23b), an almost 30-fold increase ($p<0.01$) in enzymes, and a 30- to 40-fold increase ($p<0.05$) in MT and insolubles fractions respectively (Figs. 23d-e). As the exposure concentration was increased to 2.4mg/l (a 4-fold increase), organelles were the only fraction to accumulate ($p<0.01$) more Ag (Fig. 23b). As the exposure concentration of Ag was doubled to 4.8mg/l, only the enzyme fraction accumulated more Ag ($p<0.05$) (Fig. 23c). The increase in Ag exposure concentrations to 9.6 and 16.0mg/l did not result in any significant increase ($p>0.1$) in Ag levels in any fraction (Figs. 23a-e).

In conclusion, the background Ag concentrations were similar in all fractions ($p>0.05$) at 5ppt and 30ppt. The exposure of crabs to 0.48 and 2.4mg/l Ag (NOAEL), and

to 4.8mg/l (LOAEL) Ag at both salinities did not affect the bioaccumulation ($p>0.05$) of the metal in organelles, enzymes and MT (Figs. 23b-d). The only fraction where increase in salinity had a slight significant effect ($p=0.06$) in reducing Ag accumulation is the insoluble fraction (Fig. 23e).

IV. Discussion

A. Introduction

Competitive foraging and dominance behaviors in fiddler crabs *Uca pugnax* were used as the endpoints to determine the toxic effects of Ag. A behavioral toxicological response is believed to occur when the exposure to a pollutant leads to a significant deviation in an organism's normal behavior (Depledge *et al.* 1995). This current research showed that the exposure of fiddler crabs to aqueous Ag at nominal concentrations ≤ 22.3μM had no short-term effects on fiddler crabs' competitive behavior. In addition, salinity of exposure did not affect the toxicity of Ag at or below 22.3μM. Also, dominant crabs exposed to Ag at or below the no-observed-adverse effect concentration of 22.3μM consistently forage more than submissive crabs at both salinities. It was demonstrated that, prior to exposure to Ag, salinity had no effect on competitive foraging as well as dominance behaviors of both dominant and submissive fiddler crabs.

B. Effects of Ag on the behavior of fiddler crabs: Protective effect of salinity

As with exposure to Cd, exposure of fiddler crabs to 44.5μM Ag (nominal concentration)(LOAEL) led to a greater than 40% drop in foraging behavior parameters of dominant fiddler crabs at both salinities. But unlike the case with Cd exposure -where only at 5ppt did submissive crabs forage more after the exposure of their counterparts at 44.5μM - the foraging parameters of submissive crabs has significantly increased after

exposure of dominant crabs to 44.5μM Ag at both 5ppt and 30ppt. It is believed that changes in foraging behavior of submissive crabs maybe impacted by the change in dominance behavior of their counterparts after exposure to either metal. It is also noticed that after the exposure of dominant crabs to 44.5μM Cd at 5ppt, the submissive crabs became as aggressive as their 'dominant' counterparts. However, the exposure of dominant crabs to 44.5μM Ag at 5ppt led to an increase in aggressiveness of 'submissive' crabs over their 'dominant' counterparts. On the other hand, the exposure of dominant crabs to 44.5μM Cd concentration at 30ppt did not impact the aggressiveness of dominant or submissive crabs. After the exposure of dominant crabs to 44.5μM Ag at 30ppt, the 'submissive' crabs became as aggressive as their 'dominant' counterparts.

It can therefore be concluded that, if after the exposure of dominant fiddler crabs to 44.5μM Cd or Ag (LOAEL's) the 'submissive' crabs became as or more aggressive than the 'dominant' crabs, then 'submissive' crabs would benefit by being able to forage more. If however, after exposure to 44.5μM Cd or Ag, 'dominant' crabs remained more aggressive than the 'submissive' crabs, the later would not be able to increase their foraging behavior parameters. This conclusion has many important implications. Firstly, not only does it suggest that dominance and foraging in fiddler crabs are interrelated, but also that any change in the dominance of one crab due to metal exposure can lead to a change in its own foraging behavior as well as its counterpart. Secondly, regardless of salinity, the exposure of dominant crabs to 44.5μM Ag has more devastating effects on their aggressive behavior than exposure to the same concentration of Cd. It is not surprising to find that Ag has a more disturbing effect on the behavior of fiddler crabs than Cd as bioassay tests conducted with estuarine invertebrates have shown that Ag is more toxic than Cd in comparable tests (Luoma *et al.* 1995). Thirdly, salinity has less of a

protective effect on dominance behavior in Ag-exposed fiddler crabs. This may be due to the higher toxicity of Ag, and to the fact that Ag chloro complex $AgCl^{\circ}$ that forms in seawater is toxic and highly bioavailable, whereas the Cd chloro complexes are not (Engel *et al.* 1981, Luoma *et al.* 1995, Peakall and Burger 2003).

The effects of exposure to 88.9μM Ag (nominal concentration) at 30ppt on competitive foraging and dominance behaviors in fiddler crabs are comparable to the effects of 44.5μM Ag at the same salinity (i.e. a drop in the foraging behavior parameters of exposed crabs with no change in their dominance behavior). One way to explain this is that the increase in the number of Ag ions at 88.9μM may have led to an increase in the formation of Ag chloro complexes, which are less toxic than free Ag ions (Hogstrand and Wood 1998, Wood *et al.* 1999). Fiddler crabs exposed to 148.0μM Ag (nominal concentration) at 30ppt showed a dramatic reduction in their foraging behavior and aggressiveness, which was paralleled by an increase in the foraging parameters and aggressiveness of unexposed 'submissive' crabs. At 148.0μM Ag, there are more free Ag ions available to react with chloride (McGeer and Wood 1998). It is suspected that as the ratio of Ag to Cl ions increases with the increase in Ag concentration, this may result in an increase in the availability of free toxic Ag ions (Ferguson and Hogstrand 1998).

Finally, it is worth mentioning that crabs exposed to 88.9μM and 148.0μM Ag at 5ppt did not survive. At this low salinity there are fewer chloride ions available to react with Ag, and most of the Ag would be present in its free ionic toxic form than in the less toxic chloro complex forms (Ferguson and Hogstrand 1998). The ability of fiddler crabs to survive Ag exposure concentrations of 88.9μM and 148.0μM at 30ppt may highlight the fact that the free Ag ions are more toxic to estuarine organisms than the Ag chloro complexes.

C. Relationship between Ag bioaccumulation and behavioral changes in *Uca* crabs:

1. Uptake and toxicity of Ag in *Uca* crabs:

Ag is one of the most toxic metals to estuarine organisms, surpassed only by Hg and Cu (Luoma *et al.* 1995). And like all other class B metals, Ag has a high affinity to S^- and N^- functional groups (Bell *et al.* 2002, Fisher and Wang 1998, Hook and Fisher 2002, Rainbow 1997, Ratte 1999, Smith *et al.* 2002, Wang *et al.* 1996). In estuarine organisms, Ag exerts its toxicity by disrupting osmoregulation through the inhibition of gill enzymatic activities responsible for Na^+ and Cl^- balance (Bianchini and Wood 2002, Brauner and Wood 2002, Grosell *et al.* 2002, McGeer and Wood 1998, Morgan *et al.* 1997, Morgan *et al.* 2004, Paquin *et al.* 2002, Ratte 1999, Skaggs and Henry 2002, Ward and Kramer 2002, Wood *et al.* 1999).

In estuarine invertebrates, the gill is the main site for active transport of ions from water into the hemolymph (Bianchini and Wood 2002). As with Cd^+, the uptake of Ag^+ across the gill occurs either passively via carrier proteins, and/or through selective ATP energy-dependent ion pumps (Bianchini and Wood 2002, Hebel *et al.* 1997, Hogstrand *et al.* 2002, Phillips 1995, Rainbow 1997, Ratte 1999). Gill pump selectivity is largely by ion charge and size. Thus, Ag^+ with an ionic radius of 1.26°A which is intermediate between Na^+ (0.97°) and K^+(1.32°) has been shown to enter through Na/K-ATPase pumps (Bell *et al.* 2002). Some neutral inorganic complexed metals can passively diffuse through the lipid bilayer; however, metal ions are too hydrophilic to be transported through lipid bilayer of gill membrane (Rainbow and Black 2002). Unlike Cd chloro complexes, the neutral Ag chloro complex ($AgCl^0$) is highly bioavailable, and can passively diffuse through the gill membrane lipid bilayer (Bell *et al.* 2002, Bury *et al.* 1999b, Campbell *et al.* 2002, Hogstrand and Wood 1998, Luoma *et al.* 1995, McGeer and

Wood 1998). Thus, salinity plays an important role in the speciation of and, possibly, in its bioavailability and toxicity (Erickson *et al.* 1998, Peng *et al.* 2002).

2. Effects of salinity on speciation and uptake of Ag by *Uca* crabs:

Ag speciation depends on both salinity and Ag concentration (Ferguson and Hogstrand 1998). In freshwater and irrespective of Ag concentration, a substantial fraction of Ag is present as free ionic form Ag^+, and the rest is present as dissolved $AgCl_{aq}$ (Morgan *et al.* 1997, Webb and Wood 1998). But as salinity increases, the concentration of free Ag^+ decreases rapidly as more Ag forms chloro complexes ($AgCl_{aq}$, $AgCl_2^-$, $AgCl_3^{-2}$, $AgCl_4^{-3}$) (Campbell *et al.* 2002, Ferguson and Hogstrand 1998, Galvez and Wood 1997, Luoma *et al.* 1995, Ratte 1999, Ward and Kramer 2002) with the negatively charged chloro complexes dominating in seawater (Wood *et al.* 1999). Undoubtedly, while the ionic form of Ag (Ag^+) is most toxic since it is the most bioavailable form of the metal (Hogstrand and Wood 1998, Karen *et al.* 1999, Phillips 1995, Rainbow 1995, Ratte 1999), the uncharged and less toxic $AgCl_{aq}$ may be almost as available as the former (Bury *et al.* 1999b, Hogstrand and Wood 1998, Luoma *et al.* 1995). In contrast, the negatively charged Ag chloro complexes are much less toxic and largely unavailable (Hogstrand and Wood 1998, McGeer and Wood 1998, Ratte 1999).

In this study, fiddler crabs *Uca pugnax* were exposed to 0.93, 4.45, 22.3, and 44.5μM Ag solutions as $AgNO_3$ at 5ppt, and to 4.45, 22.3, 44.5, 88.9 and 148.0μM Ag as $AgNO_3$ solutions at 30ppt. As expected, crab Ag total body burdens increased at both salinities with the increasing Ag concentrations as compared to controls (Campbell *et al.* 2002, Galvez and Wood 1997, Hogstrand *et al.* 2002, Morgan *et al.* 1997, Ratte 1999, Wang *et al.* 1996). Inevitably, Ag^+ will bind to the crab carapace where it largely remains adsorbed to the chitinous exoskeleton. Gills are the major sites of Ag uptake. In the body,

Ag strongly binds to the muscle, hepatopancreas, hemolymph and gill of the exposed crabs (Rouleau *et al.* 2000). Once inside the cell, Ag binds non-specifically to metal-sensitive organelles and enzymes due to its high affinity to S^- and N^- groups. However, a significant fraction of the metal can be sequestered and perhaps detoxified by MT proteins and granules (Adams and Kramer 1999, Bell and Kramer 2002, Hogstrand and Wood 1998, Wallace *et al.* 2003). Interestingly, at the 4.45, 22.4 and 44.5μM Ag exposure concentrations, Ag body burden and Ag levels in subcellular fractions (i.e., organelles, enzymes and MT proteins) were unaffected by salinity. This may be explained by the increase in the passive uptake of $AgCl^o$ across the gill lipid bilayer membrane at high salinity. Specifically, it is expected that at both salinities crabs accumulate Ag (1) actively through ion pumps as Ag^+, and (2) passively through the gill lipid membrane as $AgCl^0$. As the salinity was increased from 5ppt to 30ppt, the concentration and the uptake of $AgCl^o$ are expected that to increase, and that of free Ag^+ to decrease. Though the subcellular accumulation of total Ag (including Ag^+ and $AgCl^0$) is similar at both salinities, it would be predicted that the cellular uptake of Ag^+:$AgCl^0$ could decrease with increasing salinity. On the other hand, *Uca* crabs accumulated slightly more Ag in their insoluble fraction (granules and exoskeleton) at 5ppt than at 30ppt. This may be explained by the ability of the exoskeleton chitin to bind only the free ionic forms of metals (Benguella 2002, Evans *et al.* 2002, Rouleau *et al.* 2000). Since more Ag^+ is expected to be present at 5ppt than at 30ppt, this accounts for the increase in Ag adsorption to crab exoskeletons at low salinity.

3. Mechanisms of behavioral toxicity of Ag in *Uca* crabs:

It is not unlikely that Ag, similar to Cu and Cd, can affect the integrity of crustacean gill membrane, hence causing a disruption in its ionoregulatory, water

permeability and gas exchange functions (Lawson *et al.* 1995, Rasmussen *et al.* 1995). Moreover, studies have shown that Ag can exert its toxicity on marine organisms by inhibiting enzymatic activities (Bianchini and Wood 2002, Brauner and Wood 2002, Grosell *et al.* 2002, McGeer and Wood 1998, Morgan *et al.* 1997, Paquin *et al.* 2002, Ratte 1999, Skaggs and Henry 2002, Ward and Wood 2002, Wood *et al.* 1999).

In this study, the manifestation of behavioral toxicity of Ag in *Uca* crabs at ≥44.5μM Ag exposure level coincided with a significant increase in the partitioning of Ag bound to enzyme fractions, i.e. as Ag exposure level was increased from 22.2μM (NOAEL) to 44.5μM (LOAEL), the Ag concentrations in the enzyme fractions increased significantly. It can be speculated that there may be link between the increase in Ag in enzyme fractions and the decrease in the competitive behaviors of Ag exposed *Uca* crabs. Interestingly, This change in competitive behaviors at 44.5μM Ag exposure was more pronounced at 5ppt than at 30ppt, but the bioaccumulation of Ag in the enzyme fractions was not affected by exposure salinity. This discrepancy might be explained by the fact that at high salinity a larger portion of the bioaccumulated Ag is in the bioavailable less toxic form of Ag i.e. $AgCl^0$ and may not readily bind with enzymes, whereas, at lower salinity, a larger portion of bioaccumulated Ag is in its highly toxic form i.e. Ag^+ that is known to have high affinity to S.

The exposure of *Uca* crabs to 88.9μM Ag (as $AgNO_3$) at 5ppt resulted in the deaths of a significant number of exposed crabs. This is likely caused by the presence of large portion of Ag in its highly toxic form i.e. Ag^+. At 30ppt however, crabs survived the exposure to 148.0μM Ag with no increase in Ag total body burden. This finding emphasizes the role of salinity in reducing the toxicity of Ag by forming negatively

charged Ag chloro complexes, it also shows that these complexes may not be biologically available and are not toxic to *Uca* crabs.

In agreement with previous findings, this study shows that salinity plays an important role in protecting against Ag toxicity by altering its speciation. Studies conducted by Bury *et al.* (1999b), Karen *et al.* (1999), Wood *et al.* (1999), Galvez and Wood (1997) and McGeer and Wood (1998) all showed that the presence of chloride substantially protected rainbow trout against Ag^+ toxicity by modifying speciation. Bury *et al.* (2002) also showed that salinity protects aquatic invertebrates against Ag^+ toxicity. However, the impact of salinity on Ag toxicity is not universal. For instance, studies by Bury *et al.* (1999b) and Karen *et al.* (1999) showed that salinity had little effect on reducing Ag^+ toxicity to fathead minnows. Hence, the toxicity of Ag is species specific, and this stresses the need for more studies on Ag using different organisms.

Fig.17: Effect of exposure to 0.48mg/l Ag on competitive foraging (a, b, c, d), and on competitive dominance (e, f, g, h) of *Uca* crabs at 5ppt (left) and 30ppt (right)

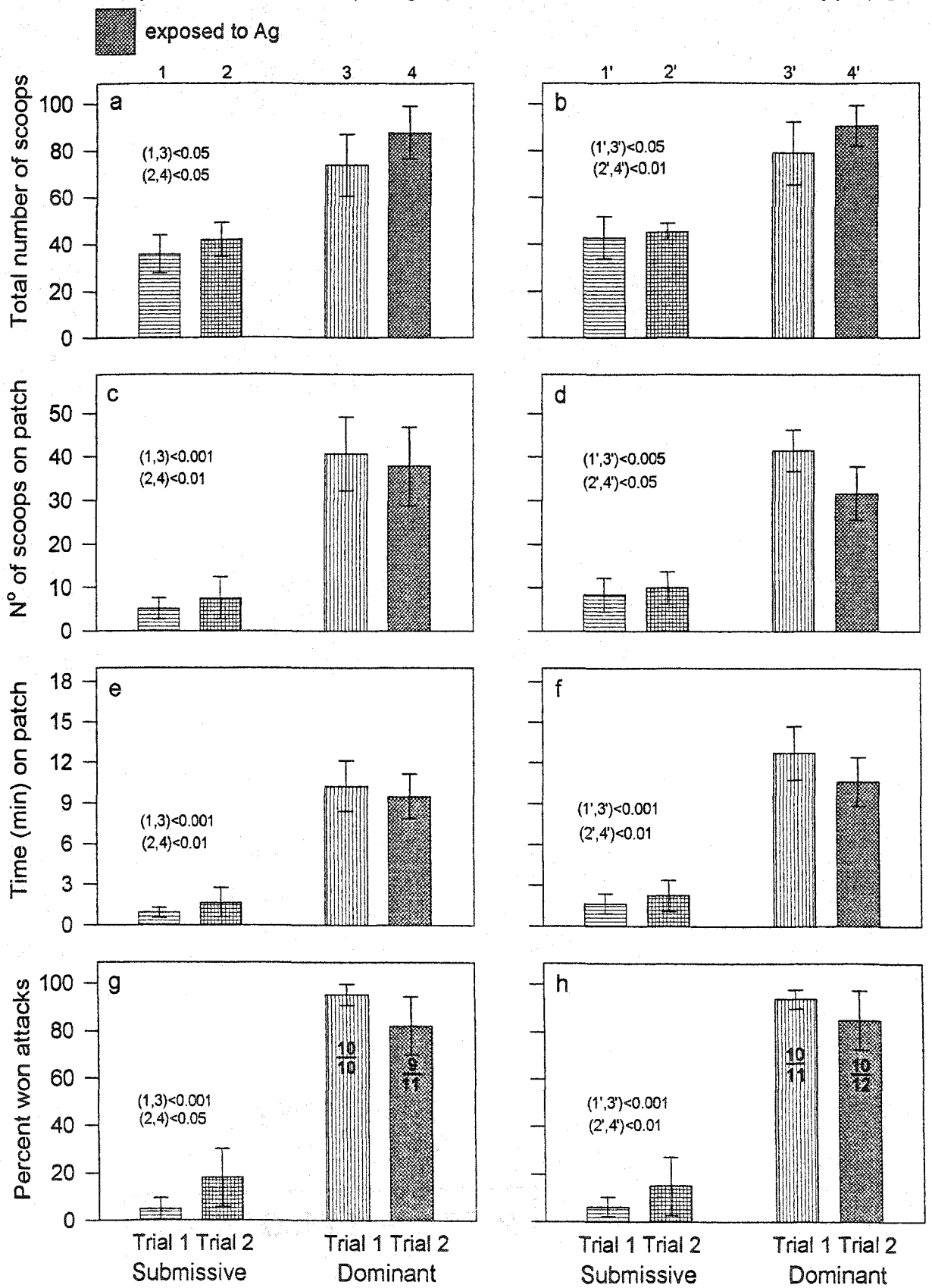


Fig.18: Effect of exposure to 2.4mg/l Ag on competitive foraging (a, b, c, d), and on competitive dominance (e, f, g, h) of *Uca* crabs at 5ppt (left) and 30ppt (right)

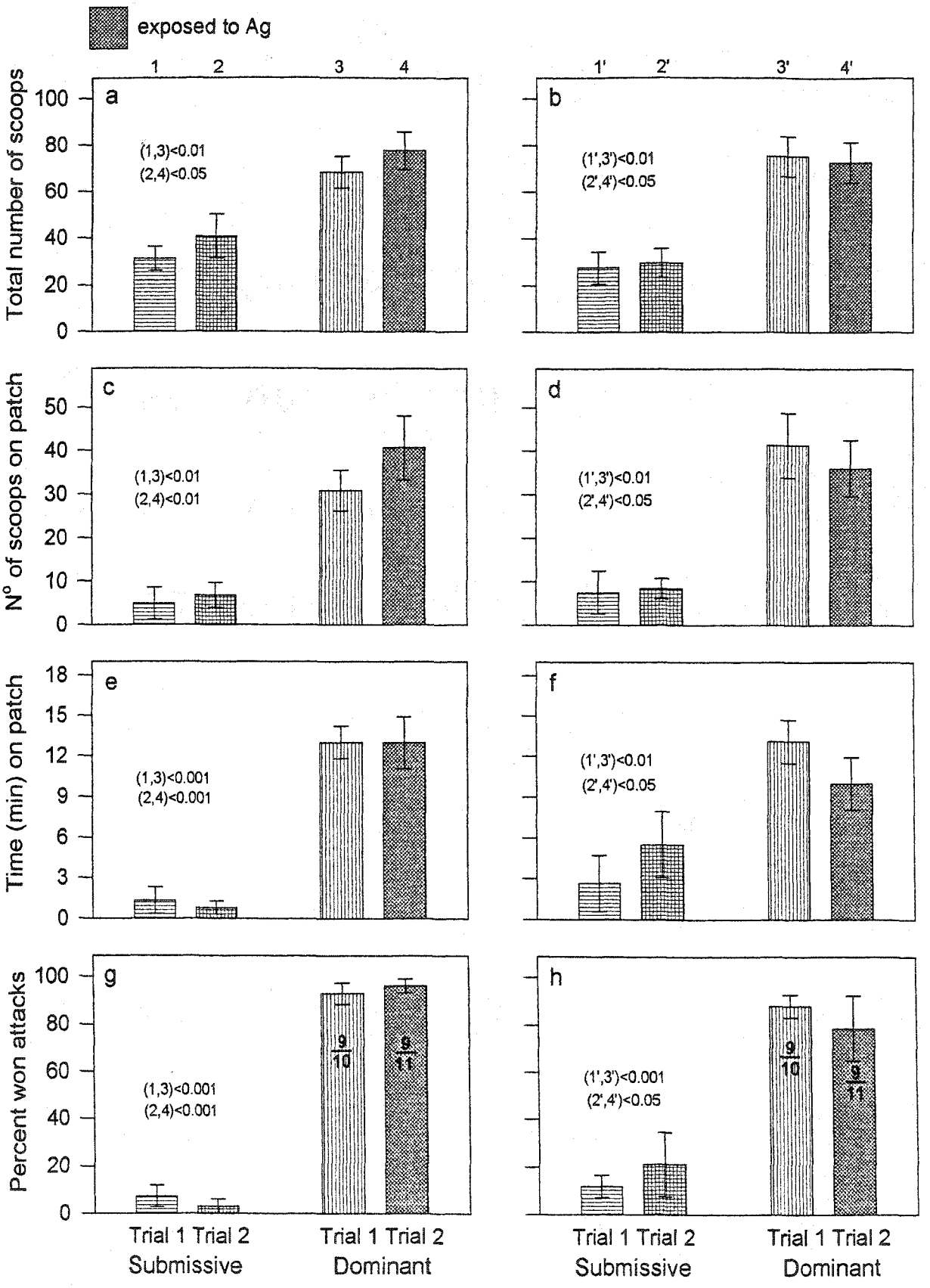


Fig.19: Effect of exposure to 4.8mg/l Ag on competitive foraging (a, b, c, d), and on competitive dominance (e, f, g, h) of *Uca* crabs at 5ppt (left) and 30ppt (right)

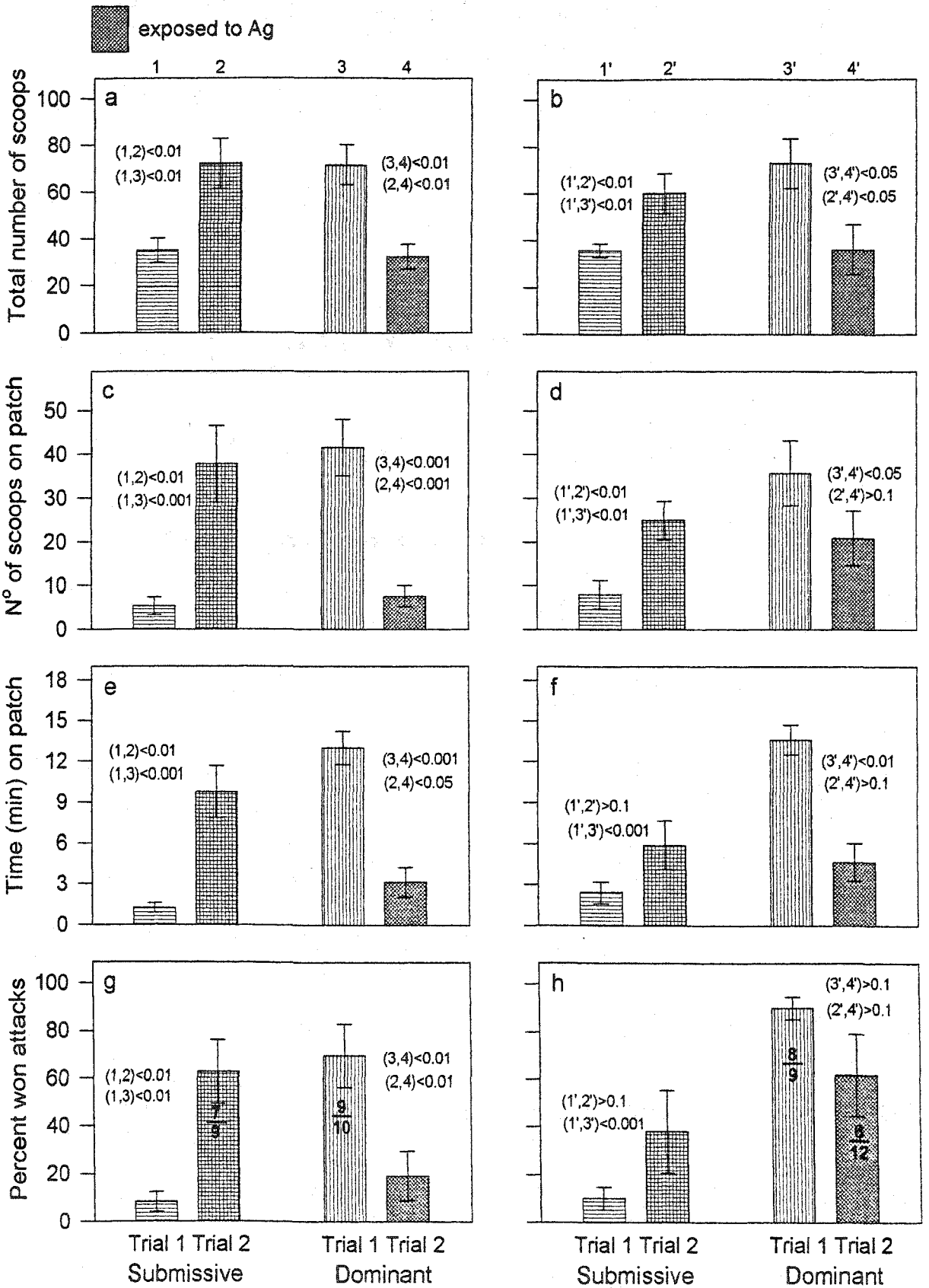


Fig.20: Effect of exposure to 9.6mg/l Ag (left) and 16mg/l Ag (right) at 30ppt on competitive foraging (a, b, c, d), and on competitive dominance (e, f, g, h) of *Uca* crabs

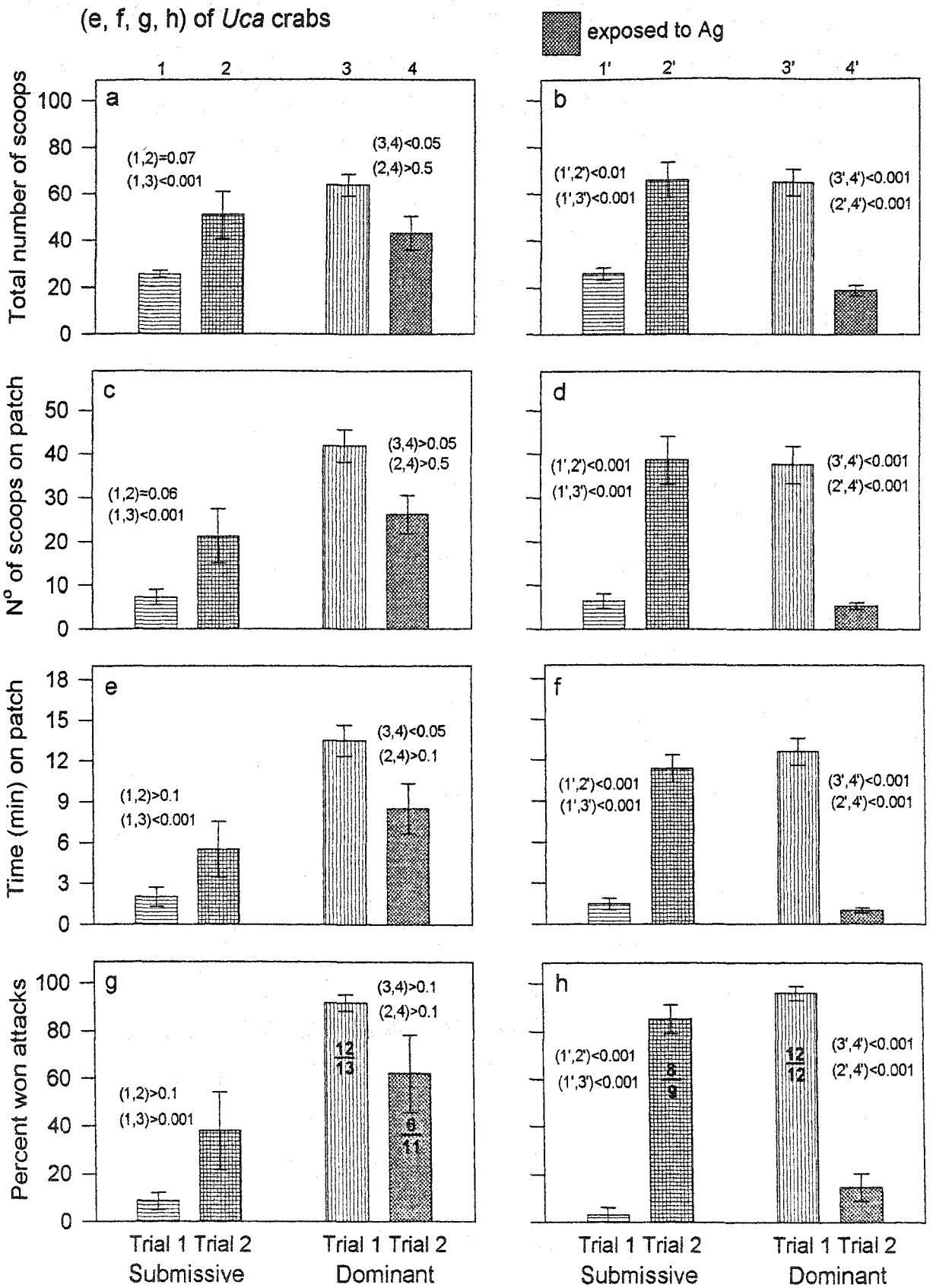


Fig. 21: Change in foraging behavior in terms of total scoops (a,b) and scoops on protein-rich patch (c,d) of submissive (gray bars) and Ag exposed dominant (black bars) *Uca* crabs, at both 5ppt (left) and 30ppt (right)

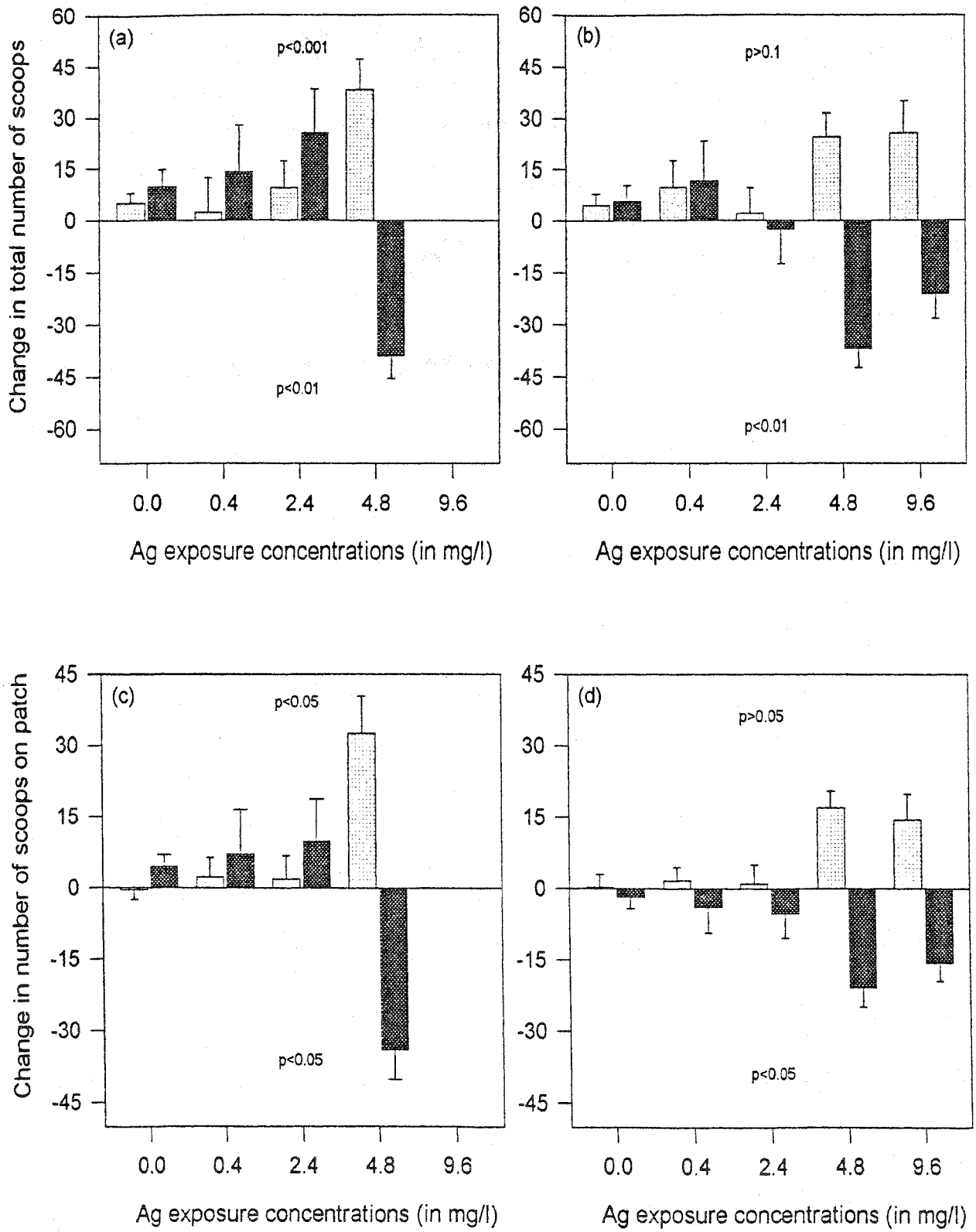


Fig. 22: Change in dominance behavior in terms of ability to control patch (a,b) and aggressiveness (c,d) of submissive (gray bars) and Ag exposed dominant (black bars) *Uca* crabs, at both 5ppt (left) and 30ppt (right)

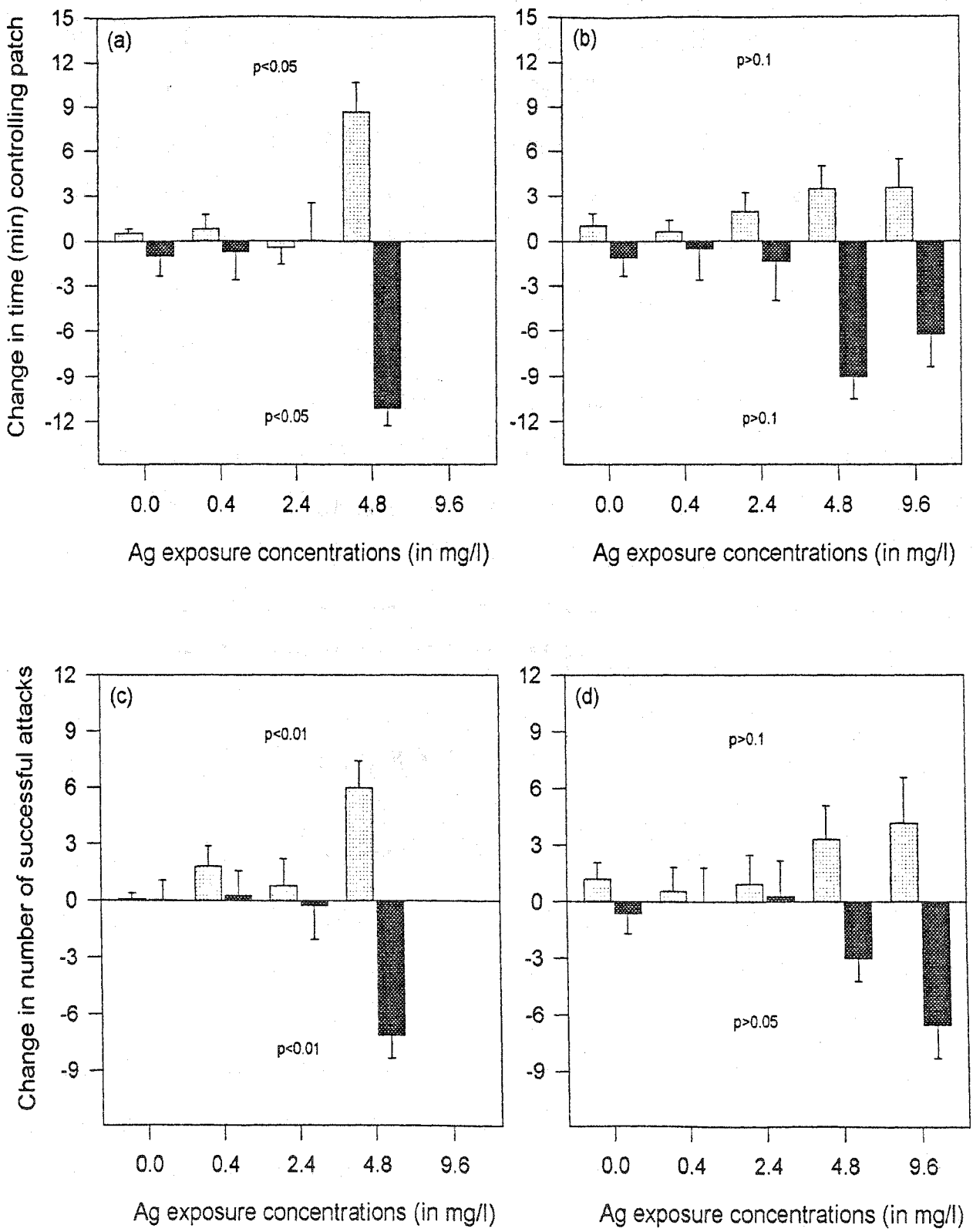
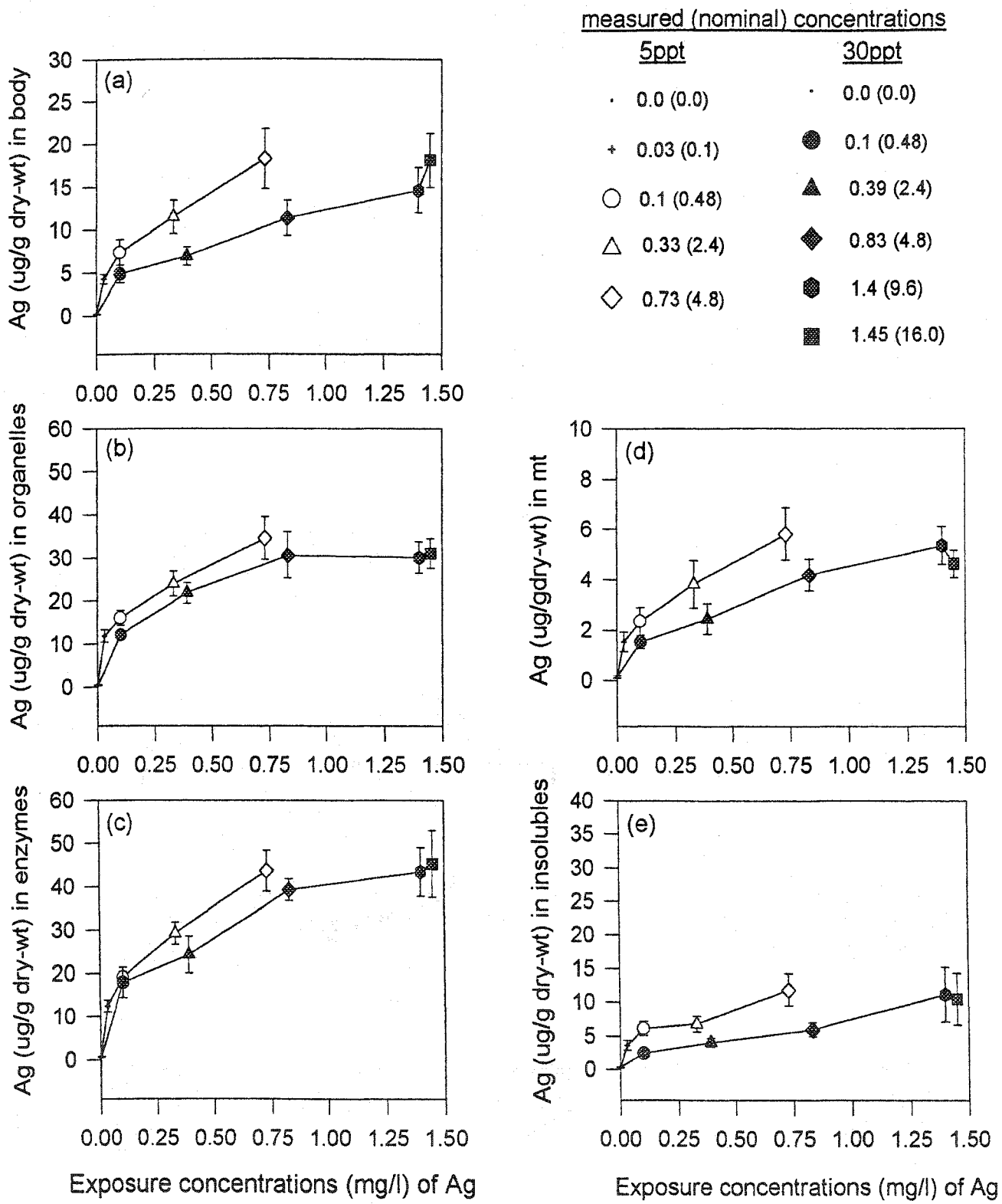


Fig. 23: Bioaccumulation of Ag at different concentrations and salinities in (a) total body, (b) organelles, (c) enzymes, (d) metallothioneins, and (e) insolubles in fiddler crabs *Uca pugnax*



§ Chapter Six §

BEHAVIORAL TOXICITY & BIOACCUMULATION OF CADMIUM AND SILVER MIXTURE IN *UCA* CRABS

I. Interactions of Metals

The toxicity of combinations of metals is of particular importance at the present time because aquatic ecosystems are often polluted with a mixture of metal pollutants rather than a single metal (Chu and Chow 2002, Preston *et al.* 2000, Segovia-Zavala *et al.* 2004). There is much less information available on the toxicity of mixtures of metals as compared to the toxicity of single metal (Preston *et al.* 2000, Schlekat *et al.* 2001). It is challenging to measure and evaluate the effects of metal mixtures because the combinations that they occur in nature are endless, and because physicochemical and physiological processes influence their effects (Peakall and Burger 2003). Although it is possible to examine the effects of two metals in the laboratory, the combination of three or more becomes problematic, particularly given the possible variations in concentrations (Peakall and Burger 2003, Schlekat *et al.* 2001).

When organisms are exposed to mixtures of metals, the interactions can be described as additive, synergistic, or antagonistic. Additive effects arise when the toxicity of mixture is equal to the sum of toxicities of the individual metals. Synergistic or antagonistic interactions arise when the toxicity of the mixture is greater than or less than the sum of the toxicities of the individual metals, respectively (Laws 1993, Preston *et al.* 2000, Ramade 1987). Toxicity of metal mixtures depends on their affinities to binding sites. For instance, if a more toxic metal in mixture has greater affinity for a key metalo-enzyme and causes greater conformational dysfunction than a competing metal, then additive or synergistic toxicity can result. But, if the more toxic metal in mixture has a strong binding affinity for metal storage proteins, this causes the less toxic metal to react in the cytosol, then antagonism can occur (Schlekat *et al.* 2001).

The toxic interaction between metals is evident but inconsistent (Khan *et al.* 1989); it varies between study organisms (Lee and Luoma 1998, Mance 1987). For instance, the exposure of the sandy shore gastropod *Nassarius festivus* to sublethal concentrations of metals (specifically Cu, Cd, Zn and Cr) had a negative effect on its feeding behavior (Cheung *et al.* 2002). However, the curlew bird, *Numenius arquata*, with elevated body burden of these metals showed no difference in foraging and breeding success when compared to birds from non-polluted areas (Currie and Valkama 1998). Hence, different species show different response to same metal exposure; this highlights the need to consider species differences in ecotoxicological risk assessment (Sauvé *et al.* 2002).

The importance of metals in estuarine environments derives from both their potential combined toxicity and excessive anthropogenic inputs. Cd and Ag can have a significant impact on estuarine ecosystems (Segovia-Zavala *et al.* 2004). The purpose of this study is to investigate the behavioral toxicity of Cd and Ag in *Uca* crabs.

II. Methods

A. Behavioral studies:

Male fiddler crabs *U. pugnax* 18-20 mm in carapace width were collected from Meredith Creek marsh on Staten Island, New York. Crabs were held in the laboratory awaiting use in behavioral studies. These crabs were used in various treatments to assess the combined effects of Cd and Ag with changing salinity. A total of 4 treatments were conducted, and each included assessment of various behavioral parameters prior to metal exposure (Trial 1), followed by exposure to metals and subsequent re-assessment of

behavior (Trial 2). The exposure conditions of each treatment are described in Table 6. All behavioral studies were conducted in a temperature-controlled room at 24°C.

Table 6: List of treatments investigating the combined effects of Cd and Ag at different salinities on behavior of fiddler crabs

Treatment #	Metal(s)	Concentration (mgl^{-1}) Nominal	Observed	Molarity ($\mu molel^{-1}$)	Salinity (ppt)
1	Cd, Ag	0.5, 0.48	0.5,0.2	4.45	5
2	Cd, Ag	0.5, 0.48	0.5, 0.4	4.45	30
3	Cd, Ag	2.4, 2.5	2.3,0.7	22.3	5
4	Cd, Ag	2.4, 2.5	2.3, 1.0	22.3	30

For each treatment, crabs (n=16) of similar carapace width were removed from tanks, and were placed individually in labeled plastic cups with mesh bottoms that were placed in a fish tank filled with filtered water (salinity ~ 15ppt). Crabs were allowed to depurate for 48 hours. At the end of depuration period, each crab was placed in a 250 ml acid-washed Erlenmeyer flask containing 100 ml of Nano® pure water of corresponding salinity for a 72-hour period. This holding was included in Trial 1 to mimic the conditions during exposure to metals in Trial 2. Crabs were then paired at random and were placed in a 15 x 20 cm opaque plastic container containing a ~1 cm layer of Meredith marsh mud. In the center of mud, a 1.5 cm diameter protein-rich patch (of crushed Freeze Dried Plankton™ with 69% crude protein) was placed on mud surface for the crabs to compete for. While in the container, each pair of crabs was videotaped for one hour. Tapes were immediately reviewed and data on competitive foraging behavior (total number of scoops and number of scoops on protein-rich patch), and on competitive

dominance behavior (time in control of the patch and aggressiveness/ number of successful attacks) of each crab were collected. When viewing video footage, it became obvious that one crab from each pair was dominant and the other was submissive. Dominance was assigned to the most aggressive crabs (i.e. the crabs that had most successful attacks are here referred to as dominant, those crabs with less or no successful attacks are referred to as submissive). At the end of the videotaping session, each crab was allowed to de-acclimate for 48 hours by feeding individually on fresh Meredith marsh mud in a labeled plastic container.

In Trial 2, crabs were transferred to labeled depuration chambers as before to allow the evacuation of gut contents. This second depuration period also lasted 48 hours. Dominant crabs were exposed in flasks containing 100-ml metal solution at the corresponding metal concentration and salinity (see Table 4). Metal solutions were prepared using Perkin Elmer ultra pure metal solution. Submissive crabs were placed in similar flasks, each containing 100 ml of metal-free solution to mimic exposure. Exposure lasted 72 hours. Subsequent to exposure, crabs were then re-paired as in Trial 1 and were videotaped for a second one-hour period. Tapes were reviewed and behavioral data were again collected. At the end of Trial 2, crabs were depurated for 48 hours. Subsequently, crabs were stored individually in zip lock bags at -80°C.

B. Fractionation procedure and sample preparation:

Metal-exposed and control crabs were subjected to subcellular fractionation as described by Wallace *et al.* 2003 (Figure 6). Briefly, crabs (four dominant and four submissive from each treatment) were thawed on ice. Then, each crab was placed in a 50 ml Nalgene® centrifuge tube containing 5.8 ml of 20 mM Tris buffer solution. Crabs were then homogenized using Polytron® PT 2100 homogenizer. Subsequent to

homogenization, a one-milliliter sample was removed from the centrifuge tube and was transferred to a pre-weighed vial for future analysis of total body burden. Four subcellular fractions were prepared from the homogenate by differential centrifugation. The resulting pellets P3) were defined as the insoluble fraction (1,950g for 15 minutes, 4,400g for 15 minutes), organelles were defined as pellets (P4) (1,950g for 15 minutes, 103,000g for 1 hour), pellets (P5) and supernatant (S5) were defined as the enzyme- and MT- containing fractions, respectively (S4 centrifuged at 20,000g for 30 minutes).

Crab tissues and subcellular fractions were digested for metals following standard protocol. Samples were then analyzed for Cd and Ag using Perkin Elmer model 3100 atomic absorption spectrophotometer.

III. Results

A. Effects of combined exposure to Cd and Ag on the behavior of fiddler crabs:

In Trial 1 (Figs. 24 and 25), dominant crabs consistently had more total scoops than submissive crabs (~80 vs. ~36 scoops, $p<0.05$), had more on-patch scoops (~42 vs. ~7 scoops, $p<0.01$), spent more time in control of the patch (~12 vs. ~2 minutes, $p<0.001$), and had a higher percentage of successful attacks (~96 vs. 4 percent of attacks, $p<0.001$). Additionally in Trial 1, there was no influence of the salinity (Appendix A, $P>0.05$) on the behavior of either dominant or submissive fiddler crabs (Figs. 24a-h and Figs. 25a-h).

Regardless of exposure salinity, competitive foraging behavior of both dominant and submissive crabs was unaltered ($p>0.1$) (Trial 1 vs. Trial 2) upon exposure of dominant crabs to a mixture of 0.5mg/l Cd and 0.48mg/l Ag (nominal concentrations) (Figs. 24a-d). In the same way, competitive dominance behavior of both dominant and

submissive crabs was unaltered ($p>0.1$) after exposure of dominant crabs to the metals mixture (Figs. 24e-h).

After exposure to a mixture of 2.5mg/l Cd and 2.4mg/l Ag (nominal concentrations) (Trial 2), dominant crabs had 60% less total scoops ($p<0.05$) at 5ppt and 30ppt than prior to exposure (Trial 1) (Figs. 25a,b). In contrast, submissive crabs had ~100% more total scoops ($p<0.05$) at 5ppt and 30ppt (Trial 1 vs. Trial 2) (Figs. 25a-b). Number of scoops on patch of the dominant crabs decreased by ~75% ($p<0.01$) at 5ppt and 30ppt (Figs. 25c,d), and alternatively, the number of scoops on patch of submissive crabs increased by 600 to 700% ($p<0.01$) at 5ppt and 30ppt respectively (Figs. 25c,d). After exposure, dominant crabs spent 65% less time ($p<0.05$) in control of the patch at both 5ppt and 30ppt. Submissive crabs spent 650% ($p<0.05$) at 5ppt and 30ppt, more time on the protein-rich patch (Trial 1 vs. Trial 2) (Fig. 25e-f). After exposure, the percentage of successful attacks by dominant crabs dropped by 60% ($p<0.01$) at 5ppt and 30ppt. The percentage of successful attacks by submissive crabs has increased by 60% ($p<0.01$) at both salinities (Figs. 25g,h).

B. Effects of exposure to Cd and Ag mixtures on bioaccumulation in fiddler crabs:

As in the case with exposure to individual Cd (Figs. 16a-e), fiddler crabs exposed to 0.5mg/l Cd in combination with 0.48mg/l Ag accumulated significantly more Cd (Appendix A, $p<0.05$) at 5ppt than at 30ppt (Figs. 26a-e). However, unlike the case with exposure to individual Cd, crabs exposed to 2.5mg/l Cd in combination with 2.4mg/l Ag accumulated as much Cd at 5ppt as at 30ppt ($p>0.1$). Also at both salinities, crabs accumulated more Cd ($p<0.05$) in all fractions as the exposure concentrations were increased to 2.5mg/l Cd in combination with 2.4mg/l Ag (Figs. 26a-e). The comparison of Figs. 16 and 26 strikingly show that, at 5ppt, crabs exposed to 0.5 and 2.5mg/l Cd in

combination with Ag accumulated less Cd ($p<0.01$) than those exposed to individual Cd. At 30ppt however, crabs accumulated as much Cd ($p>0.1$) when exposed to individual Cd as when exposed to the same Cd concentration in combination with Ag.

At 0.48mg/l Ag nominal exposure concentration, and irrespective of salinity and Cd presence, crabs accumulated the same amount of Ag (Appendix A, $p>0.05$) in total body, organelles, enzymes and MT fractions (Figs. 23a-d, Figs. 27a-d). As the Ag exposure concentration was increased to 2.4mg/l in combination with 2.5mg/l Cd, fiddler crabs accumulated more Ag ($p<0.001$) at 5ppt and 30ppt than at 0.48mg/l Ag (Figs. 27a-e). Also, fiddler crabs exposed to 2.4mg/l Ag in combination with 2.5mg/l Cd accumulated more Ag at 5ppt than at 30ppt ($p<0.01$) in their bodies, organelles and enzymes (Figs. 27a-c). It is worth mentioning that crabs exposed to 2.4mg/l Ag in combination with Cd accumulated more Ag ($p<0.05$) in all fractions (especially in the enzyme') as compared to crabs exposed to the same concentration of individual Ag (Figs. 23a-e, 27a-e).

IV. Discussion

A. Effects of exposure to Cd and Ag mixture on the behavior of fiddler crabs:

Any attempt to evaluate toxicities of metal mixtures is challenging. Simply because metal combinations that occur in nature are endless, and because physical and biological processes influence their effects (Chu and Chow 2002, Peakall and Burger 2003). Literature review revealed limited studies on the toxicity of Cd and Ag in combination at different salinities. Voyer *et al.* (1982) showed that exposure to nanomolar Cd solution reduced the swimming activity and feeding rate of winter flounder embryos. The toxicity of Cd decreased with increasing salinity. Ag by itself was not toxic

to fish embryos at the nanomolar level. The addition of Ag to Cd solutions, however, decreased the toxicity of the latter metal significantly.

In this study, the exposure of dominant fiddler crabs to 22.3μM Cd and 22.3μM Ag (nominal concentrations) (LOAEL) in a 1:1 mixture had a negative impact on the competitive foraging and dominance behaviors of exposed crabs. This impact was counterbalanced by an increase in the competitive behaviors of unexposed 'submissive' crabs. Clearly, however, metal-induced changes in the competitive behavioral parameters of both 'dominant' and 'submissive' crabs were similar at both salinities (5ppt and 30ppt). At LOAEL, The impact of exposure to metal mixture however was much more pronounced at 30ppt, as compared with impacts of individual metals. Specifically, the comparison of the effects of exposure to 44.5μM Cd or Ag individually to that of a 1:1 metal mixture clearly shows similarities as well as differences. At 5ppt, the exposure of fiddler crabs to Cd, Ag and their mixture had similar negative impacts on the competitive behaviors of exposed 'dominant' crabs and positive impacts on that of non-exposed 'submissive' crabs. However, at 30ppt, the effects of exposure of fiddler crabs to metal mixture of Cd and Ag (at LOAEL) significantly altered the dominance behavior of both exposed and non-exposed crabs. However, the exposure to 44.5μM Cd or Ag individually did not affect the dominance behavior of crabs. In fact, the level of impact on crabs resulting from an exposure to the 1:1 mixture of Cd and Ag at 30ppt is comparable to that resulting from exposure to double the concentration (i.e. 88.9μM) of individual metals. This may be related to the change in Ag speciation in presence of Cd. At 30ppt, the observed concentrations of Ag in solution increased significantly in presence of Cd as compared to that of individual Ag (see Appendix B). This may explain the increase in bioaccumulation and toxicity of Ag in mixture in fiddler crabs at 30ppt.

B. Relation between Cd and Ag bioaccumulation and behavioral changes in crabs:

A literature review showed that few studies have been conducted on the combined toxicities of Cd and Ag. For instance, Voyer *et al.* (1982) showed that Ag chloro complexes ($AgCl_n^{1-n}$) reduced the toxicity of Cd to winter flounder embryos at different salinities. However, Fraysse *et al.* (2002) showed that Cd increased the uptake of Ag in zebra mussels. Segovia-Zavala *et al.* (2004) suggested that bioaccumulation of Cd and Ag in mussels collected from coastal waters is correlated with anthropogenic discharges.

In this study, when compared to the level of accumulated metal after exposure to 22.3μM (LOAEL) individual metals, exposure of *Uca* at 5ppt to a mixture of 22.3μM Cd and 22.3μM (LOAEL) resulted in a 2-4 fold decrease in Cd accumulation, and in a 2-5 fold increase in Ag accumulation in body burden, as well as in subcellular fractions. The most pronounced change was observed in the enzyme fraction. It is believed that Ag^+ reduces the bioaccumulation of Cd because the former has a higher affinity for sulfur groups in proteins (Bell *et al.* 2002, Wang *et al.* 1996) and to enzymes in particular (Hook and Fisher 2002). Hence, Ag^+ can compete with Cd ions at the ion pump sites, which makes Ag^+ more available. When comparing bioaccumulation of individual metal and mixture of metals, the exposure of *Uca* to Ag in mixture at 30ppt did not affect the bioaccumulation of Cd but did lead to a 2-fold increase in the accumulation of Ag in all subcellular fractions. Here, Ag did not reduce the bioaccumulation of Cd probably because at this high salinity there are less Ag^+ to compete with Cd at the ion pump sites. Still, at this salinity as well as at 5ppt, crabs accumulated more Ag in the presence of Cd than they did when exposed to Ag alone. It can be concluded that, in agreement with the results of Fraysse *et al.* (2002) results, Cd can have a synergistic effect on Ag uptake in *Uca*; however, a mechanism for this however is yet to be identified.

The behavioral toxicity of exposure to Cd and Ag mixture (at LOAEL) on *Uca* crabs is similar to the effects of exposure to 88.9μM of either Cd or Ag individually. Since Cd increased the uptake of Ag, it can be concluded that Cd has a synergistic effect on Ag behavioral toxicity. These results show the complexity of examining the toxicity of metal mixtures; although it is possible to study the interactions of two metals in controlled experiments, the combination of three or more under the same experimental conditions becomes problematic (Chu and Chow 2001, Peakall and Burger 2003, Schlekat *et al.* 2001).

C. Future directions:

This study showed that exposure of dominant male *Uca* crabs to Cd and Ag (individually or in combination) may affect their foraging and dominance behavior. This may be, primarily, due to the bioaccumulation of these metals in the subcellular fractions of exposed crabs. Kim *et al.* (2004) demonstrated that exposure of olive flounder to Cd resulted in an increase Cd accumulation in the tissues of exposed fish. Following the end of exposure, tissue Cd concentration of exposed flounder decreased immediately. Sloman *et al.* (2003) showed that rainbow trout exposed to sublethal concentrations of Cd for 24 hours, followed by a 1-3 day depuration period, had a decreased ability to compete with non-exposed fish. However, the competitive ability of exposed fish given a 5-day depuration period was not significantly impaired. It would be interesting to investigate the elimination of Cd (and Ag) in exposed *Uca* crabs, and its consequences on their foraging and dominance behavior.

Fig.24: Effect of exposure to a mixture of 0.5mg/l Cd and 0.48mg/l Ag on competitive foraging (a, b, c, d), and on competitive dominance (e, f, g, h) of *Uca* crabs at 5ppt (left) and 30ppt (right)

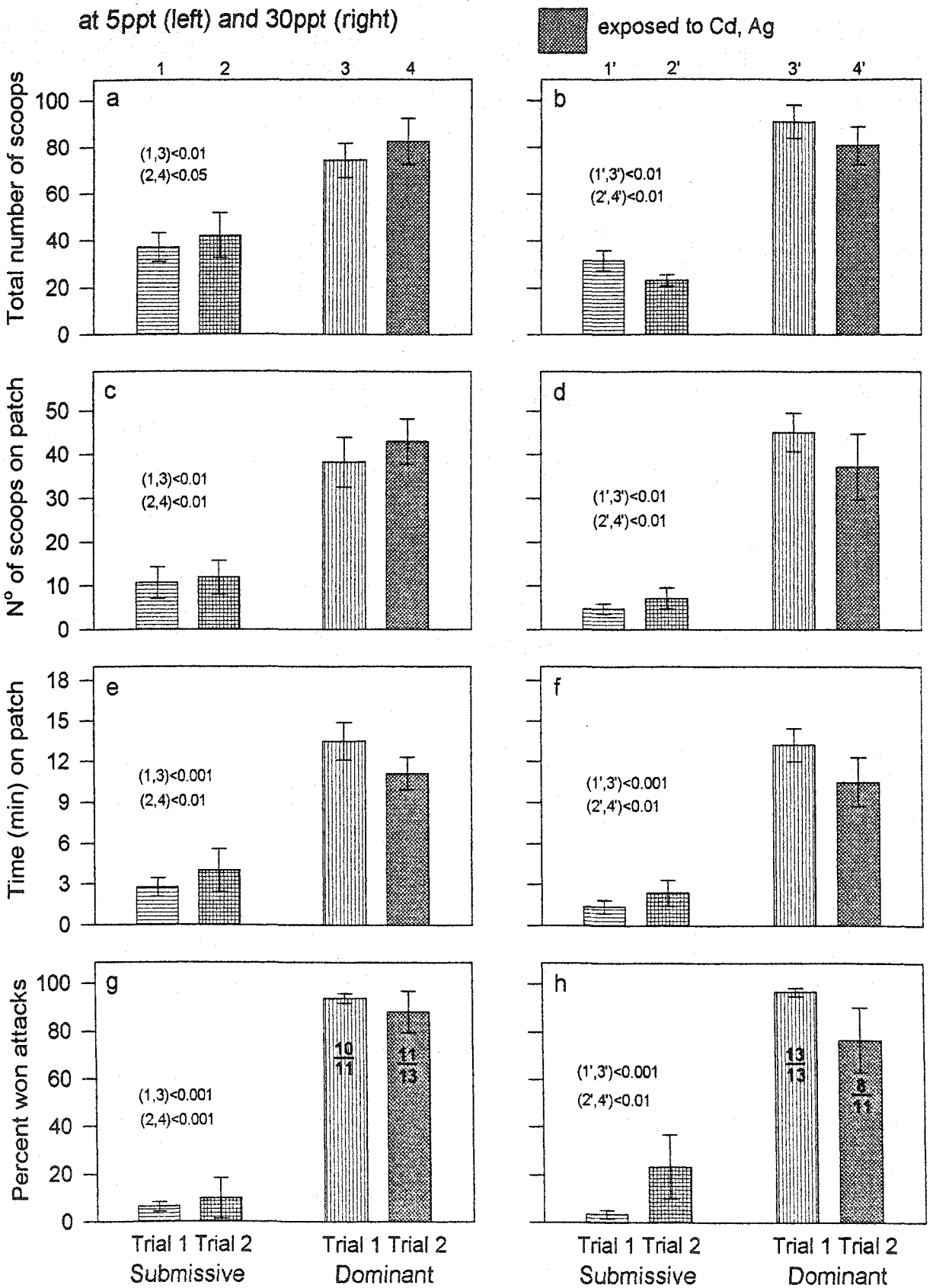


Fig.25: Effect of exposure to a mixture of 2.5mg/l Cd and 2.4mg/l Ag on competitive foraging (a, b, c, d), and on competitive dominance (e, f, g, h) of *Uca* crabs at 5ppt (left) and 30ppt (right)

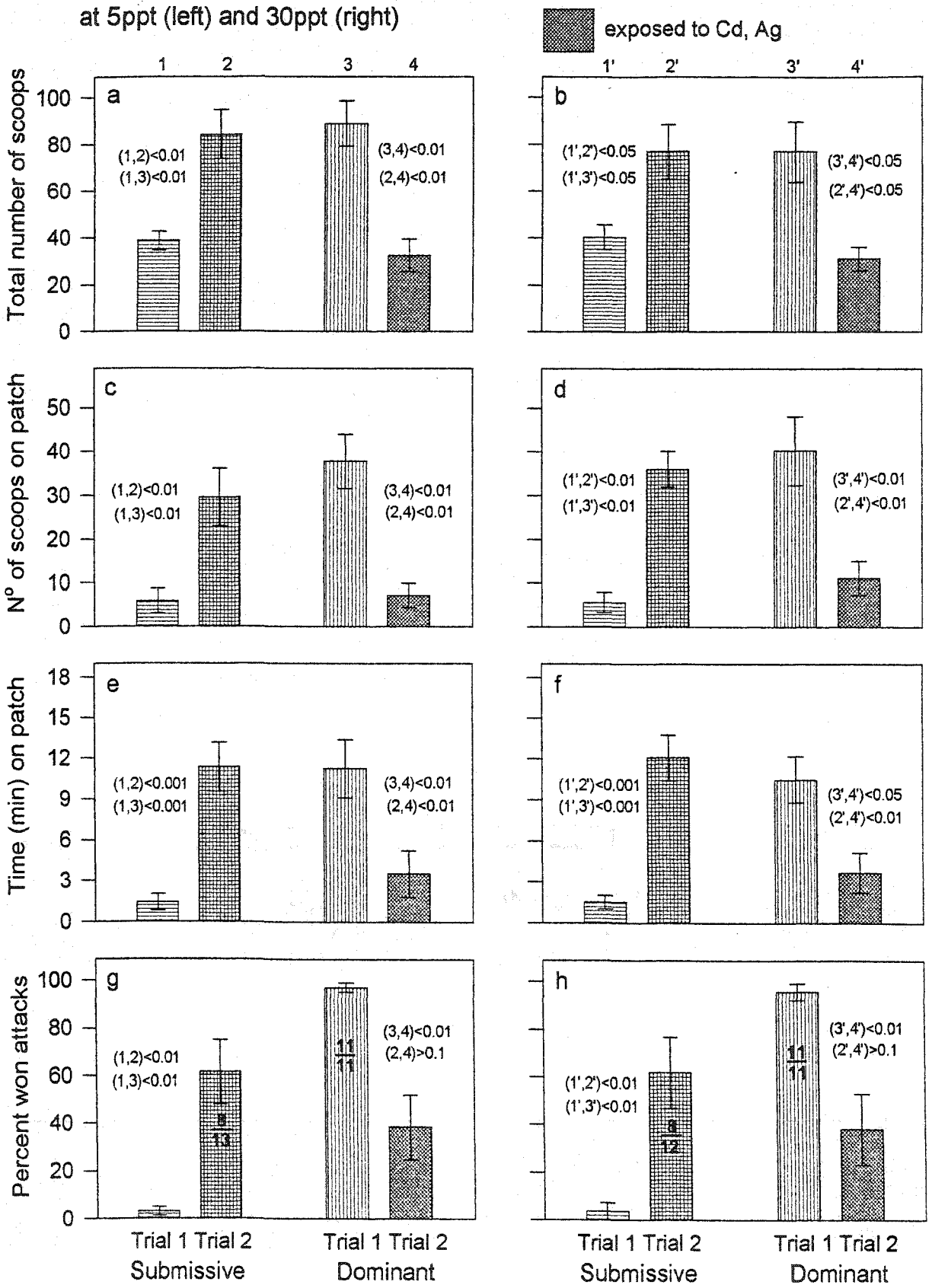


Fig. 26: Bioaccumulation of Cd, when combined with Ag, at different concentrations and salinities in (a) total body, (b) organelles, (c) enzymes, (d) metallothioneins and (e) insoluble fractions in fiddler crabs *Uca pugnax*

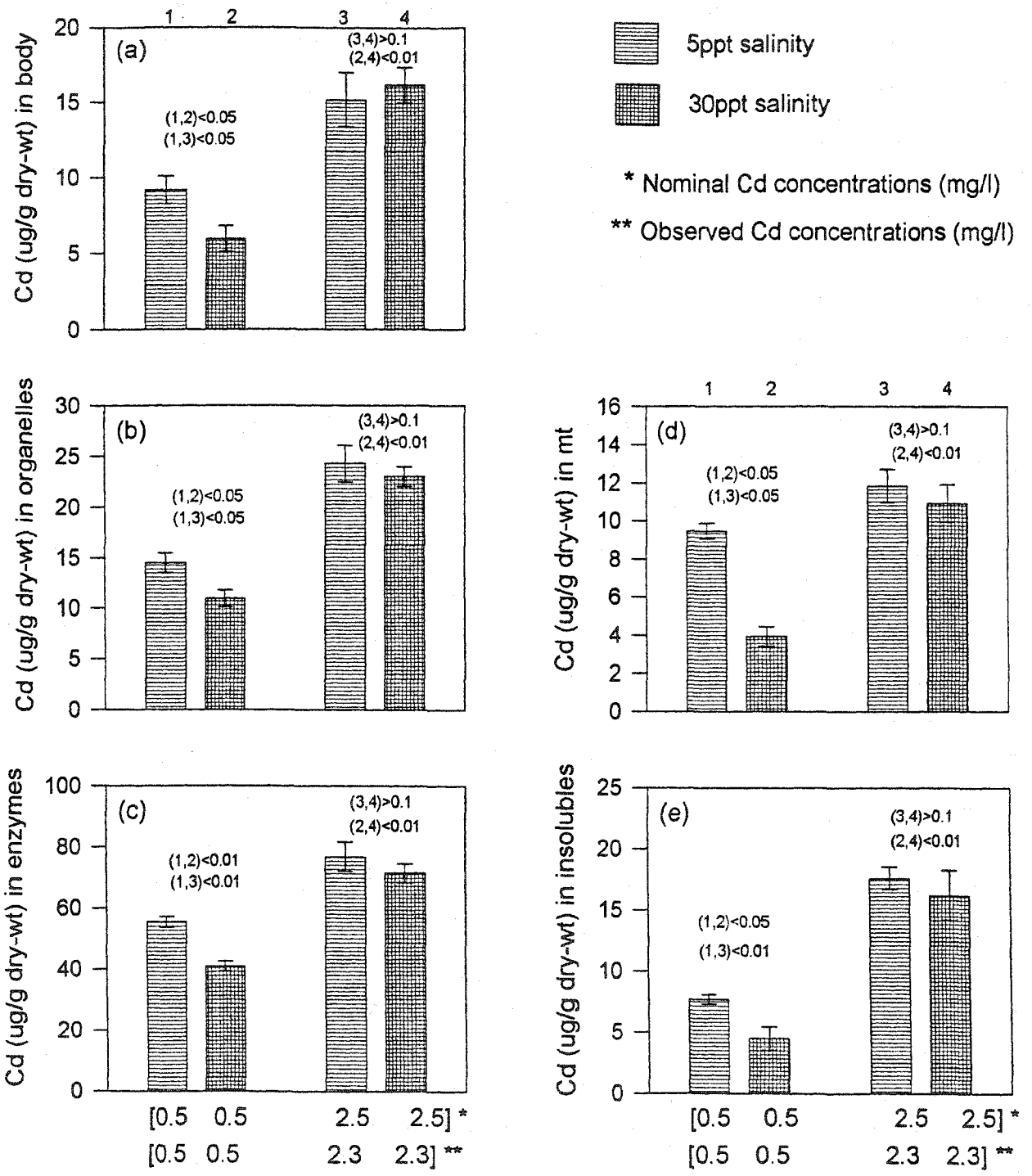
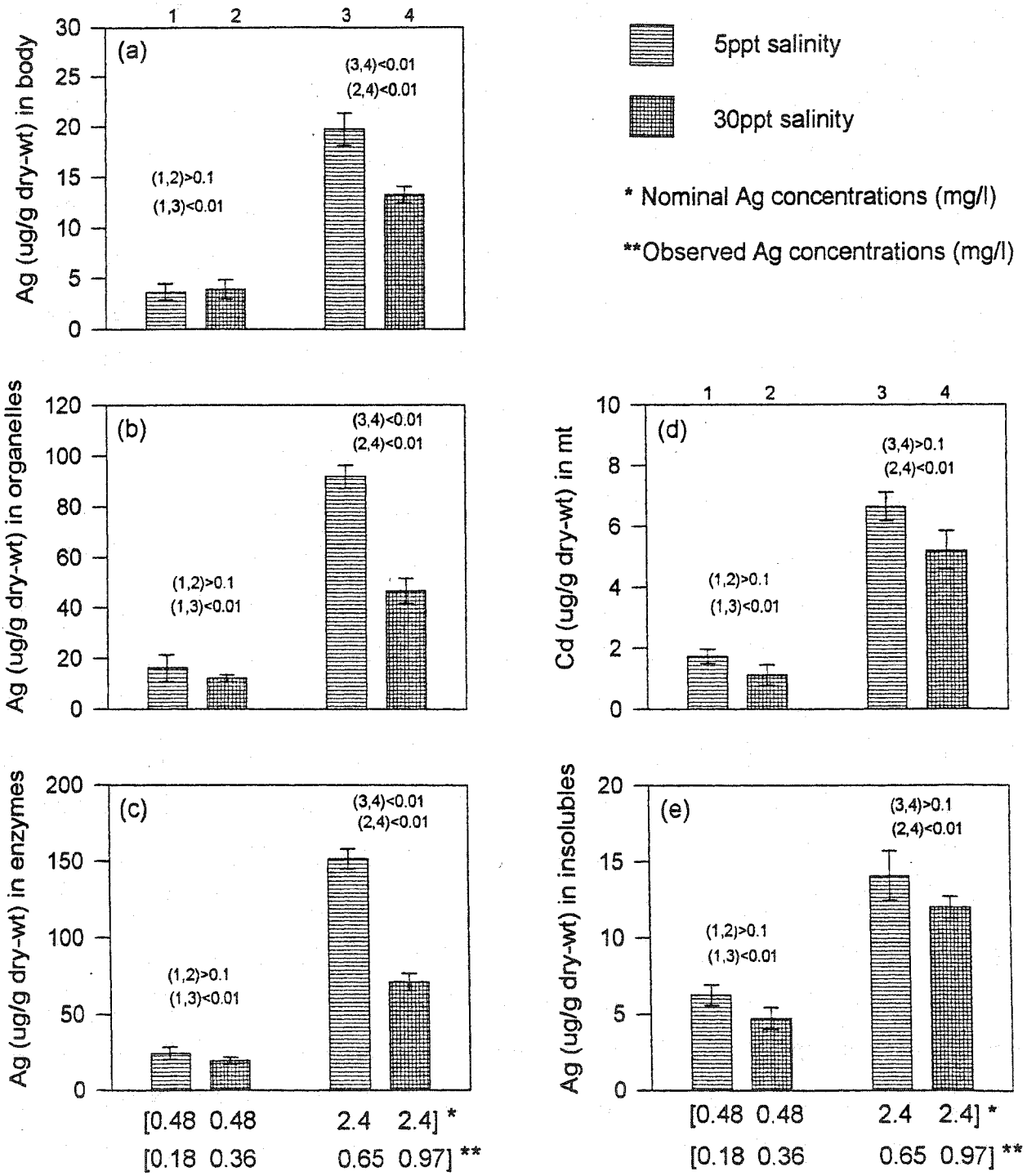


Fig. 27: Bioaccumulation of Ag, when combined with Cd, at different concentrations and salinities in (a) total body, (b) organelles, (c) enzymes, (d) metallothioneins and (e) insoluble fractions in fiddler crabs *Uca pugnax*



§ Chapter Seven §

A FIELD APPLICATION: THE EFFECTS OF METAL POLLUTANTS IN *UCA* CRABS

I. Introduction

A field study was carried out to verify if *Uca* crabs from a polluted site behave differently than those collected from a cleaner site. First, foraging and dominance behavior of crabs from both sites was investigated. Then, metal contents of various subcellular fractions of 'impacted' and 'reference' crabs were compared. The polluted site chosen for *Uca* crabs collection is Meredith Creek marsh in Staten Island. This creek is connected to the Arthur Kill that is a part of New York-New Jersey Harbor estuary. The Harbor is one of the most polluted estuaries in the world; it is surrounded by a population of more than 20 million people and concentrated refining industries and wastewater treatment facilities (N.Y.C. DEP 1996, 1997). The Arthur Kill itself is surrounded by a dense urban population, heavy industries and major shipping lanes. Its sediments are contaminated with an array of metals and organic pollutants, which moderately-to-highly impact benthic organisms (U.S. EPA 1995, 1998), whereas the reference site is Rutgers' Marine Research station in Tuckerton located in a natural reserve in southern NJ.

II. Methods

A. Behavioral studies:

This experiment was conducted to verify that environmental pollution might alter foraging and dominance behaviors of *Uca* crabs. For this study, crabs were collected from two separate sites: the polluted Meredith marsh in Staten Island, New York and the cleaner Rutgers Marine Research station marsh in Tuckerton, New Jersey. Crabs were collected from both sites on the same day in July of 2002. For each site, eight pairs of

crabs with similar carapace width (18-20 mm) were selected for the study. The crabs were placed in depuration chambers for 48 hours immediately after they were collected from the field. Crabs from both sites were paired at random; each pair was placed in a 15 x 20 cm opaque plastic container containing a thin layer of Meredith mud. In the center of mud, a protein-rich patch was placed on mud surface for the crabs to compete for. While in the container, each pair of crabs was videotaped for one hour. Tapes were reviewed and data on competitive foraging behavior (total number of scoops and number of scoops on protein-rich patch), and on competitive dominance behavior (time in control of the patch and aggressiveness/ number of successful attacks) of each crab were collected. Crabs were depurated for 48 hours before being stored individually at -80°C while awaiting fractionation and subsequent metal analyses.

B. Fractionation procedure and sample preparation:

Metal-exposed and control crabs were subjected to subcellular fractionation as described by Wallace *et al.* 2003. Briefly, crabs (four dominant and four submissive from each treatment) were thawed on ice. Then, each crab was placed in a 50 ml Nalgene® centrifuge tube containing 5.8 ml of 20 mM Tris buffer solution (prepared by adding 3.152g of J.T. Baker Ultrapure Grade TRIS Hydrochloride to 1 liter of Nano® pure water and adjusted to pH of 7.6). Crabs were then homogenized using Polytron® PT 2100 homogenizer. Subsequent to homogenization, a one-milliliter sample was removed from the centrifuge tube and was transferred to a pre-weighed vial for future analysis of total body burden. Homogenized crabs were first centrifuged in a Sorval® (RC 5C Plus model) at 1,950g for 15 minutes. Supernatants (S1) were transferred to a set of centrifuge tubes, which were further centrifuged in Beckman centrifuge (L8-M Ultracentrifuge

model) at 103,000g for one hour. After adding 5.0 ml of Tris buffer to pellets (P1), they were placed in water bath at 100°C for 2 minutes; 5.0 ml of 1N NaOH was added to each tube before being vortexed and placed again in a water bath at 65°C. After one hour, pellets (P1) were removed from water bath; they were vortexed and spun in Sorvall centrifuge at 4,400g for 15 minutes. Supernatants (S2) were transferred to pre-weighed vials; these are the cellular debris. A 5 ml of 1N NaOH was added to pellets (P2), pellets were then vortexed and centrifuged in Sorvall centrifuge at 4,400g for 15 minutes. Supernatants (S3) were transferred to the same vials containing the cellular debris. Pellets (P3) were transferred to a set of pre-weighed vials after being resuspended in 4 ml of Nano® pure water; this constitutes the insoluble fraction (intracellular granules plus carapace). After centrifuging supernatants (S1), supernatants (S4) were transferred to another set of Beckman centrifuge tubes that were placed in water bath at 80°C for 10 minutes, then on ice for 45 minutes. Pellets (P4) contain the organelles; they were transferred to a set of pre-weighed vials after being resuspended in 2 ml of Nano® pure water. After supernatants (S4) cooled on ice, they were centrifuged in Beckman centrifuge at 20,000g for 30 minutes. Supernatants (S5), which contain MT proteins, were transferred to a set of pre-weighed vials. Pellets (P5) contain enzymes, were transferred to pre-weighed vials after being resuspended in 2 ml of Nano® pure water.

Crab tissues and subcellular fractions were digested for metals following standard protocol. Vials containing subcellular fractions were dried in a drying oven at 65°C, and then they were weighed. Concentrated HNO_3 (5 ml) was then added to each vial. Samples, covered with reflux bulbs, were refluxed until tissues dissolved. Samples were then evaporated to dryness, resuspended in 10 ml of 2% Ultragrade HNO_3 and filtered through .45 μm Millipore filters. Filtered samples were then analyzed for Cd and Ag

using Perkin Elmer model 3100 atomic absorption spectrophotometer. Quality Control and Quality Assurance samples included (1) standard tissue samples, (2) fractionation procedural blanks, (3) digestion blanks and (4) filter blanks. During analysis, background and standards were periodically run to verify the accuracy of the instrument. If background or standard samples did not meet QA/QC the instrument was recalibrated.

III. Results

A. Population-specific differences in fiddler crabs' competitive behavior:

The competitive behaviors of two populations of crabs collected from different sites were compared. The purpose of this treatment was to show that long-term exposure of two fiddler crab populations to different levels of metals in nature might alter their competitive behaviors. For this purpose, crabs from Tuckerton (a relatively clean site in south New Jersey) were paired with crabs from Meredith Creek marsh (a polluted site connected Arthur Kill) were compared. Results showed that Tuckerton crabs had a higher number of total scoops ($p<0.01$) than those from Meredith marsh (Figs. 28a-b). Tuckerton crabs were also able to dominate the protein patch ($p<0.01$) (Fig. 28c). Aggressiveness, however, of Tuckerton and Meredith crabs was the same since both crabs were able to win ~50% of attacks ($p>0.5$) (Fig. 28d).

B. Background metal levels in the two *Uca* populations:

Crabs collected from Meredith creek and Tuckerton were analyzed for metal content. These analyses showed that Meredith crabs have higher concentrations ($p<0.05$) of Ag, Cd, Cu, Mn, Ni, and Se in at least one of their metal-sensitive fraction (Figs. 29a, 29b). Interestingly, Tuckerton crabs showed elevated metal content ($p<0.05$) of As in all

fractions. There was no difference in Co, Mo and V contents between the two crab populations.

IV. Discussion

Trace metals, which are common contaminants of aquatic ecosystems, are classified as either Class B (sulphur- and nitrogen-seeking, e.g. Ag, As, Cd, Cu), borderline (e.g. Co, Cr, Mn, Ni, V, Zn), or Class A (oxygen-seeking, e.g. Mo, Se) (Nieboer and Richardson 1980). Many trace metals are essential for metabolic activities, and at high levels all have the potential to cause toxicological effects (Rainbow 1995). The toxicities of trace metals to estuarine organisms are often affected by the chemical characteristics of the exposure media (e.g. pH and salinity), competition among metals for sites of uptake, and species-specific susceptibility (Baker *et al.* 2003, Erickson *et al.* 1998).

In this study, fiddler crabs *Uca pugnax* collected form Tuckerton, NJ (a relatively unpolluted site) and from Meredith Creek, NY (a polluted site) were analyzed for metal contents in their various fractions (total body burden, organelles, enzymes, metallothioneins and insoluble fractions). When compared to crabs from the unpolluted site, crabs from the polluted site had higher levels of almost all Class B metals analyzed (e.g. Ag, Cd, Cu) in their enzyme fraction, higher levels of Borderline metals (Mn, Ni, Zn) in their total body burden, and elevated levels of Se (a Class A metal) in their enzymes and MT fractions.

Laboratory investigation showed that Tuckerton crabs had at least twice as many scoops than Meredith crabs and it is suspected that the elevated levels of trace metals in the enzyme fraction of Meredith crabs may be related to this reduced foraging behavior.

Studies showed that the exposure of aquatic organisms to metals in the environment affects their health by reducing their abilities to carry their physiological functions and metabolic activities. For instance, crabs collected from polluted sites, as compared to crabs from unpolluted sites, showed elevated levels of Cu, Cd and Zn and reduced abilities to hypoosmoregulate in high salinity (Harris and Santos 2000). Cheung *et al.* (2002) showed that the exposure of sandy shore gastropods to Cr significantly reduced their feeding behavior, as compared with the control, by affecting their chemoreception. Smith and Weis (1997) showed that the exposure of mummichogs fish to Hg caused a reduction in their abilities to capture grass shrimp and an increase in their brain Hg. Finally, Sauvé *et al.* (2002) showed that the exposure of bivalves to Ag, Cd Zn and Hg suppressed their phagocytic activities.

Fig. 28: Differences in competitive foraging behavior (a, b), and in competitive dominance (c, d) between Tuckerton and Meredith *Uca* crabs

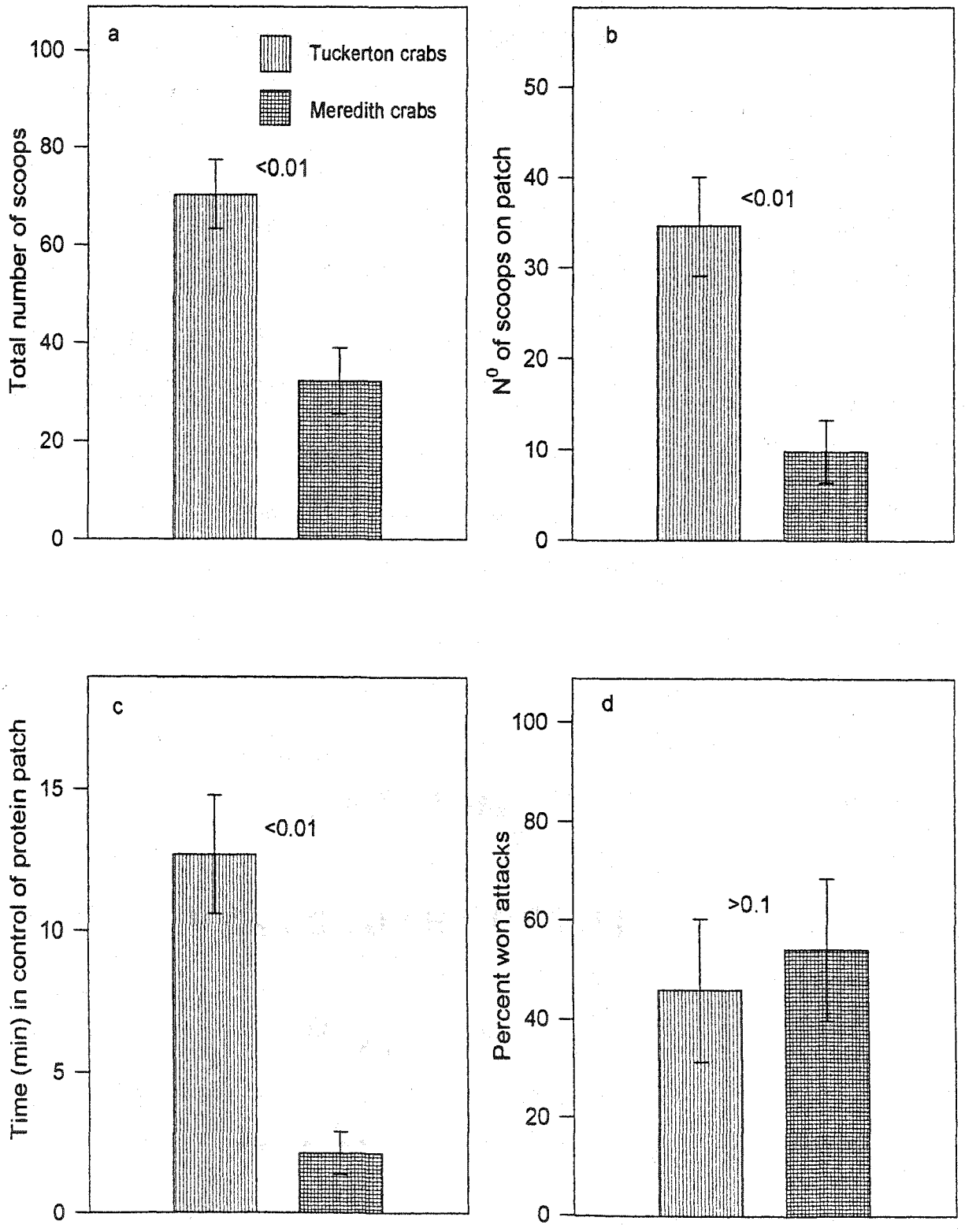


Fig.29a: Distribution of metals in cellular fractions of two *Uca* populations collected from Tuckerton, New Jersey and Meredith marsh on Staten Island, New York

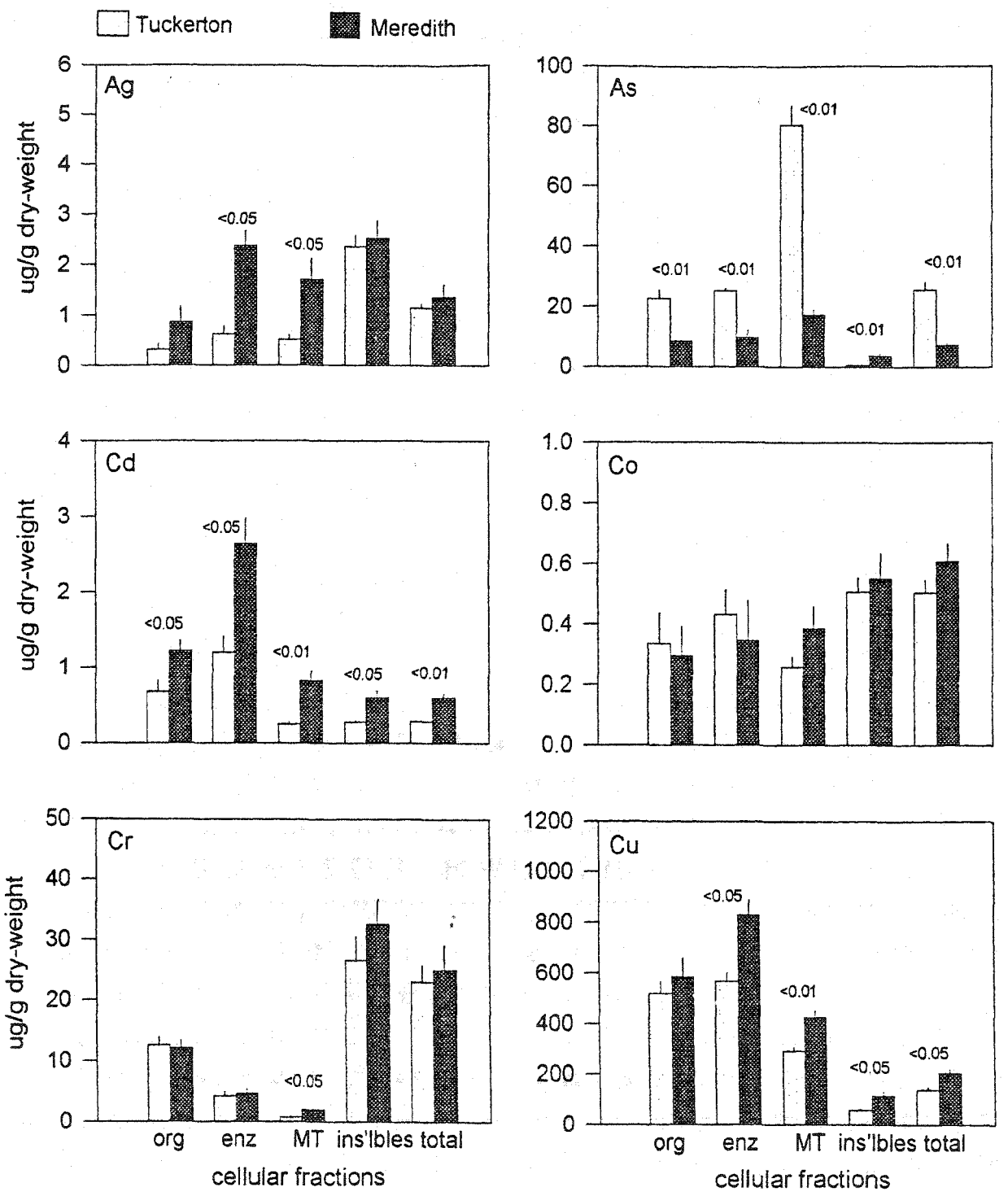
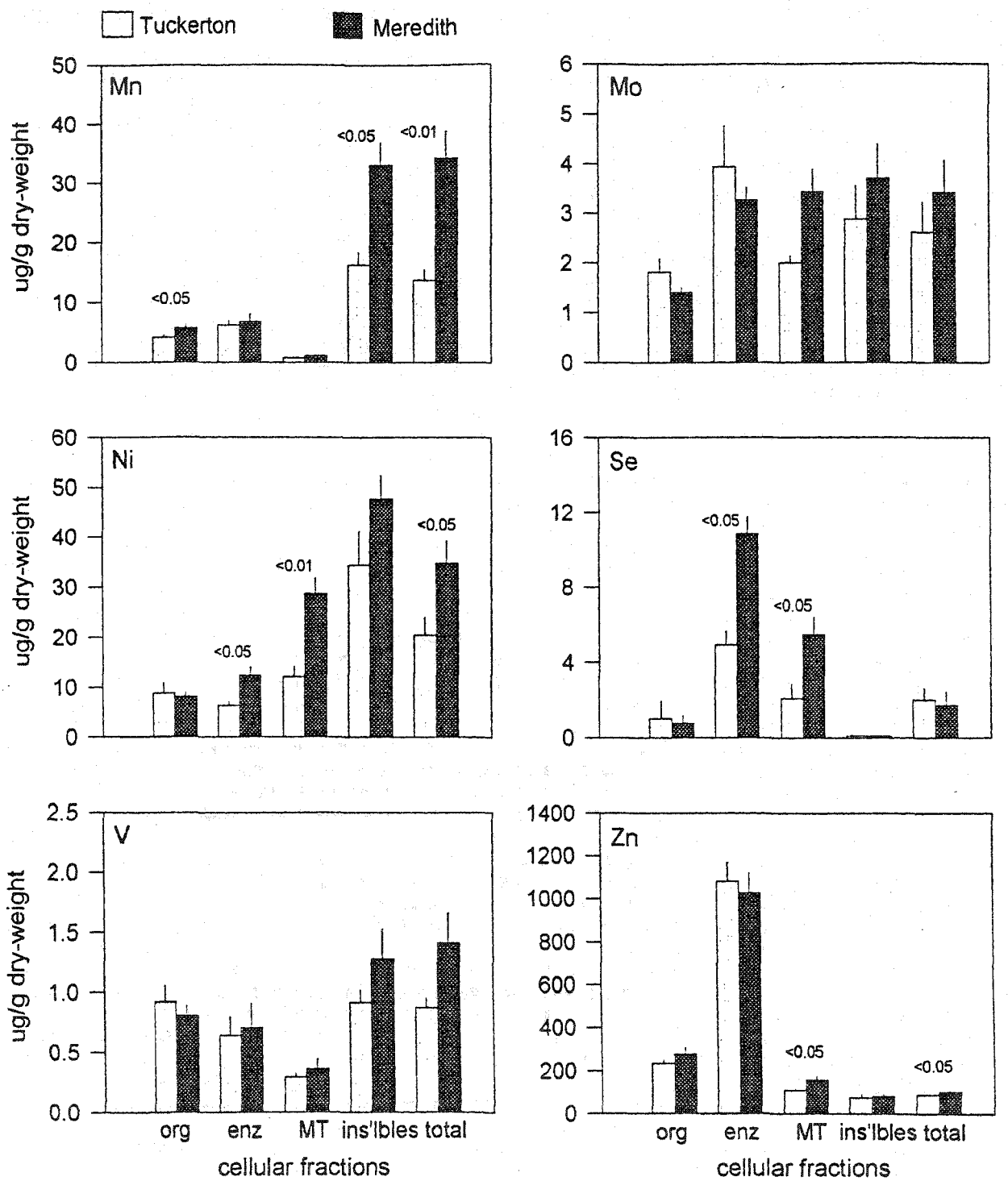


Fig.29b: Distribution of metals in cellular fractions of two *Uca* populations collected from Tuckerton, New Jersey and Meredith marsh on Staten Island, New York



§ Chapter Eight §

CONCLUSION

This study revealed that the exposure of *Uca* to Cd or Ag individually at LOAEL had more severe effect on foraging than on dominance behavior of exposed crabs. Therefore, foraging behavior is a more sensitive measure of metal toxicity. A demonstration of this effect was specifically seen when the competitive foraging and dominance behaviors of *Uca* crabs collected from polluted (Meredith) and unpolluted (Tuckerton) sites were compared. This study showed that Tuckerton crabs foraged more, but were as dominant as Meredith crabs. This indicates that foraging behavior in fiddler crabs is more vulnerable to metal pollutants. This is an important finding as it shows that dominant crabs from polluted sites were still able to express their dominance behavior, and perhaps suggesting that competition for mates, food and/or shelter is not impacted. This would ensure their survival even though their metal body burden may have reached a critical level.

Since changes in the competitive behaviors of fiddler crabs was impacted at roughly the same level of Cd and Ag, behavioral toxicities of Cd and Ag in *Uca* crabs may be comparable. However, when considering an important environmental parameter (i.e. salinity), this study shows that at the 'lowest observable adverse effect concentration', the interference of Cd on dominance is more severe at 5ppt than at 30ppt. For Ag, salinity did not have a similar effect. The decrease in Cd toxicity with the increase in salinity may be due to the formation of Cd chloro complexes that may not be biologically available for uptake by *Uca* crabs (Burke *et al.* 2003, Peakall *et al.* 2003). In contrast, the formation of Ag neutral chloro complexes at high salinity does not seem to protect, as they may not be bioavailable and toxic as well to *Uca* crabs.

This study showed that, at low salinity, *Uca* crabs were able to survive a higher exposure level of Cd as compared with Ag. But at high salinity, crabs survived a higher

Ag exposure level. Since, at low salinity, these metals are mostly present in their free ionic forms, it may be inferred that Ag ions are more toxic than Cd ions (Hogstrand *et al.* 1998, Luoma *et al.* 1995). Hence, Ag may be more of a concern in freshwater (Bury *et al.* 2002, Fisher *et al.* 1998, Galvez *et al.* 1997, McGeer *et al.* 1998). However, as salinity increases, Cd and Ag ions react with chloride to form various chloro complexes; the ability of fiddler crabs to survive an elevated Ag concentration at high salinity suggests that total Ag is less toxic than total Cd, it also indicates that Cd may be more toxic than Ag in saline waters (Peakall *et al.* 2003). This also reveals the importance of salinity and speciation in metal toxicity.

The effect of a simultaneous exposure to Cd and Ag on the competitive behavior of *Uca* crabs at lower salinity is similar to the impact of these metals individually. However, at higher salinity, the effects of exposure to combined metals surpassed that of individual Cd or Ag. This suggests that the effect of combined exposure to Cd and Ag on crabs' competitive behavior is only additive at 5ppt, but synergistic at 30ppt. The enhanced toxicity of metal mixtures may be due to the observed increase in uptake of the more toxic metal (i.e. Ag) in presence of Cd (Voyer *et al.* 1982). This more pronounced toxicity of combined metals at higher salinity may also be due to increased damage effects of metal mixtures on the gill membranes of exposed crabs (Chu *et al.* 2002, Schlekat *et al.* 2001). Pervious studies have shown that exposure to metals in solution causes more damage to the gills of euryhaline crabs at higher salinity (Lawson *et al.* 1995, Soegianto *et al.* 1999), but there is still need for further research to be conducted on the toxicity of various metals on the gill of fiddler crabs at different salinities. When considering metal body burden, the onset of metal behavioral toxicity occurred when fiddler crabs accumulated more Ag when combined with Cd than when exposed to Ag

individually. It is reasonable to believe that, at both salinities, Cd enhanced the uptake of Ag at the gills of fiddler crabs. Although Ag has higher affinity to S^- and N^-, and binds more strongly than Cd to cellular proteins, this does not explain the enhanced uptake of Ag in the presence of Cd at toxic exposure level; a mechanism therefore needs to be proposed. This study reveals the complexity of metal interactions and shows the importance of environmental factors in affecting synergistic toxicities of metals.

In euryhaline crabs, gills are the major route of metal uptake. Salinity of exposure medium influences the bioaccumulation of metals through physiological effect i.e. changes in the osmoregulatory activities of gill enzymes, as well as physicochemical effects i.e. changes in metal speciation with increasing salinity. In this study, fiddler crabs accumulated more Cd at low salinity, but there was no salinity-dependent change in Ag accumulation. The increase in Cd accumulation at a lower salinity may be attributed to the increase in gill Na/K-ATPase activity (Skaggs *et al.* 2002), and the higher concentration of the available free Cd^+ present at low salinity (Rainbow 1997, Wright 1995). As salinity increases, chloro-complexes dominate and that cannot be accumulated by these organisms (Burke *et al.* 2003, Luoma *et al.* 1995). The accumulation of Ag in fiddler crabs was not affected by salinity; this may be due to the increase in the formation and uptake of neutral Ag chloro complexes ($AgCl^0$) with increasing salinity. In other words, fiddler crabs may be able to compensate for decrease in the uptake of free Ag^+ by increase in the uptake of neutral Ag chloro complexes with increasing salinity.

It is widely believed that metals exert their toxic effects on aquatic organisms by binding to their enzymes, hence altering or reducing their functions (Holiday 1985, Skaggs *et al.* 2002). In this study, the onset of Cd toxicity coincided with an increase in Cd associated with enzyme fractions of fiddler crabs. Given that the level of Cd in the

enzyme fraction was 2.5-fold greater at 5ppt than at 30ppt, this may partly explain the higher Cd behavioral toxicity on dominance in fiddler crabs at lower salinity. The enzyme fraction, however, of *Uca* crabs accumulated as much Ag at 5ppt as at 30ppt. This may be caused by the reduction in the uptake of free Ag^+ at high salinity due to the increase in the formation of $AgCl^0$ and other chloro complexes. The uptake of this neutral Ag chloro complex sheds light on its behavioral toxicity. Since the change in dominance behavior of exposed fiddler crabs is more pronounced at low salinity where Ag^+ prevails, it is believed that the reduction in total Ag behavioral toxicity at high salinity is due to the increase in the uptake of the neutral, but less toxic, $AgCl^0$.

In most toxicological studies, the usual approach is to demonstrate that a given metal causes adverse effects in controlled laboratory experiment. This study went one step further, by attempting to relate behavioral changes to metal in subcellular fractions. It showed that subcellular and behavioral changes in estuarine organisms could be used to assess metal toxicity especially because these changes can be measured in exposed organisms. These variations provide evidence of exposure to environmental metal pollutants; they are considered a sign of progressive deterioration in the health of exposed organisms, which inevitably would reduce their survival potential.

The objective of ecotoxicological testing is to assess the possible adverse effects of environmental pollutants on ecosystems. The environment is continuously loaded with metal pollutants released by urban communities and industries, and the ultimate sink of these pollutants is the aquatic environment. The presence of metals in aquatic ecosystem does not, by itself, indicate deteriorative effects. In environmental risk assessment, connections must be established between exposure levels, metal accumulation in organisms and the effects of metal internalized by exposed organisms. These effects -

whether molecular, cellular, physiological or behavioral- are used as biomarkers of metal exposure, which in turn are used to assess metal toxicity to studied organisms in particular, and to the whole population in general.

APPENDIX A

P-Values of Behavioral Data (U-Test)

Figure 7

	Fig. 7a	Fig. 7c	Fig. 7e	Fig. 7g		Fig. 7b	Fig. 7d	Fig. 7f	Fig. 7h
Bar (1,2)	0.12	0.95	0.67	0.63	**Bar (1',2')**	0.59	0.87	0.40	0.64
Bar (3,4)	0.20	0.24	0.52	0.63	**Bar (3',4')**	0.27	0.83	0.17	0.46
Bar (1,3)	0.016	0.016	0.0007	0.0005	**Bar (1',3')**	0.0008	0.0007	0.0007	0.0005
Bar (2,4)	0.0007	0.0008	0.0007	0.0004	**Bar (2',4')**	0.0007	0.0008	0.0006	0.0006

	Figs. 7a & 7b	Figs. 7c & 7d	Figs. 7e & 7f	Figs. 7g & 7h
Bar (1,1')	0.67	0.52	0.30	0.85
Bar (2,2')	0.29	0.63	0.75	0.23
Bar (3,3')	0.40	0.37	0.96	0.86
Bar (4,4')	0.53	0.92	0.63	0.21

Figure 10

	Fig. 10a	Fig. 10c	Fig. 10e	Fig. 10g		Fig. 10b	Fig. 10d	Fig. 10f	Fig. 10h
Bar (1,2)	0.75	0.46	0.26	0.80	**Bar (1',2')**	0.87	0.56	0.52	0.44
Bar (3,4)	0.39	0.64	0.29	0.83	**Bar (3',4')**	0.90	0.46	0.50	0.63
Bar (1,3)	0.0008	0.006	0.0007	0.0004	**Bar (1',3')**	0.018	0.001	0.0007	0.0004
Bar (2,4)	0.0016	0.008	0.0007	0.0004	**Bar (2',4')**	0.006	0.0008	0.0007	0.0005

	Figs. 10a & 10b	Figs. 10c & 10d	Figs. 10e & 10f	Figs. 10g & 10h
Bar (1,1')	0.79	0.87	0.91	0.78
Bar (2,2')	0.75	0.95	0.59	0.44
Bar (3,3')	0.49	0.29	0.54	0.78
Bar (4,4')	0.67	0.09	0.20	0.44

Figure 11

	Fig. 11a	Fig. 11c	Fig. 11e	Fig. 11g		Fig. 11b	Fig. 11d	Fig. 11f	Fig. 11h
Bar (1,2)	0.17	0.10	0.30	0.78	**Bar (1',2')**	0.90	0.83	0.91	0.52
Bar (3,4)	0.90	0.92	0.59	0.80	**Bar (3',4')**	0.83	0.63	0.22	0.52
Bar (1,3)	0.0016	0.0008	0.0007	0.0004	**Bar (1',3')**	0.008	0.005	0.003	0.0005
Bar (2,4)	0.0008	0.003	0.005	0.003	**Bar (2',4')**	0.027	0.002	0.001	0.0004

	Figs. 11a & 11b	Figs. 11c & 7d	Figs. 11e & 11f	Figs. 11g & 11h
Bar (1,1')	0.59	0.26	0.21	0.52
Bar (2,2')	0.37	0.63	0.44	0.78
Bar (3,3')	0.63	0.46	0.31	0.52
Bar (4,4')	0.46	0.95	0.52	0.78

Figure 12

	Fig. 12a	Fig. 12c	Fig. 12e	Fig. 12g		Fig. 12b	Fig. 12d	Fig. 12f	Fig. 12h
Bar (1,2)	0.001	0.038	0.003	0.035	**Bar (1',2')**	0.27	0.59	0.40	0.18
Bar (3,4)	0.0027	0.0009	0.004	0.005	**Bar (3',4')**	0.086	0.006	0.0015	0.37
Bar (1,3)	0.0008	0.0010	0.0004	0.0003	**Bar (1',3')**	0.0045	0.0008	0.0007	0.0003
Bar (2,4)	0.003	0.0016	0.39	0.34	**Bar (2',4')**	0.75	0.15	0.088	0.0005

	Figs. 12a & 8b	Figs. 12c & 12d	Figs. 12e & 12f	Figs. 12g & 12h
Bar (1,1')	0.87	0.34	0.003	0.92
Bar (2,2')	0.01	0.003	0.90	0.03
Bar (3,3')	0.71	0.67	0.004	0.94
Bar (4,4')	0.10	0.12	0.13	0.03

Figure 13

	Fig. 13a	Fig. 13c	Fig. 13e	Fig. 13g		Fig. 13b	Fig. 13d	Fig. 13f	Fig. 13h
Bar (1,2)	0.02	0.009	0.008	0.006	**Bar (1',2')**	0.003	0.0001	0.049	0.002
Bar (3,4)	0.009	0.008	0.007	0.006	**Bar (3',4')**	0.0007	0.0008	0.03	0.003
Bar (1,3)	0.02	0.009	0.008	0.005	**Bar (1',3')**	0.0016	0.0007	0.02	0.0003
Bar (2,4)	0.009	0.008	0.007	0.007	**Bar (2',4')**	0.0008	0.0008	0.06	0.046

	Figs. 13a & 13b	Figs. 13c & 13d	Figs. 13e & 13f	Figs. 13g & 13h
Bar (1,1')	0.80	0.34	0.29	0.72
Bar (2,2')	0.82	0.55	0.06	0.67
Bar (3,3')	0.61	0.34	0.14	0.65
Bar (4,4')	0.03	0.08	0.03	0.67

Figure 17

	Fig. 17a	Fig. 17c	Fig. 17e	Fig. 17g		Fig. 17b	Fig. 17d	Fig. 17f	Fig. 17h
Bar (1,2)	0.70	0.90	0.95	0.30	**Bar (1',2')**	0.52	0.45	0.70	0.80
Bar (3,4)	0.30	0.90	0.91	0.26	**Bar (3',4')**	0.29	0.20	0.46	0.79
Bar (1,3)	0.04	0.0007	0.0007	0.002	**Bar (1',3')**	0.035	0.001	0.0007	0.0005
Bar (2,4)	0.01	0.0006	0.0045	0.015	**Bar (2',4')**	0.0027	0.013	0.003	0.008

	Figs. 17a & 17b	Figs. 17c & 17d	Figs. 17e & 17f	Figs. 17g & 17h
Bar (1,1')	0.52	0.51	0.51	0.37
Bar (2,2')	0.83	0.28	0.29	0.65
Bar (3,3')	0.63	0.91	0.23	0.85
Bar (4,4')	0.90	0.79	0.56	0.65

Figure 18

	Fig. 18a	Fig. 18c	Fig. 18e	Fig. 18g		Fig. 18b	Fig. 18d	Fig. 18f	Fig. 18h
Bar (1,2)	0.56	0.29	0.79	0.30	**Bar (1',2')**	0.59	0.25	0.15	0.80
Bar (3,4)	0.36	0.14	0.95	0.90	**Bar (3',4')**	0.75	0.083	0.18	0.40
Bar (1,3)	0.004	0.004	0.0008	0.0005	**Bar (1',3')**	0.0038	0.004	0.006	0.0006
Bar (2,4)	0.011	0.009	0.0007	0.0003	**Bar (2',4')**	0.010	0.013	0.1	0.019

	Figs. 18a & 18b	Figs. 18c & 18d	Figs. 18e & 18f	Figs. 18g & 18h
Bar (1,1')	0.32	0.61	0.61	0.49
Bar (2,2')	0.56	0.45	0.054	0.24
Bar (3,3')	0.37	0.29	0.87	0.48
Bar (4,4')	0.95	0.34	0.37	0.90

Figure 19

	Fig. 19a	Fig. 19c	Fig. 19e	Fig. 19g		Fig. 19b	Fig. 19d	Fig. 19f	Fig. 19h
Bar (1,2)	0.006	0.0015	0.0006	0.0018	**Bar (1',2')**	0.008	0.008	0.20	0.50
Bar (3,4)	0.003	0.0008	0.0009	0.0029	**Bar (3',4')**	0.02	0.02	0.0015	0.53
Bar (1,3)	0.004	0.0007	0.0007	0.0054	**Bar (1',3')**	0.01	0.003	0.0007	0.0006
Bar (2,4)	0.005	0.0005	0.017	0.001	**Bar (2',4')**	0.027	0.46	0.56	0.17

	Figs. 19a &19b	Figs. 19c & 19d	Figs. 19e & 19f	Figs. 19g & 19h
Bar (1,1')	0.79	0.55	0.46	0.77
Bar (2,2')	0.34	0.40	0.10	0.16
Bar (3,3')	0.87	0.46	0.79	0.29
Bar (4,4')	0.79	0.09	0.52	0.07

Figure 20

	Fig. 20a	Fig. 20c	Fig. 20e	Fig. 20g		Fig. 20b	Fig. 20d	Fig. 20f	Fig. 20h
Bar (1,2)	0.07	0.056	0.28	0.38	**Bar (1',2')**	0.001	0.0009	0.0006	0.0004
Bar (3,4)	0.02	0.50	0.038	0.38	**Bar (3',4')**	0.0008	0.0007	0.0005	0.0004
Bar (1,3)	0.0007	0.0007	0.0007	0.0006	**Bar (1',3')**	0.0007	0.0007	0.0007	0.0002
Bar (2,4)	0.87	0.67	0.20	0.32	**Bar (2',4')**	0.0008	0.0007	0.0005	0.0006

	Figs. 20a & 20b	Figs. 20c & 20d	Figs. 20e & 20f	Figs. 20g & 20h
Bar (1,1')	0.67	0.42	0.74	0.20
Bar (2,2')	025	0.0058	0.02	0.07
Bar (3,3')	0.83	0.29	0.32	0.65
Bar (4,4')	0.01	0.001	0.0007	0.07

Figure 24

	Fig. 24a	Fig. 24c	Fig. 24e	Fig. 24g		Fig. 24b	Fig. 24d	Fig. 24f	Fig. 24h
Bar (1,2)	0.87	0.75	0.90	0.23	**Bar (1',2')**	0.10	0.45	0.42	0.58
Bar (3,4)	0.75	0.37	0.22	0.53	**Bar (3',4')**	0.20	0.43	0.26	0.58
Bar (1,3)	0.006	0.002	0.0007	0.0007	**Bar (1',3')**	0.0011	0.0008	0.0007	0.0005
Bar (2,4)	0.013	0.0016	0.008	0.0008	**Bar (2',4')**	0.0007	0.006	0.004	0.0067

	Figs. 24a & 24b	Figs. 24c & 24d	Figs. 24e & 24f	Figs. 24g & 24h
Bar (1,1')	0.50	0.40	0.14	0.26
Bar (2,2')	0.21	0.45	0.55	0.67
Bar (3,3')	0.08	0.17	0.87	0.25
Bar (4,4')	0.83	0.59	0.79	0.76

Figure 25

	Fig. 25a	Fig. 25c	Fig. 25e	Fig. 25g		Fig. 25b	Fig. 25d	Fig. 25f	Fig. 25h
Bar (1,2)	0.002	0.002	0.0007	0.004	**Bar (1',2')**	0.03	0.001	0.0006	0.0027
Bar (3,4)	0.001	0.0007	0.009	0.003	**Bar (3',4')**	0.01	0.006	0.015	0.004
Bar (1,3)	0.001	0.001	0.0006	0.0004	**Bar (1',3')**	0.01	0.0013	0.0007	0.0003
Bar (2,4)	0.0002	0.008	0.007	0.20	**Bar (2',4')**	0.007	0.0018	0.003	0.19

	Figs. 25a & 25b	Figs. 25c & 25d	Figs. 25e & 25f	Figs. 25g & 25h
Bar (1,1')	0.39	0.91	0.82	0.64
Bar (2,2')	0.53	0.14	0.75	0.95
Bar (3,3')	0.67	0.92	0.95	0.98
Bar (4,4')	0.83	0.48	0.83	0.95

P-Values of Bioaccumulation Data (unpaired t-test)

Figure 16

	Fig. 16a		Fig. 16b		Fig. 16c		Fig. 16d		Fig. 16e	
	5ppt	30ppt	5ppt	30ppt	5ppt	30ppt	5ppt	30ppt	5ppt	30ppt
Conc. 0.0 – 0.5mg/l	0.000	0.005	0.000	0.015	0.000	0.02	0.000	0.005	0.000	0.000
Conc. 0.5 – 2.5mg/l	0.003	0.017	0.01	0.07	0.000	0.09	0.000	0.02	0.000	0.001
Conc. 2.5 – 5.0mg/l	0.58	0.03	0.98	0.15	0.02	0.008	0.70	0.06	0.88	0.003
Conc. 5.0 – 10.0mg/l	0.20	0.000	0.87	0.027	0.66	0.001	0.60	0.001	0.41	0.006

	Fig. 16a 5ppt vs. 30ppt	**Fig 16b** 5ppt vs. 30ppt	**Fig. 16c** 5ppt vs. 30ppt	**Fig. 16d** 5ppt vs. 30ppt	**Fig. 16e** 5ppt vs. 30ppt
Control	0.058	0.17	0.87	0.39	0.68
0.5mg/l	0.0008	0.002	0.0002	0.001	0.0001
2.5mg/l	0.0000	0.0008	0.0000	0.0000	0.0000
5.0mg/l	0.0009	0.008	0.0001	0.0004	0.0004
10.0mg/l	0.18	0.08	0.09	0.30	0.075

Figure 23

	Fig. 23a		Fig. 23b		Fig. 23c		Fig. 23d		Fig. 23e	
	5ppt	30ppt	5ppt	30ppt	5ppt	30ppt	5ppt	30ppt	5ppt	30ppt
Conc. 0.0 – 0.1mg/l	0.000	---	0.000	---	0.000	---	0.01	---	0.003	---
Conc. 0.1 – 0.48mg/l	0.09	---	0.10	---	0.01	---	0.27	---	0.08	---
Conc. 0.0 – 0.48mg/l	---	0.003	---	0.000	---	0.002	---	0.002	---	0.004
Conc. 0.48 – 2.4mg/l	0.10	0.20	0.05	0.007	0.01	0.20	0.22	0.25	0.60	0.08
Conc. 2.4 – 4.8mg/l	0.10	0.10	0.10	0.10	0.03	0.02	0.20	0.30	0.10	0.16
Conc. 4.8 – 9.6mg/l	---	0.36	---	0.90	---	0.50	---	0.20	---	0.20
Conc. 9.6 – 16.0mg/l	---	0.42	---	0.85	---	0.85	---	0.40	---	0.90

	Fig. 23a 5ppt vs. 30ppt	Fig. 23b 5ppt vs. 30ppt	Fig. 23c 5ppt vs. 30ppt	Fig. 23d 5ppt vs. 30ppt	Fig. 23e 5ppt vs. 30ppt
Control	0.16	0.3	0.70	0.17	0.07
0.5mg/l	0.2	0.07	0.76	0.23	0.01
2.5mg/l	0.08	0.57	0.35	0.56	0.02
5.0mg/l	0.13	0.60	0.45	0.22	0.06

Figure 26

	Fig. 26a	Fig. 26b	Fig. 26c	Fig. 26d	Fig. 26e
Bar (1,2)	0.04	0.03	0.001	0.0001	0.02
Bar (3,4)	0.66	0.54	0.39	0.50	0.55
Bar (1,3)	0.02	0.003	0.006	0.04	0.0000
Bar (2,4)	0.0004	0.0002	0.0000	0.0007	0.002

Figure 16 vs. Figure 26

	0.5mg/l		2.5mg/l	
	5ppt	**30ppt**	**5ppt**	**30ppt**
Figure a	0.001	0.1	0.000	0.09
Figure b	0.004	0.46	0.001	0.26
Figure c	0.000	0.41	0.000	0.69
Figure d	0.01	0.47	0.000	0.39
Figure e	0.000	0.34	0.000	0.80

Figure 27

	Fig. 27a	Fig. 27b	Fig. 27c	Fig. 27d	Fig. 27e
Bar (1,2)	0.8	0.49	0.32	0.10	0.17
Bar (3,4)	0.01	0.0005	0.0000	0.10	0.28
Bar (1,3)	0.0001	0.0000	0.0000	0.0000	0.004
Bar (2,4)	0.0002	0.0005	0.0001	0.001	0.0003

Figure 23 vs. Figure 27

	0.48mg/l		2.4mg/l	
	5ppt	**30ppt**	**5ppt**	**30ppt**
Figure a	0.06	0.50	0.01	0.003
Figure b	0.98	0.96	0.000	0.004
Figure c	0.30	0.81	0.000	0.000
Figure d	0.33	0.36	0.03	0.04
Figure e	0.90	0.04	0.01	0.000

APPENDIX B

Nominal versus Measured Concentrations of Cd

	5ppt		30ppt	
Cd nominal concentration	**Measured pre-exposure conc. (mg/l)**	**Measured post-exposure conc. (mg/l)**	**Measured pre-exposure conc. (mg/l)**	**Measured post-exposure conc. (mg/l)**
0.5mg/l Cd	0.45 ± 0.03	0.34 ± 0.01	0.47 ± 0.03	0.34 ± 0.03
0.5mg/l Cd*	0.49 ± 0.04	0.44 ± 0.12	0.51 ± 0.06	0.44 ± 0.01
2.5mg/l Cd	2.46 ± 0.18	2.09 ±0.12	2.45 ± 0.08	2.21 ± 0.17
2.5mg/l Cd*	2.27 ± 0.10	2.39 ± 0.10	2.33 ± 0.15	2.41 ± 0.06
5.0mg/l Cd	4.49 ± 0.30	4.19 ± 0.26	4.78 ± 0.10	4.29 ± 0.03
10.0mg/l Cd	9.38 ± 0.39	6.85 ± 0.23	9.41 ± 0.44	10.14 ± 0.16

* Cd in combination with Ag

Nominal versus Measured Concentrations of Ag

	5ppt		30ppt	
Ag nominal concentration	**Measured pre-exposure conc. (mg/l)**	**Measured post-exposure conc. (mg/l)**	**Measured pre-exposure conc. (mg/l)**	**Measured post-exposure conc. (mg/l)**
0.10mg/l Ag	0.03 ± 0.00	0.001 ± 0.00	----------	----------
0.48mg/l Ag	0.10 ± 0.03	0.08 ± 0.01	0.1 ± 0.02	0.07 ± 0.03
0.48mg/l Ag*	0.18 ± 0.02	0.17 ±0.02	0.36 ± 0.03	0.29 ± 0.01
2.4mg/l Ag	0.33 ± 0.04	0.15 ± 0.01	0.39 ± 0.06	0.41 ± 0.05
2.4mg/l Ag*	0.65 ± 0.08	0.80 ± 0.21	0.97 ± 0.05	1.40 ± 0.04
4.8mg/l Ag	0.73 ± 0.16	0.49 ± 0.05	0.83 ± 0.04	0.81 ± 0.04
9.6mg/l Ag	----------	----------	1.40 ± 0.08	1.76 ± 0.07
16.0mg/l Cd	----------	----------	1.45 ± 0.02	1.13 ± 0.05

* Ag in combination with Cd

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