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SOME EFFECTS OF CADMIUM ON SELECT CRAYFISH IN THE FAMILY CAMBARIDAE

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ABSTRACT OF DISSERTATION

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The Graduate School

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ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Arts and Sciences
at the University of Kentucky

By
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A series of acute (96h) toxicity tests were conducted on six species of crayfish in the family Cambaridae. Toxicity values fell into a sensitive group comprising *Orconectes placidus* and *Procambarus acutus* (LC 50= 0.368mg Cd/L - 0.487mg Cd/L; LC10= 0.048 mg Cd/L - 0.092 mg Cd/L) and tolerant group comprising *O. juvenilis*, *O. virilis*, *P. alleni,* and *P. clarkii* (LC 50= 2.44 mg Cd/L - 3.30 mg Cd/L; LC10= 0.386 mg Cd/L - 0.947 mg Cd/L). For juvenile crayfish, the LC50 and LC10 values were as follows: *O. juvenilis*, 0.060 and 0.014 mg Cd/L; *O. placidus*, 0.037 and 0.002 mg Cd/L; *P. clarkii*, 0.624 and 0.283 mg Cd/L. Cd exposure decreased molting success highlighting the importance of this sensitive process. Behavioral responses were assessed in *O. placidus*, *O. virilis*, *P. acutus*, *P. alleni*, and *P. clarkii*. The tail-flip predator avoidance behavior was significantly reduced by cadmium exposure. In most species tested, the claw raise defensive behavior was significantly increased by Cd exposure. Between species, as body mass increased, the tail-flip response frequency decreased, and the claw-raise response increased in frequency. *P. clarkii* was also analyzed for the effect of Cd exposure on heart rate and response to two stimuli. The data indicate that heart rate may be a useful

physiological marker of Cd toxicity. The major organ systems were dissected from survivors of four adult crayfish toxicity tests (*O. juvenilis*, *O. placidus*, *P. acutus*, *P. clarkii*) and analyzed for metal content. Cadmium tissue content correlated with Cd exposure. Cadmium accumulated more in the hepatopancreas, gills and green glands of sensitive species than in tolerant species. Zn showed negative correlations with Cd exposure in the hepatopancreas. Cu increased in green glands, gills, and hemolymph and, in some cases, decreased in the hepatopancreas. Ca, Fe, Mg, and Zn also showed significant trends. Zn accumulated in the exposure water over 24h. These data indicate that Cd may displace Cu and/or Zn in the hepatopancreas and the displaced metal then may move into other tissues, especially the gills and green glands, possibly to be excreted.

Key words: Crayfish, Cadmium, Toxicity, Tissue Accumulation, Behavior

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For Lucia, the carnation in my wilderness.

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Chapter 1: Introduction.

1.1 Problem formation

Crayfish are common and unmistakable residents of fresh waters. They resemble small lobsters and possess jointed appendages, the most obvious of which are their large pincers or chela which "those who handle them incautiously will discover that their grip is by no means to be despised" (Huxley 1880). They are common stream inhabitants in Kentucky and most of the United States (Taylor and Schuster 2004; Hobbs 1976). In fact, North America has the greatest diversity of crayfish species of anyplace in the world. More than 320 of the worldwide total of approximately 520 species are found here (Taylor *et al*. 1996; Master *et al*. 2000).

A very large proportion of the crayfish fauna are considered to be imperiled or vulnerable to extinction. Some authors estimate that about 50% of U.S. species are threatened at some level (Taylor *et al*. 1996; Master *et al*. 2000). The World Conservation Union (IUCN) Red List of Threatened Species, which is somewhat more conservative in its approach, still lists 34% of U.S. crayfish species as vulnerable or threatened (Master *et al*. 2000; IUCN 2005).

Despite the general consensus that a large proportion of crayfish species are at risk of extinction, only 4 species (about 1%) are protected under the U.S. Endangered Species Act (U.S. ESA). Currently, no other species are proposed for listing or are candidates for listing (U.S. Fish and Wildlife Service 2005). By comparison, 37% of U.S. freshwater fish are considered to be imperiled, threatened, or vulnerable, and 16% of all U.S. species are listed or are proposed or candidates for listing under the U.S. ESA (Stein *et al*. 2000; U.S. Fish and Wildlife Service 2005). The only group of North American freshwater animals that seem to be more imperiled than crayfish are the freshwater

mussels. About 69% of these molluscs are considered vulnerable, threatened, endangered, or extinct (Master *et al*. 2000). However, they are more rigorously protected. More than 29% of U.S. species are listed under the U.S. ESA, or are proposed or candidates for listing (U.S. Fish and Wildlife Service 2005). While proposed and candidates species do not receive any legal protection under the U.S. ESA, they are at least in the process of becoming protected, which may take many years to complete. Thus, compared to other endangered freshwater groups, crayfish are poorly protected.

It has been estimated that 28% of imperiled, threatened, or vulnerable crayfish species are affected by pollution. This is the second most serious threat after habitat loss/degradation (Wilcove *et al*. 2000). It ranks ahead of alien species introduction, perhaps because of the accumulation of pollutants in aquatic ecosystems and the increased importance of the respiratory route of exposure.

Cadmium, a common and widespread toxicant, is found in 776 of 1476 hazardous waste sites on past and present United States Environmental Protection Agency's (U.S. EPA) National Priorities Lists (Taylor *et al.* 1999). It is one of the most toxic metals and is listed 7th of 275 substances on the current CERCLA Priority List of Hazardous Substances (ATSDR 2005). It enters the environment primarily through mining activities and fossil fuel combustion. Commercially available Cd is typically recovered as a byproduct from zinc, copper, and lead mining. Globally, 25,000 to 30,000 tons of Cd are released into the environment annually, with perhaps 4,000 to 13,000 tons of that total coming from anthropogenic sources (Taylor *et al.* 1999). The Cd^{2+} ion, the most bioavailable form and the most toxic to aquatic organisms, is thought to be taken up through the gills and intestine by the same mechanism as calcium and Zn (Verbost *et al.* 1989; Bondgaard and Bjerregaard 2005). This nonessential metal can cause a wide range

of effects in aquatic organisms, including slowed growth, reduced reproductive performance, impaired Ca metabolism, and outright mortality (Albert *et al.* 1992; Wright and Welbourne 1994; Ahearn *et al*. 2004). The impact of Cd on Ca metabolism makes Cd a toxicant of special interest because of its implications for the molting process, a time of greatly increased Ca metabolism (Wheatly and Ayers 1995).

Despite the imperiled status of so many crayfish species, acute toxicity tests, which are important for determining toxicant criteria, have been conducted on only a few crayfish species. Only four toxicity tests, each involving a different species, have been included in the derivation of freshwater criteria values as reported in the U.S. EPA ambient water quality criterion document for Cd (Boutet and Chaisemartin 1973; Phipps and Holcombe 1985; Mirenda 1986; Naqvi and Howell 1993; U.S. EPA 2001). Only one of these tests included a sensitive early life stage. None of the species tested were threatened or endangered species. In contrast, twenty-four species of fish were tested, many several times, and included in the derivation of freshwater Cd criteria. Thus, crayfish are poorly represented in the regulatory literature whose purpose is to protect them. Additionally, no studies have assessed the relative tolerance of different species of crayfish, an important consideration given the potential for a related group of organisms such as crayfish to have different tolerances (Hobbs and Hall 1974).

Despite the wide use of crayfish in behavioral studies (*e.g.* Huxley 1880; Hayes 1975; Hayes 1977; Bruski and Dunahm 1987; Listerman *et al*. 2000; Kellie *et al*. 2001; Schapker *et al*. 2002), there is relatively little data on how heavy metal exposure affects crayfish behavior. The effects of heavy metals, including Cd, on feeding behaviors and shelter use by crayfish have been examined, as has whether Cd can elicit avoidance or attraction responses (Maciorowski *et al.* 1980; Steele *et al*. 1992; Misra *et al.* 1996;

Alberstadt *et al.* 1999; Sherba *et al*. 2000). However, no work has been done on important defensive behaviors such as the claw-raise behavior or the tail-flip escape behavior.

A small amount of work has been done on the fate of Cd in crayfish tissues. Thorp *et al*. (1979) and Mirenda (1986) examined the pattern of Cd deposition in various crayfish organs. Thorp and Gloss (1986) compared laboratory and field studies of acute Cd exposure to crayfish. Many studies have been done on wild caught crayfish and their uptake of heavy metals including Cd. (*e.g.* Anderson 1977; Dickson *et al*. 1979; Bagatto and Alikhan 1987a; Ricon-Leon *et al*. 1988; Bendell-Young and Harvey 1991; Naqvi *et al*. 1993; Khan *et al*. 1995; Schilderman *et al*. 1999; Sanchez-Lopez *et al*. 2004). However, no studies have examined how Cd may affect other metals in the bodies of crayfish, or compared multiple species to determine how consistent are the pattens of deposition.

The work included in this manuscript has six major objectives:

1. Expand the database of crayfish toxicology.
2. Assess the effects of Cd on crayfish molting success.
3. Compare the differences in Cd accumulation between sensitive and tolerant species of crayfish.
4. Explore the effects of Cd exposure on the distribution of other, nutritionally important, metals in the crayfish's body.
5. Investigate the effects of Cd on crayfish defensive behaviors
6. Examine the usefulness of crayfish heart rate as a physiological marker of Cd stress.

To elaborate, this dissertation presents toxicology data for five species that have not been tested previously, using a consistent methodology so that the interspecific variability of Cd tolerance could be assessed. It also presents new data on the toxicity of Cd to the juveniles of two, previously untested crayfish species. To assess how these toxicity tests compare to values reported in the literature, two additional assays, one on adults and one on juveniles, were conducted to allow results from this methodology to be compared to literature values. Accumulations of Cd in the whole body and organ systems of sensitive and tolerant crayfish species were measured. The primary organs of Cd accumulation are determined for each species and the differences between the accumulation patterns of these two groups are compared. Finally, the tissue residue patterns of selected nutritionally important metals was measured in various organs. This is used to investigate the changes caused by Cd exposure in various organs, giving clues about how the organism as a whole is responding to Cd. This work also presents new findings about the effects of Cd on two important defensive behaviors in crayfish, the claw-raise behavior and tail-flip escape behavior. It also assesses the utility of crayfish heart rate as a physiological marker of Cd stress.

1.2 Crayfish biology

1.2.1 Taxonomy and evolution

Debate exists regarding the evolutionary origins of crayfish. One area of contention regards the proposed monophyly of crayfish and another whether the ancestors of modern crayfish invaded freshwater once or several times. Evidence for the polyphyly of crayfish includes zoogeography, morphology, ontology, paleontology, physiology, and differences in commensal organisms (Huxley 1880; Smith 1912; Gurney 1935; Gelder 1999; Scholtz 2002). Ortmann (1902) was the first person to propose the

monophyletic origin of crayfish. Recent work in embryology, juvenile anatomy, various aspects of adult anatomy, and molecular biology together support his hypothesis and strengthen the case for monophyly (Scholtz and Richter 1995; Hasiotis 1999; Skiebe 1999; Scholtz 1999; Scholtz and Kawai 1999; Crandall *et al*. 2000; Scholtz 2002; Scholtz and Kawai 2002; Sinclair *et al*. 2004).

Current taxonomy indicates that three families of crayfish exist within the infraorder Astacida, which itself is within the order Decapoda. This order is within the subphylum Crustacea and phylum Arthropoda. Two crayfish families, the Astacidae and Cambaridae are placed within the superfamily Astacoidea, while family Parastacidae resides within the super family Parastacoidea (Scholtz 2002; Taylor and Schuster 2004). The Astacidae are found in Europe, western Asia, and western North America. The Cambaridae are found in eastern North America and eastern Asia, while the Parastacidae live exclusively in the southern hemisphere in South America, Australia, New Zealand, and Madagascar (Hobbs 1988). The clawed lobsters (*e.g. Homarus americanus*) were once considered to be either ancestral or closely related to the freshwater crayfish (Hobbs 1988). Now, they are thought to be more distantly related and that all resemblance between these groups is ancient (Scholtz 2002).

The hypothesis that crayfish invaded freshwater multiple times gains much of its support from the widely separated distributions of the Parastacidae, Cambaridae and Astacidae (Starobogatov 1995; Scholtz 2002). Recent fossil evidence has opened the possibility of a more ancient common ancestor for crayfish that only entered freshwater once (Hassiotis 1999). Currently, the oldest fossil crayfish date from the Triassic period (245-200 million years ago). However, fossil burrows that are attributed to ancient crayfish have been dated to the Pennsylvanian period (320-268 million years ago), thus

predating the breakup of the supercontinent of Pangea. Having already invaded freshwater, different populations could have been carried on Gondwanaland and Laurasia when Pangea broke up at the end of the Triassic period (Hassiotis and Mitchell 1989; Hassiotis 1999; Scholtz 1999; Scholtz 2002). This, with current interpretations of various crayfish anatomical and physiological characteristics, has increased the overall level of support for the single invasion theory (Scholtz 2002).

Given that the number of crayfish species is great in North America and that the overall mophology of most species is similar, identifying individual species can be difficult. Many of the most reliable keys rely upon the gonopods of Form I males to give accurate assessments. Particularly useful keys include Hobbs 1976, Hobbs and Jass 1988, Hobbs 1989, Jezerinac *et al*. 1995, Pflieger 1996, Taylor 2000, and Taylor and Schuster 2004. The key by Rhoades (1944) is historically important as the first comprehensive key of Kentucky crayfish.

1.2.2 Crayfish anatomy and physiology

The anatomy of crayfish is relatively well understood, in large part because of the widespread use of crayfish as instructional tools in schools around the world since the eighteenth century (Vogt 2002). Their convenient size and relative abundance make themideally ssuited for laboratory exercises. Another reason for the instructional and research popularity of crayfish is Thomas Henry Huxley's classic, well illustrated textbook, *The Crayfish: an Introduction to the Study of Zoology*, published in 1880. Since that time, crayfish have become a widely used species for behavioral research (*e.g.* Hayes 1977; Bruski and Dunham 1987; Keller and Moore 2000; Listerman *et al*. 2000; Schapker *et al*. 2002), neurological and neuromuscular studies (*e.g*. Cooper *et al*. 1995;

Johnstone *et al*. 2002; Forgue *et al*. 2001; Patullo and Macmillan 2004), and many other topics.

As crustaceans, crayfish possess a heavy exoskeleton or cuticle composed largely of chitin and calcium carbonate ($CaCO_3$). This structure is secreted in layers, the outermost layer is the epicuticle and is composed of lipids, proteins, and calcium salts, but lacks chitin. Beneath it lies the exocuticle, composed mostly of chitin-protein microfibrils with a strengthening matrix of $CaCO_3$, and may contain pigments. The endocuticle is similar, but unpigmented. It is secreted by the animal after molting while the previous two layers are laid down before molting occurs underneath the old exoskeleton. Pore canals extend outward through the endocuticle and exocuticle allowing cells of the epidermis to extend into the cuticle and help regulate calcification. All the preceding layers are secreted by the epidermis, a single layer of columnar or cuboidal epithelium attached to a basement membrane (Felgenhauer 1992).

Tegumental glands are located just below the epidermis. Some secrete materials that help tan (harden) the cuticle while other types secrete mucus. The glair glands of the female, used to attach eggs to the pleopods, are another example of tegumental glands. Antibacterial and antifouling functions have also been proposed for some of these glands (Felgenhauer 1992).

Setae also are present on the surface of the crayfish cuticle and can have multiple forms and functions. Setal morphology can range from simple shafts to shafts with various arrangements of setules. Setae can be involved in feeding, grooming, mechanoreception, and chemoreception. Other cuticular sensory structures include cuticular articulated peg organs (CAP organs), funnel canal organs, and hair-fan organs. CAP organs are either mechanosensory or propriosensory in nature. Funnel canal organs

are stress sensors in the flexible cuticle of pereopods. Hair fan organs sense low frequency vibrations and some researchers liken them to the lateral line system of fish (Felgenhauer 1992).

A crayfish's body is segmental like all arthropods. Usually, each segment possesses a pair of appendages which are biramous (*i.e.* having two portions), an endopod and exopod attached to a basal protopod. However, a dramatic amount of specialization can alter this basic design. The head of crayfish, like all crustaceans, have 5 fused segments. Anteriorly to posteriorly, the appendages of the head include the antennules, the antennae, the mandible, the first maxilla, and the second maxilla. The antennules are chemosensory and mechanosensory in function. The antennae are mechanosensory and function in certain behaviors. The short, heavily calcified mandibles masticate food items. The 1st maxillae help to manipulate food, abrade it, and seal the gill chambers. The 2nd maxillae have similar functions to the 1st maxillae, but the exopod on each side is modified into a scaphognathite, or gill bailer which beats to generate a current through the gill chamber (Holdich 2002a). The anterior most structure on the head is the rostrum, which serves to protect the eyes, antennae, and antennules. The head may be ornamented with various spines and grooves that can be helpful in identifying species. The posterior margin of the head is demarcated by the cervical groove (Holdich 2002a).

The thoracic region is composed of 8 segments. The first three segments possess the 1st through 3rd maxillipeds, respectively. The 1st maxillipeds compliment the function of the scaphognathites, manipulate food, filter water, and help provide a protective cover for the gill chamber. The 2nd and 3rd maxillipeds both manipulate and process food and are active in filter feeding. Both possess gills as well, which, like all the gills, are covered

by the carapace. This constitutes the outer shell of the thorax and forms protective gill chambers on each side. The 3rd maxillipeds are often used to clean other appendages (Holdich 2002a). The next appendages are the greatly enlarged 1st pereopods known as the chelipeds or claws. They may take a variety of shapes depending upon species, but are always more robustly constructed than the other appendages and possess a chela with a movable and an immovable finger. They are used in predation, offensive and defensive behaviors, courtship, mating, chemoreception, station keeping in swift currents, burrowing, egg laying, climbing, and righting the animal if overturned. They also provide a counterbalance to the weight of the abdomen and have gills. The 2nd and 3rd pereopods have a similar structure and function to each other. They possess a small chela at the tip and, in addition to walking, aid in grooming, collect food particles, and help with egg attachment during spawning. The 3rd and 4th pairs of pereopods are most used in walking although all others contribute. In females, the 3rd pereopods have the gonopores at their base. The 4th and 5th pereopods lack the small chela found on the 2nd and 3rd pereopods and function in walking and mating. The male's gonopores are located at the base of the 5th pair of pereopods. Gills are present on all five pairs of pereopods.

Externally, the end of the carapace marks the end of the thorax. The 6 abdominal segments are covered by curved plates called pleonites. Unlike the previous segments, these are unfused, allowing the organism to flex its abdomen. The first five abdominal segments possess appendages called pleopods or swimmerets. They are small and beat in sequence. Typically they are biramous and covered with setae. They serve to help the animal maintain station and create a water current when inside a burrow. In females, the pleopods are larger and are used to carry eggs during the breeding season. The female can move the pleopods carrying the egg mass to promote water circulation around the

eggs. In the males of the Astacoidea, the 1st pleopods are highly modified as a sperm transfer organ. They are nearly tubular in cross-section and often possess intricate ornamentations at their terminal ends that are extremely important in identifying species. Also in the male, the 2nd pair of pleopods are modified to act as pistons to push the spermatophore through the 1st pleopods and section it into short lengths. In the female, the 1st pair of pleopods are vestigial. In the parastacid crayfish, the 1st pair of pleopods is vestigial as well, so the mechanism of sperm transfer is different, but is not known. The last segement of the crayfish's body comprises the tail fan used in the backward swimming that is characteristic of crayfish, lobsters, and shrimp. The central portion is called the telson and the appendages called the uropods. These appendages are modified into flattened, but slightly curved plates. In addition to swimming, they may aid in backward burrowing and help to protect the egg mass of the female (Holdich 2002a).

The musculature of crayfish is generally similar to other animals, although a few significant differences do exist. All crayfish muscle is striated, including that of the heart and digestive system. Typically, muscles operate in flexor/ extensor pairs that are either attached to the exoskeleton, the endophragmal skeleton, or apodemes. The endophragmal skeleton consists of internal skeletal members that are projections of the external skeletal plates, while apodemes are chitinous tendons. Muscles are not directly attached to apodemes or the skeleton. Attachment is achieved by bundles of tendonous cells that have arrays of microtublues within them and external fibers extending out from them (Holdich and Reeve 1988; Vogt 2002).

The cellular apparatus of contraction, the sarcomere, is very similar to that found in vertebrates. The sequence of events and processes involved in sarcomere contraction are also quite similar. In general, three types of muscle fibers have been described based

upon sarcomere length. Fibers with long sarcomeres are tonic in nature. Termed Group A fibers, they have slow contraction times and high strength and are used to maintain posture or for slow, powerful movements. More rapid movements are handled by fibers with short sarcomeres (*i.e.* phasic fibers). They are also known as Group C fibers and have lower strength, but a faster contraction time and are used for quick bursts of activity. Group B fibers possess a structure and physiological characteristics that are intermediate between the other two types. All three types are often present in a given muscle. As with other animals, crayfish muscles receive both excitatory and inhibitory innervation. Glutamate serves as the excitatory neurotransmitter while γ- aminobutyric acid (GABA) serves as the inhibitory neurotransmitter (Vogt 2002)

One unique aspect of crustacean musculature relates to how the animal accommodates the molting process (ecdysis). In a normal, healthy individual, the muscles of some appendages, especially the chela, are much larger in diameter than the aperture in the old exoskeleton through which the limb must pass when the old exoskeleton is finally shed. Cells are not lost through apoptosis, but the size of each cell is reduced by the breakdown of some of the sarcomeres. Muscles remain functional, but weakened, and the cellular machinery is reassembled after ecdysis has been completed. This process also seems to also help accommodate the increase in size brought about by molting (Vogt 2002).

In a broad sense, the nervous system of crayfish is generally similar to other animals, with sensory neurons, motor neurons, and interneurons. The cell bodies, or soma, of nerve cells tend to be accumulated in either sense organs or in ganglia. The brain, or supraesophageal ganglia, consists of a pair of ganglia located dorsally to the esophagus. A pair of connecting nerve cords attach the brain to the subesophageal

ganglion which gives rise to the ventral nerve cord. This structure possesses a series of ganglia down its length, five in the thorax and 6 in the abdomen. While through most of its length a single nerve cord, it is derived from an ancestral paired nerve cord (Vogt 2002).

The crayfish brain can be divided into three regions. The protocerebrum handles visual input and regulates the endocrine system. The deutercerebrum processes chemosensory information, especially from the antennules. It also serves as an integration center. The tritocerebrum receives information from the antennae and may also have integrative functions (Vogt 2002). The ganglia of the ventral nerve cord control appendicular movement and the swimming movement of the abdomen (Holdich and Reeve 1988). An unusual feature of the crayfish nervous system, although shared with a few other invertebrate groups, is the existence of giant neuron fibers. These allow for faster transmission of impulses and thus faster responses to stimuli (Holdich and Reeve 1988; Vogt 2002).

Crayfish sensory systems include the intero- and exteroreceptors one might expect from an active omnivore. They typically possess compound eyes with hundreds to thousands of individual ommatidia. Under low light conditions a superpositional image can be formed whereby light entering multiple adjacent ommatidia can shine through the walls of each individual ommatidum to strike several of these structures at once. While this lowers visual accuity, it increases the light gathering power of each ommatidium (Vogt 2002). Eyes can be reduced, nonfunctional, or lost in cave crayfish (Pflieger 1996; Ziemba *et al*. 2003; Taylor and Schuster 2004).

Crayfish possess a pair of statocysts, one in the base of each antennule. They consist of a concretion of small sand grains that are lost and replaced with each molt.

These structures sense gravity, high frequency vibrations, and movement of the organism (Vogt 2002).

Various chemoreceptive structures can be found on crayfish, especially on the antennules, oral region, and pereopods. The antennules seem to be tuned to sense distant sources, thus acting as a sense of smell, while those in other locations are used in feeding, thus functioning more like a sense of taste. Hydrodynamic sensors can be found across the crayfish's body and serve to keep the organism aware of anything that may be moving in the water around it, be it another crayfish, a predator, or prey. Crayfish also possess various interoceptors such as chemoreceptors, baroreceptors, mechanoreceptors, and nerve cord stretch receptors to monitor the internal physiological status of the organism (Vogt 2002).

Crayfish possess an open circulatory system. Broadly, it consists of a heart, seven main arteries, minor arteries arising from them, sinuses that penetrate the tissues, and the hemolymph that fills these spaces. The heart is a single chambered sac that resides within a larger chamber, the pericardium. The heart sometimes contains internal structures that may help to channel the hemolymph. The heart possesses six ostia in three pairs, on the dorsal, ventrolateral, and caudolateral, aspects of the organ. The ostia possess no valves, but do have muscular pads that seem to close these apertures during heart contraction (systole). However, the arteries leaving the heart do have valves to prevent the back flow of hemolymph during diastole. The pericardium is under muscular control to expand during systole to draw hemolymph out of the nearby sinuses to help fill the heart during diastole (Vogt 2002).

The arteries of the circulatory system consist of five anterior and two posterior arteries. Anteriorly, there are two antennary, two hepatopanreatic, and one opthalmic

arteries, which supply many portions of the cephalothorax, including sense organs and cephalic appendages. The opthalmic artery has a special auxilliary pumping structure called the *cor frontale*, a muscular, contractile expansion of the artery itself. The posterior arteries are the sternal artery, which has branches to supply the mouthparts, esophagus, maxillipeds, pereopods, gonopods, and the thoracic and abdominal musculature. The dorsal abdominal artery primarily supplies hemolymph to the abdominal musculature, gonads, pleopods, and uropod (Vogt 2002).

After hemolymph is delivered into the target organs it is released into expanded, unlined sinuses that bathe and penetrate the tissues. After passing over the tissues, much of the hemolymph is collected in the sternal sinus. From there it is circulated up into the branchial sinuses, then into the gills where oxygen can be obtained, and carbon dioxide and ammonia released. Thence, the hemolymph returns to the pericardium through the branchiopericardial vessels (Vogt 2002).

Since crayfish have an extensive calcified exoskeleton that strongly resists diffusion over most of the body, specialized respiratory structures are required. The gills are typically associated with the thoracic appendages or are in close proximity to them. While the gills are covered with the exoskeleton, its thickness can be as little as 1μm. The general structure of crayfish gills is termed tricobranchiate as each gill is made of a central axis with up to 300 filaments arising from it. Each segment may have up to 4 individual gills associated with it although usually there are fewer than this maximum. Podobranchs are found attached to the base of an appendage and only one may be present per segment. Arthrobranch are found on the arthrodial membrane that is between the appendage and the body wall. There can be up to two of these per segment. Single pleurobranchs may be found on the epimeral wall (*i.e.* the inner wall of the carapace) that

faces each segmental appendage. In the Astacoidea, the podobranchs tend to be somewhat modified having a wing like lamina covered by branchial filaments (Vogt 2002).

A flow of water is maintained over the gills by the beating of a modified extension of the 2nd maxilla, the scaphognathite (*i.e.* gill bailer). Cleaning of the gills occurs through several mechanisms. Long filaments called setobranchs and the setae of the branchiostegite (*i.e.* the plate that forms the carapace on each side of the body) help to clean the gills. Normal limb movements function in gill cleaning by moving these structures across the gill filaments. Specialized leg rocking behaviors exists that enhance the cleaning of the gills. While these mechanisms are effective against particulate matter, they are less effective against bacterial and protozoan infestations. Typically, only molting can remove those pests. Crayfish do not possess the direct method of cleaning the gills using the chela that some shrimp possess (Felgenhauer 1992; Batang and Suzuki 2000; Vogt 2002).

In general, the hemolymph flows from the branchial sinuses up the axis of the gill and into the filaments through small sinuses arranged to establish a countercurrent exchange of gases. In addition to acquiring O_2, the waste products CO_2 and NH_3 are released from the gills. While many crabs have gills that are segregated into either ion regulating or respiratory structures, most all gills in crayfish possess both abilities. However, individual filaments may be specialized into the exchange of either gases or ions. Respiratory epithelia tend to be poor in mitochondria and rather thin. The cuticle is also thinner over respiratory cells, with the total thickness of the epithelium with cuticle is approximately 2μm. Ionoregulatory cells tend to be thicker, as does the cuticle that lies over top of them. They also have many more mitochondria and possess folds in the apical

and basal membranes. Crayfish gills also seem to contain specialized excretory cells called nephrocytes that seem to play a part in ridding the crayfish of unwanted particulate matter such as viruses, and various waste products (Taylor and Taylor 1992; Vogt 2002).

Oxygen readily diffuses across the crayfish gill, sometimes having an efficiency of 71%, which is nearly comparable to fish and cephalopods (Wolvekamp and Waterman 1960). Carbon dioxide and ammonia are also lost across the gill surface. Carbonic anhydrase may help reduce carbon dioxide levels by converting HCO_3^- into CO_2, which can pass across the outer membrane more easily. However, transmembrane transport proteins probably play a major role in removing NH_3 and CO_2, when these chemicals are dissolved as HCO_3^- or NH_4^+ and used as counter ions for ionoregulatory exchange (Vogt 2002).

Ionoregulation is the other major function of crayfish gills. This is especially important given that crayfish are strong osmoregulators and freshwater concentrations of various important ions are very low compared to crayfish tissue concentrations. As previously mentioned, ionoregulatory cells are rich in mitochondria. This is because much of the ionic uptake is accomplished through the action of transmembrane transport ATPase proteins, such as Na^+/K^+ ATPase and Ca^{2+} ATPase. In the apical surface of the gills there are $2Na^+(Ca^{2+})/H^+(NH_4^+)$ exchangers (antiporters), Cl^-/HCO_3^- exchangers, and Ca^{2+} channels. Another Ca^{2+} antiporter also may exist, this one being a nonelectrogenic $Ca^{2+}/2Na^+(2H^+)$ exchanger. These are driven by iongradients established by two enzymes in the basolateral membrane. Na^+/K^+ ATPase powers the $2Na^+(Ca^{2+})/H^+(NH_4^+)$ exchangers and Cl^-/HCO_3^- exchangers. Ca^{2+} ATPase helps to draw ions through the Ca^{2+} channels, although Na^+/K^+ ATPase can also help with Ca^{2+} uptake. There is also evidence for a H^+ ATPase that enhances the activity of the Cl^-/HCO_3^- exchanger. These are assisted

by the electrochemical gradient established by the Cl^-/HCO_3^- exchangers. A $3Na^+/Ca^{2+}$ exchanger and Cl^- channel also exist in the basolateral membrane. (Flik *et al*. 1993; Rainbow 1995; McMahon 2002; Vogt 2002; Ahearn *et al*. 2004).

Other important ionoregulatory organs include the antennal glands and hepatopancreas. The antennal glands or green glands share some structural similarities to the vertebrate kidney. There is a filtration structure associated with the circulatory system, the coelomosac which is supplied by the antennary artery. It is composed of podocytes that form a basolateral membrane and interdigitating network of cellular extensions known as the slit diaphragm which together form a filter matrix capable of retaining molecules from 70 to 150 kDa in size or larger (Fingerman 1992; McMahon 2002). However, larger waste products, such as organic acids and damaged proteins, may be actively secreted by cells of the coelomosac. The ultrafiltrate produced here then moves to the labyrinth, which consists of a complex network of parallel and interconnected channels that can secrete various metabolic wastes, such as organic acids, into the urine. The next structure in the green gland is the nephridial tubule. It possesses a proximal and distal member. The latter is heavily involved in ion reabsorbtion as it possesses Na^+/K^+ ATPase and Ca^{2+} ATPase and has an ultrastructure that resembles other ion transporting cells. The bladder is a storage site for the urine but also is involved in the reabsoprtion of ions. The green glands are known to reabsorb various useful materials such as glucose and amino acids, but the exact location of this uptake and its mechanism is not know (Vogt 2002). As a whole, the green glands are known to recover 90-95% of electrolytes from the urine (Wheatly and Gannon 1995; Wheatly 1999).

The green glands also play a major role in the detoxification of various heavy metals and organic pollutants. Lead administered by injection was found to accumulate in

the green glands of the crayfish *Orconectes propinquus*. Both the coelomosac and labyrinth were found to accumulate Pb from the hemolymph, perhaps using endocytosis. The heavy metal then was accumulated in electron dense granules or in vacuoles. Eventually these were eliminated from the green glands by apocrine secretion for final elimination with the urine (Roldan and Shivers 1987). The antennal glands also possess a suite of cytochrome P450 mixed function oxidase enzymes. This broad family of enzymes is known to act in the metabolism of steroids and fatty acids as well as to detoxify certain xenobiotic organic chemicals. Benzo[a]pyrene induced P450 enzyme activity in the green glands to a greater extent than in the hepatopancreas, the other major organ responsible for the detoxification of xenobiotic compounds. This is despite the fact that total cytochrome P450 quantities were higher in the hepatopancreas. Also, the suite of metabolites produced from benzo[a]pyrene degradation was different, indicating that the cytochrome P450 enzyme systems may have different functions in the hepatopancreas and green glands (Jewell and Winston 1989; Jewell *et al*. 1997).

The digestive system of crayfish consists of three major regions, the foregut, midgut, and hindgut. The foregut consists of the esophagus and stomach and is lined with the cuticle. The midgut is not lined with cuticle and consists of the midgut canal, midgut cecum, and hepatopancreas. The hind gut is lined in a similar fashion to the fore gut and consists solely of the intestine that continues on to the anus to discharge wastes from the body. The cuticle of both the hindgut and foregut is shed with the rest of the exoskeleton during a molt (Vogt 2002).

When food is consumed, the maxillipeds, maxillae, and mandibles tear food into small pieces that can pass into the stomach via the esophagus. Food particles are lubricated with mucus to ease the transfer. The stomach consists of two major sections,

the cardiac stomach and pyloric stomach. The cardiac stomach contains teeth (ossicles), a pair of opposed lateral teeth and a single dorsal tooth that squeeze and grind food into tiny particles. These heavily calcified portions of cuticle, plus some other minor ossicles that may be present, constitute the gastric mill. This processed food or chyme is filtered by a set of setae, the cardiac filter, that are arranged across one of the canals leaving the cardiac stomach, the cardiopyloric filter canal. This canal passes beneath the medial pyloric chamber to ventral chambers which contain the pyloric filters, also arrays of setae, which are capable of retaining particulates larger than 50nm in diameter. These filters guard the antechamber of the hepatopancreas, from which food particles move out into the hepatopancreas where nutrients are absorbed (Vogt 2002).

In addition to the cardiopyloric filter canal and ventral chambers, the pyloric stomach includes the medial chamber, dorsal chamber and dorsolateral canals. The medial chamber compacts undigested food into fecal pellets. The dorsal chamber and dorsolateral canals may function in returning the water removed from the fecal pellet to the cardiac stomach, but this has not been confirmed (Vogt 2002).

The midgut has some ability to absorb nutrients, but is too small to make a major contribution to this task. It may also function in the coating of fecal pellets with the peritrophic membrane before the pellets are sent along the hindgut and expelled from the body. The hindgut has no function in nutrient absorption, but can act in the uptake of water by allowing the crayfish to drink through its anus (Vogt 2002).

The hepatopancreas is a paired structure with one half on each side of the pyloric stomach. The tubules that leave the antechamber of the hepatopancreas divide many times into several hundred blind ending sacks. The hepatopancreas contains five major cell types: embryonic (E) cells, resorptive (R) cells, “blister-like” (B) cells, fibrillar (F)

cells, midget (M) cells. The E-cells serve as a reservoir of undifferentiated stem cells that differentiate into any of the other types of cells, except M cells which seem to migrate into the hepatopancreas from other tissues. The R, B, and F cells are unique to the hepatopancreas. They tend to get shed into the lumen of the tubules of the hepatopancreas as they become senescent, or, in the case of B cells, earlier in a poorly understood digestive function (Vogt 2002).

The function of M cells has not been firmly established. They may be endocrine since they resemble vertebrate endocrine cells in certain respects. They may also serve to regulate the function of the hepatopancreas, its musculature, or both. F cells show characteristics of protein secreting cells. R cells have a rather bipolar distribution of cellular contents, in contrast to the uniform distribution of F cells. R cells seem to function in the uptake, processing, and storage of nutrients. They also may have a role in the production of various proteins such as vitellogenins and hemocyanin, but this has not be conclusively proven. F cells produce and secrete digestive enzymes, including astacin which has the ability to cleave collagen without pH denaturation. B cells have a distinctive appearance because of a large central vacuole (blister) that seems to store waste products bound for expulsion from the body in the feces. This is accomplished when the entire cell is released from its hepatopancreatic tubule into the lumen. B cells may also have some function in the synthesis of compounds, but this has not been well characterized (Vogt 2002).

The hepatopancreas is an important ionoregulatory organ, along with the gills and green glands. The complement of transport membranes is in some ways similar to those found in gills. In the brush border membrane of the hepatopancreas there are $2Na^{+}(Ca^{2+})/H^{+}(NH_4^{+})$ exchangers (antiporters), nonelectrogenic $Ca^{2+}/2Na^{+}(2H^{+})$

exchangers, and Ca^{2+} channels. A SO_4^{2-}/Cl^- exchanger also exists in the brush border membrane. The basolateral membrane has Na^+/K^+ ATPase, Ca^{2+} ATPase, $3Na^+/Ca^{2+}$, Cl^-/HCO_3^-, SO_4^{2-}/Oxalate and SO_4^{2-}/HCO_3^- exchangers (Ahearn *et al*. 2004). A Ca^{2+} channel that may function in removing Ca from the hemolymph during the molt cycle is also present in the basolateral membrane (McMahon 2002; Vogt 2002; Ahearn *et al*. 2004). Other transport proteins may exist that can transport metals, especially nutritionally important ones like Zn and Cu using Ca to drive an exchange of ions (Chavez-Crooker *et al*. 2001).

The hepatopancreas has a major role in the crayfish's ability to detoxify heavy metal and organic pollutants. While the antennal gland also has a role in these activities, it seems that the function of the two organs may be different so that they are not simply redundant parts of the same system. For example, it has been shown that a nutritionally important metal, Fe, is accumulated in the F cells, and to a lesser extent the R cells, of the hepatopancreas after Fe injection, but Pb, a non-nutritional metal, injected in the same way, tends to accumulate in the antennal gland (Roldan and Shivers 1987; Vogt 2002). Two other nutritionally important metals, Zn and Cu, tend to accumulate in the R cells of the hepatopancreas. There they are incorporated into lysosomes as a sulphite mineral, rendering them inert (Lyon and Simkiss 1984). However, initial accumulation of heavy metals is likely caused by binding to glutathione, one or more metallothioneins, and possibly other metal transport or storage proteins (Lyon *et al*. 1983; Del Ramo et. al. 1989a; Del Ramo et. al. 1989b; Ahearn *et al*. 2004). From there they can then move to the long-term storage sites in granules, lysosomes, or mitochondrial granules (Ahearn et al. 2004). Eventually these metals will be lost when the cells containing them are sloughed into the gut's lument to be lost with the feces as they near death (Vogt 2002).

The most potent known inducer of cytochrome P450 CYP 1A1 is 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) which was found to induce 7-ethoxyresorufin O-deethylase (EROD) activity in the crayfish *Procambarus clarkii*. This enzyme is a common biomarker for planar polyaromatic hydrocarbon exposure (Ashely *et al*. 1996). However, usually this activity indicates Cytochrome P450-1A1 activity, an enzyme subfamily that has not been conclusively found in crustaceans (James and Boyle 1998). Levels of EROD activity in crayfish were much lower than those typically found in vertebrates for the injection doses used (Ashley *et al*. 1996). The pesticide fenitrothion was also found to induce both total cytochrome P450 levels and EROD activity in *P. clarkii* (Escartín and Porte 1996; Porte and Escartín 1998). Elevated P450 levels were also seen in *P. clarkii* taken below a sewage treatment plant in Spain (Fernandes *et al*. 2002). A less potent P450 inducer, the commercial PCB mixture Aroclor 1248, was not found to induce statistically significant levels of EROD activity in *Procambarus acutus* (Brammel and Wigginton, unpublished data). The members of the P450 family that are induced may be different between the hepatopancreas and the antennal gland indicating that the two organs are not two parts of a single homogenous response mechanism to xenobiotics, but two related, but different systems (Jewell and Winston 1989; Jewell *et al*. 1997; Vogt 2002).

Crayfish are dioecious animals, possessing male and female individuals. As with most animal species, a very small percentage of a population may be intersex. The gonads can be found directly under the mid-gut. The testes, which consists of a paired anterior lobe and unpaired posterior lobe, may be seen when the midgut is lifted. Often they seem to be clinging to the bottom of the alimentary canal. The vas deferens from the testes are long and convoluted, serving to package sperm into spermatophores used

during mating and exit the body through a gonopore on the base of the 5th pereopod. The ovaries, which are trilobed, are large enough, except in sexually immature specimens, to be seen in the pericardial cavity without moving the mid-gut. Immature ovaries have numerous tiny white eggs, while a sexually mature female has many large brownish eggs that take up a significant portion of the pericardial cavity. These eggs are macrolecithal and centrolecithal. The oviducts are relatively straight tubes and terminate at the base of the 3rd pereopods (Pennak 1991; Reynolds 2002; Vogt 2002).

Sperm packaged in spermatophores is transferred from the male to the female using the gonopods, at least in the Astacoidea, whose function has been detailed previously. In cambarids, the spermatophore is attached to a specialized structure called the *annulus ventralis* on the ventral surface of the female crayfish. The spermatophore can be stored for several months and maintain viability. When the female lays her eggs, the spermatophore degenerates, allowing the sperm to fertilize the eggs. The eggs are affixed to the pleopods by means of a special tegumental gland (glair gland) secretion called glair. From a few dozen in smaller individuals, to several hundred eggs in the larger individuals of more fecund species may be produced in each reproductive cycle. The most fecund crayfish may lay in excess of 700 eggs (Reynolds 2002; Vogt 2002). In warmer areas, such as the southern United States or Central America, crayfish typically lay eggs during mid to late summer and young hatch during the late summer to early fall. In cooler areas, such as Kentucky and up into Canada, crayfish may mate in the fall or early spring, bear eggs during the fall or spring and hatch their eggs in mid to late spring (Hamr 2002). In truly tropical regions, crayfish can reproduce several times in a given year (Reynolds 2002).

Crayfish possess a well developed endocrine system. The pericardial organ, post-commisural organ and X-organ-sinus gland system are all derived from neural tissues. They possess neurosecretory cells that have processes that extend into neurohemal organs to secrete hormones into the hemolymph. The androgenic gland, mandibular organ and Y-organ are derived from modified epithelial cells. Hormones produced by them are released into local hemolymph sinuses (Vogt 2002).

The pericardial organ lays in the wall of the pericardium and secretes several notable hormones that affect heart rate, including serotonin, dopamine, and octopamine (Vogt 2002). In addition to modulating heart rate, these hormones can affect other tissues as well and can act to broadly alter the response patterns of crustaceans. For example, in several decapods including *H. americanus*, *P. clarkii*, *Astacus astacus*, and *A. fluviatilis*, octopamine injection will decrease the aggressiveness of individuals, making them more docile, and cause them to display a more submissive posture, while serotonin injection will increase aggression, cause the display of a more aggressive posture, and increase the duration of intraspecific fighting (Livingstone *et al.* 1980; Antonsen and Paul 1997; Huber *et al*. 1997; Huber and Delago 1998). However, this pattern is not true for all decapods as the Australian crayfish *Cherax destructor* shows the opposite pattern of response (McRae 1996).

The postcommissural organ envelopes the axon bundle of the postesophageal commisure. The hormones that this structure secretes have not been identified yet although the structure is typical of a neurohemal organ. In shrimp, this organ is known to produce darkening hormone (Vogt 2002).

The X organ-sinus gland system is located inside the eye stalks and is well known for its role in the regulation of molting. It consists of two neurosecretory organs, the X

organ and the sinus gland. The latter is innervated by enlarged axon terminals from the X organ, other parts of the eye stalk and brain. The X organ has ultrastructure indicative of its protein synthesis activities. The X organ- sinus gland system produces crustacean hyperglycemic hormone (CHH), molt inhibiting hormone (MIH), Gonad/vitellogenesis inhibiting hormone (GIH/VIH), mandibular organ inhibiting hormone (MOIH), red pigment concentrating hormone (RPCH) and pigment dispersing hormone (PDH). Other factors are likely produced by the X organ-sinus gland system as well (Vogt 2002).

The Y organs are paired epidermal organs located near the attachment point of the posterior dorsoventral muscle and caudolaterally to the esophagus. Their cells possess an ultrastructure indicative of steroid production. Indeed, they produce the steroid molting hormone ecdysone and its derivatives (Vogt 2002).

The mandibular organs are located to either side of the esophagus, near the apodeme serving the mandibular adductor muscle (Vogt 2002). This organ secretes methyl farnesoate a hormone similar in structure to the juvenile hormone of insects (Chang 1995). The androgenic glands are only found in male crayfish and seem to regulate the masculinization of the animal and spermatogenesis in the testes (Vogt 2002).

Two peculiarities of crayfish physiology, along with other decapoda, are the processes of autotomy and ecdysis or molting. Autotomy is a process whereby a crayfish can loose a limb to escape predation or some other hazard, such as a trapped chela. In this process, a specialized muscle contracts to press the ischium of the appendage against the coxa of the same. A fracture forms along the ischium breaking the limb free. A membrane will rapidly form across the break to prevent the loss of hemolymph. Crayfish can regenerate lost limbs over subsequent molts until the new limb is nearly the same

size as the original and is fully functional. Usually, not all appendages are necessary, so an individual may loose several limbs but still be able to move and feed (Holdich 2002a).

Molting is a consequence of the strong protective exoskeleton possessed by crayfish. An individual cannot grow larger while within the same shell, so it must be shed and replaced. During replacement, water is absorbed to allow the crayfish to expand. Then the exoskeleton hardens, finalizing the animal's new body size. Molting stages can be measured using several anatomical features, especially the setae of the pleopods and how they appear in relation to the cuticle (Aiken 1973; Huner 1980). This process is tightly controlled by the interplay of hormones from several organs.

The Y-organ secretes ecdysone, a steroid derived hormone, which is typically hydroxylated into 20-hydroxyecdysone or molt stimulating hormone (MSH) in various tissues (Chang 1995). The molt inhibiting hormone (MIH) is secreted by the X-organ into the sinus gland (Reynolds 2002; Vogt 2002). Since this organ complex is located in the eye stalks, removal of the eye stalks can alter the molt cycle by destroying the inhibitory system, thus accelerating the occurrence of the next molt (Chang 1995). However, this practice can reduce the likelihood of surviving the process (Huner 1980). The hormone methyl farnesoate (MF) seems to be involved in the ecdysal process as well, acting to stimulate ecdysteroid production. This hormone is very similar in structure to insect juvenile hormone and is produced by the mandibular organ (Chang 1995). A mandibular organ inhibiting hormone (MOIH) may be produced by the same cells in the X organ that produce MIH. MOIH serves as an inhibitor for MF production. Thus far it has only been isolate from the crab *Cancer pagurus*, but its presence in other decapods seems likely (Vogt 2002).

During the process of ecdysis, Stage C is considered the normal intermolt period when the exoskeleton is fully formed and fully calcified. Stage D begins the preparations for the molting process as the epidermis begins to separate from the endocuticle. At the same time, the enzymatic breakdown of the endocuticle begins and Ca begins to be reabsorbed into the body (Reynolds 2002). This Ca is stored in the gastroliths, also called calcium stones, that lie in the lateral walls of the cardiac stomach. Smaller crayfish are able to store a greater proportion of their Ca in these structures relative to larger crayfish. Based on experimental data, a hypothetical 1.0g crayfish may retain about 25% of its intermolt Ca in its gastroliths while a hypothetical 30g crayfish can retain less than 10% of its intermolt Ca. Larger crayfish may be able to dissolve a greater proportion of the Ca from their exoskeleton, but they loose much more through excretion as well (Wheatly and Ayers 1995). More Ca may be stored in the hepatopancreas and hemolymph (Huner 1980). The muscles in the limbs tend to degenerate somewhat, as mentioned previously, to allow them to be successfully drawn through the narrow opening of limb base (protopod) of the old exoskeleton.

As the old endocuticle is being dissolved, and new epicuticle and exocuticle are being secreted by the epidermis. Eventually, enough $CaCO_3$ has been removed from the old exoskeleton to make it brittle. The crayfish will stop feeding and seek shelter. The shell is quite delicate at this point and will split between the cepahlothorax and abdomen. This the actual ecdysis event (Stage E). The crayfish will move its body to loosen the old exoskeleton (Huner 1980; Reynolds 2002). The final release from the exoskeleton can be quite rapid as the crayfish may use a tail-flip to jerk itself free. Also at this time, the gastroliths, along with the cuticle of the stomach, are shed into the lumen of the that organ where they can be digested (Reynolds 2002).

Obviously, the hardening of the new exoskeleton is a high priority to Stage A crayfish because they are highly vulnerable to any number of predators, including other crayfish, while the shell is soft. The stored Ca is used to calcify the exoskeleton, but especially the mouthparts so that feeding can resume. The exocuticle is calcified at this time and the endocuticle begins to be secreted (Huner 1980; Reynolds 2002). Smaller crayfish may have stored as much as 50% of the Ca that they will need internally (Huner *et al.* 1978). However, this quantity is much smaller for larger crayfish which may retain only 15% of its premolt Ca (Wheatly and Ayers 1995). Usually, a crayfish will attempt to consume part or all of its shed exoskeleton to recover the Ca and chitin to continue the calcification process. Other food items may be taken as well once the exoskeleton has achieved sufficient strength. However, much of the remaining Ca is obtained through branchial uptake (Huner 1980; Wheatly and Ayers 1995). The hardening of the exoskeleton continues through stage B and finishes in stage C (Huner 1980; Reynolds 2002).

Juvenile crayfish require 10-15 molts typically to reach maturity, although the exact number varies according to nutrition and species (Pennak 1991). In many species, individuals reach sexual maturity in 1-2 years, although some of the larger or troglobytic species can require many more years to mature (Reynolds 2002). Once mature, typically crayfish will molt twice a year. Both adult male and female cambarids exhibit a type of form alternation by molting from a non-reproductive form (Form II) to a reproductively active form (Form I) during the breeding season, then back to Form II (Wetzel 2002). While some decapods can only reproduce soon after the female finishes molting, while the exoskeleton is still soft, this does not seem to be a requirement for crayfish (Reynolds 2002). For species that hatch eggs in the spring, most molting occurs during the spring

and fall (Wetzel 2002). It has been observed that in the spring sometimes a large proportion of the individuals in a stream reach will molt at very nearly the same time (Taylor and Schuster 2004).

1.2.3 Crayfish behavior

The behavioral patterns of freshwater crayfish have been extensively studied for many years. In general, crayfish are considered to be primarily nocturnal, although juveniles may be more active during the day than adults (Pennak 1991). Activity pattern observations and physiological measures, such as heart rate, indicate that crayfish are most active when it is dark and that even moonlight can reduce activity. This pattern may exist to reduce predation, or may mimic nocturnal patterns seen in prey items (Gherardi 2002). Activities are often temperature related and warmer temperatures are often associated with greater activity. However, there are many confounding factors to this general trend, such as photoperiod, feeding status, and molt status.

Crayfish tend to select habitats based on food availability, temperature, and cover availability. Often, temperature is of special importance because of the over-all effect it has on crayfish metabolism. Different species are adapted to better take advantage of more specialized habitats. These crayfish include troglobytic, cool water, warm water, fast water, slow water, and burrowing types (Gherardi 2002). Shelter is also of vital importance for protection during periods of inactivity and during molting. Stream and lake dwelling crayfish select a variety of natural structures for shelter, such as drift wood, rocks, and aquatic plants. Many species of crayfish burrow to some extent, either to provide additional shelter, or as refuges from unfavorable environmental conditions. Tertiary burrowers may occasionally construct simple tube burrows in the lakes or streams where they live, often only when environmental conditions are not favorable and

rarely during the breeding season. Secondary burrowers usually live in areas that suffer periods of dessication, but are flooded at other times. The structure of their burrows may be somewhat more complex, showing a spiraling path downwards and occasionally side tunnels, and can often be in stream or lake banks as well as the bottoms of water bodies. More time is spent in the burrow, especially during seasons of drought. It is common to find multiple crayfish in each of these burrows. Primary burrowers construct much more elaborate excavations. There may be multiple chambers with several passageways to the surface. Usually, these are constructed well away from streams or lakes in areas were the water table is close enough to the surface for the crayfish to reach, sometimes more than 4m deep. The total length of the burrow can exceed 10m. The exits of these burrows are often marked by mud chimneys constructed by the crayfish. Many of these species spend the majority of their time in the burrow, emerging only to feed, locate new habitats, and find mates, typically on moist nights or during rain to avoid desiccation (Hobbs 1976; Gherardi 2002).

Crayfish possess two primary behavioral mechanisms for defense when presented with a predator. They can either take an aggressive stance with claws raised to attempt to grapple with the predator, or use the well muscled abdomen and uropod to escape by swimming backwards (Stein and Magnusson 1976; Hayes 1977; Stein 1977). Smaller individuals tend to swim backwards a shorter distance and hide more than larger individuals. Females can typically swim faster than males because of their wider abdomen. Females also tend to hide more often after a swim than similar sized males (Stein 1977).

As crayfish body size increases, they become more likely to defend themselves with their chelae (Keller and Moore 2000; Kellie *et al*. 2001). This behavior, whereby the

crayfish raises it chelae to either side in front of it, often with a general raising of the body posture, has been described as the predator response posture or claw-raise behavior (Hayes 1977). Other decapods (*H. americanus*) have also been shown to increase the frequency of their more aggressive defensive responses, and reduce the frequency of tail flipping as they grow in size (Lang *et al.* 1977; Wahle 1992).

Crayfish have a variety of behaviors used to interact with conspecifics. Broadly, this behavior can be divided into agonistic behavior and mating behaviors. Agonistic behaviors arise from the interaction of two crayfish regarding some resource, such as a shelter or food. Within these interactions, certain well defined sequences of events have been identified (Hayes 1975; Bruski and Dunham 1987). Typically, interactions between two crayfish result in either one crayfish retreating before the dominant member of the pair, or a physical contest between them to establish dominance. In *Orconectes rusticus*, a typical bout includes an approach phase whereby the crayfish move toward each other and orient themselves head to head several cm apart. The individuals may perform one or more meral spreads, a spread of the chelae to the sides. Then bouts of striking with chelae, pushing, raising the body into an elevated posture, antennule flicks, and antennae taps on the opponent occur. Sometimes they interlock their chelae and push back a forth or walk side to side. Eventually, one individual will attempt to flee by tail flipping or walking away. The other may pursue and reinitiate conflict. Eventually, the loser will wave its antennae and adopt a more submissive body posture. The winner may release its opponent and either ignore it or continue to pursue and reinitiate conflict. This elicits additional antennal waving and submissive posturing from the defeated animal. Eventually, the dominant individual will stop pursuing the other and the beaten individual will actively avoid contact with the winning individual (Bruski and Dunham

1987). Loss of chelipeds tends to reduce an animal's ability to compete with conspecifics in agonistic contests. While higher than individuals with a missing claw, individuals with a regenerated claw tended to have lower social status than individuals with two original chela (Gherardi *et al*. 2000).

Crayfish seem to be able to sense various chemical cues from nearby members of the same species. They can sense alarm signals from damaged individuals and stress chemicals from undamaged crayfish undergoing stress. These chemicals encourage a conspecific to move away from the source of these chemicals, probably to avoid the stressor acting upon the original animal (Schneider and Moore 2000; Gherardi *et al.* 2002).

During mating, typically the male will seek out receptive females, perhaps drawn by pheromones. When one is found, the male may pursue her until she ceases to evade him, although in some species, the female will actively resist copulation until subdued by the male. Then, in members of the Astacoidea, the male will flip the female on her back and will align himself above her while holding her in position with his chela. Spermatophore transfer will occur via the gonopods. In parastacids, the male will invert himself and slide beneath the female. Sometimes the mating process can be rather rough and cause injury to the female (Huner and Barr 1991; Pennak 1991; Gherardi 2002).

After mating, the laying of eggs may occur soon thereafter or months later. When it happens, the female will secrete the adhesive glair from special glands on the swimmerets and uropod that attach the eggs to the pleopods. The eggs may be carried for just a few weeks to more than a year by the female. Eggs are groomed by the pereopods and dead or infected eggs can be removed. The pleopods can be moved to fan water over the eggs. In some species, the female may remain more sheltered during the period of egg

carrying, called “in berry” for the blackberry-like appearance of the egg mass. Sometimes the female will remain in a burrow. In *O. virilis*, the male may construct a burrow that the female will use to brood the eggs (Gherardi 2002). When the eggs hatch, during the first instar of the Astacoidea, the juveniles remain attached to their egg cases by means of telson threads. The egg cases remain attached to the female at this time. The second instar juveniles will remain attached using their chela, which possess special hooks at this stage. Starting with instar 3, the juveniles may begin to leave the female for short periods. However, this soon after hatching, juveniles will return to the mother when disturbed. Sometimes the mother will walk about to collect her young. Females secrete a pheromone that helps the juveniles move towards her. The juveniles will leave the female permanently at stage 3 to 7 depending upon species. The physical contact of young on the pleopods serves to inhibit the female from consuming juvenile crayfish. Once, the young have gone from her, the female will begin consuming juveniles within a few days (Pennak 1991; Gherardi 2002).

1.2.4 Crayfish ecology

Crayfish are usually considered to be omnivorous (Nyström 2002). They are mostly predacious under favorable conditions (Momot 1995; Whitledge and Rabeni 1997). However, even then, some plant material is still consumed possibly for its micronutrient content. Vegetation and algae may be eaten as incidental material taken along with prey items (Goddard 1988; Momot 1995). However, herbivory and detritivory can be more important feeding strategies under less favorable conditions (France 1996). Crayfish can provide a large component of the animal biomass in an aquatic ecosystem. Sometimes they comprise half or more of the macroinvertebrate biomass in lakes or streams (Momot 1995). Crayfish also serve as important food items to a variety of

aquatic and terrestrial predators. These include aquatic insects, other crayfish, many fish species, some amphibians and reptiles, and a variety of birds and mammals (Huner and Barr 1991).

When multiple species occupy the same range, they can be isolated from each other either ecologically or behaviorally. For example, *Orconectes virilis* and *O. immunis* have overlapping ranges and are both plentiful species. However *O. virilis* are more aggressive than *O. immunis* and will exclude them from habitats. *O. immunis* is more tolerant of low oxygen conditions and is a more capable burrower, allowing it to survive drought by constructing tunnels. Thus they can utilize habitats unsuitable for *O. virilis* within the same range (Bovbjerg 1970). Introduced *O. rusticus* have been shown to be more aggressive than *O. virilis* or *O. propinquus* and can effectively exclude both species from some habitats (Hill and Lodge 1994). Similarly, *Pacifastacus leniusculus* has been shown to be dominant to *Cambaroides japonicus* and capable of excluding this species from shelters (Nakata and Goshima 2003). *P. leniusculus* also has shown the ability to dominate various European crayfish species such as *A. astacus*, *Austropotamobius pallipes* and *A. torrentum* (Vorburger and Ribi 1999).

Given that many crayfish species can burrow to escape drought, sometimes an intermittent stream is able to support a larger number of crayfish than a nearby permanent one. This may be a result of reduced fish predation (Flinders and Magoulick 2003).

1.2.5 Ranges, habitat preferences, and burrowing habits of study species

Orconectes juvenilis is primarily a stream dwelling crayfish restricted to the central portion of Kentucky in the Salt River and lower Kentucky River drainage basins and nearby Ohio river and minor tributaries, including some in southern Indiana. A small

region of the upper Cumberland River also contains this species. It is typically found on gravel, cobble, boulder, or fractured bedrock substrates (Taylor 2000; Taylor and Schuster 2004).

O. placidus is also a stream dwelling crayfish from the lower Tennessee and Cumberland and middle Green river drainages. It occurs in flowing streams and rivers, most commonly with gravel, cobble, or boulder substrates, although sometimes on sand. Neither this species nor *O. juvenilis* is known to burrow extensively (Taylor and Schuster 2004).

O. virilis has a much broader home range extending from Hudson Bay in Canada south to Oklahoma, southern Illinois and Indiana and from Montana and Colorado in the west through the Great Lakes region to New England with isolated populations outside of this range. It can be found in clear, swiftly running waters to highly turbid waters with no measurable flow. Only rarely does this species construct the simplest of burrows in stream banks (Hobbs and Jass 1988; Jezerinac *et al.* 1995).

Procambarus acutus also has a very wide distribution. It occurs on the Atlantic coast from Georgia to Maine and on the gulf coast from Texas to the western edge of Florida and north to Ohio, Indiana, Illinois, and Wisconsin in the central U.S. Its range in Kentucky is restricted to the far western portion of the state. This crayfish can be found in a wide variety of habitats, including swamps, seasonal pools, streams and moderate-sized rivers, on substrates ranging from mud to rock. It is a competent burrower of relatively simple vertical shafts or angled tunnels, used to escape dry conditions (Pflieger 1996; Taylor and Schuster 2004).

P. alleni is restricted to central and southern Florida. It lives in seasonal wetlands, sloughs, and pools, often in areas with clay or marl substrates. This species frequently

burrows to stay below the watertable when its habitat drys. In the Everglades, they can be found in areas of up to 18ppT salinity (Hendrix and Loftus 2000; Acosta and Perry 2002).

P. clarkii can be found in abroad area centered on the southern portion of the Mississippi River and Gulf Coast and extended north to far western Kentucky and southern Missouri. They can occasionally be found in flowing waters, but more commonly in pools, ditches, and swamps, including seasonally dry habitats. They commonly burrow in the winter and at other times of the year to escape dessication (Pflieger 1996).

Three of these species are very wide ranging (*O. virilis*, *P.acutus*, *P. clarkii*) while two are more moderate in range (*O. placidus*, *P. alleni*) while *O. juvenilis* has a relatively small range. However, they all may be quite common within their ranges.

1.3 Economic importance

Crayfish have been of considerable gastronomic and subsequently, economic importance for thousands of years. Crayfish remains have been found in 28,000 year old aboriginal Australian cooking hearths (Kefous 1981, quoted in Sokol 1988). In Europe, they have been collected for food for at least 2000 years (Skurdal and Taugbøl 2002). Currently they are gathered from natural sources and widely used in aquaculture (Holdich and Lowery 1988; Huner 1994; Holdich 2002b).

European harvests of all crayfish species amount to some 7000-8000 metric tons per year (Skurdal and Taugbøl 2002). However, much of this total is from introduced species, especially *P. clarkii* and *P. leniusculus*, as European crayfish species have suffered greatly from the accidental introduction of an oomycete fungus, *Aphanomyces astaci*, the crayfish plague (Evans and Edgerton 2002). Originally imported with infected

North American crayfish, this fungus is responsible for 75% to 90% reductions in harvest of native species, such as *A. astacus*, *A. leptodactylus*, and *A. pachypus*, from many infected water bodies (Ackefors and Lindqvist 1994; Skurdal and Taugbøl 2002).

In the United States more than 50,000 metric tons of crayfish are harvested each year, either from wild collection or aquaculture. The bulk of this harvest is from *P. clarkii* and *P. zonangulus* with *P. clarkii* making up 70-80% of the total. East-Asia and especially China have recently increased their production of crayfish, specifically the introduced *P. clarkii*. Currently, production is high enough to support exports of some 70,000 metric tons per year to the United States. Kenya maintains a fishery with exports as high as 500 metric tons per year, again from introduced *P. clarkii* as Africa has no native crayfish species, except for a few in Madagascar (Huner *et al*. 1994; Huner 2002; Lewis 2002). Other North American species, such as *O. rusticus*, *O. limosus*, *Cambarus bartoni* and *C. robustus* have some economic potential, but thus far have mainly been raised or caught for the bait-fish industry with only minor production for human consumption (Guiaşu 2002; Hamr 2002).

Australian crayfish of the genus *Cherax* are widely used in aquaculture in Australia with harvests of all species ranging from 300-450 tons per year. Production is generally increasing and several *Cherax* species have been exported for aquaculture. However, only Ecuador has been successful with these crayfish thus far with an annual production in excess of 100 metric tons per year (Mills *et al*. 1994; Lawrence and Jones 2002).

1.4 Mechanisms of cadmium uptake and toxicity to crayfish

Crayfish typically take in metal toxicants through their digestive system via their food or through their gills. Uptake through the integument is relatively minor because of

their impermeable, hardened exoskeleton. Cadmium, in particular, enters the gills through the apical calcium channel (Verbost *et al.* 1989; Bondgaard and Bjerregaard 2005). Transport through the basolateral membrane can occur through the Ca^{2+} ATPase ion pump or facilitated diffusion, probably via the $3Na^{+}/Ca^{2+}$ ion exchanger (Verbost *et al.* 1989; Rainbow 1995; Silvestre *et al.* 2004). A wide variety of metal transport proteins, such as members of the SLC30 (formerly CDF, it includes ZnT proteins) and ZIP transport protein families, have been characterized in vertebrates, especially mammals (*e.g.* Palmiter and Huang 2004; Eide 2004). Members of the ZnT family are often Zn exporters from cells while ZIP proteins include Zn importers. A few mammalian SLC30 and ZIP proteins have been implicated in zinc and cadmium, uptake, regulation, and detoxification. The importance of ZnT-1 to Zn exposure has been demonstrated in mammalian cells (Palmiter 2004). Also ZIP-8 has been shown to increase Cd uptake in mouse testes (Dalton *et al.* 2005). MTF-1 can induce ZnT-1 protein, and both ZnT-1 and MTF-1 are induced by Cd (Langemade *et al.* 2000; Hasumi *et al.* 2003). However, their role in invertebrate metal ion regulation and metabolism has not yet been characterized (Hogstrand, personal communication). Cadmium uptake by the intestinal epithelium is thought to be mediated by a divalent cation exchange mechanism at the brush border membrane, as are the uptakes of Cu and Zn. It may be transported through the basolateral membrane of the intestines in a similar manner as through the gills, although the complete mechanism is not known (Ahearn *et al*. 2004). The increased uptake of Ca immediately after molting occurs can increase the uptake of Cd after molting (Bondgaard and Bjerregaard 2005).

Metals that are moved through the basolateral membrane are then transported through the crayfish's body bound to hemolymph proteins, such as the respiratory

pigment hemocyanin (Roesijadi and Robinson 1994), and also in hemocytes. Hemocyanin seems to be the primary Cu carrying protein (Rtal and Truchot 1996), although glutathione plays a role as an intermediary, transferring Cu to apohemocyanin (Brouwer and Hoexum-Brouwer 1992). An iron binding protein has been isolated from the hemolymph of two marine crabs (Landry and Topham 1990). The roles of these and other hemolymph proteins in heavy metal transport in invertebrates has not been firmly established (Ahearn *et al.* 2004). When it reaches a target tissue, Cd in particular, may cause various negative effects by binding to proteins and altering their functional efficiency, changing the induction of assorted genes, or by promoting reactive oxygen species formation (Verbost *et al.* 1989; Almeida *et al.* 2002; Hartwig *et al.* 2002; Rainbow 2002). A detailed model of enzymatic disruption caused by heavy metals can be seen in Gomis-Rüth *et al*. (1994). The endopeptidase astacin uses Zn in its catalytic center, but when different metals are substituted, the conformation of the metal binding area changes in each case, as does the activity of the enzyme. Cu, Hg, and Ni reduce the activity of the enzyme considerably. Interestingly, the addition of Co increases the activity of the enzyme.

In aquatic invertebrates, three general categories of response to heavy metal insult exist. First, the species may regulate the metal by excreting all the metal that is in excess of metabolic needs. Second, the organism may sequester the metal permanently and excrete very little. The third option involves the sequestration of metals that are subsequently eliminated from the body, so body burden may decrease if exposure stops (Rainbow 2002). Crayfish would seem to fall into this third category, at least with regards to Cd (Roldan and Shivers 1987; Vogt 2002).

When Cd reaches a target tissue, it will bind to molecules, especially those having amino ($-NH_2$) or sulfhydryl (-SH) groups (Pedersen *et al*. 1996). Glutathione is a simple tripeptide containing cysteine and may be involved in the detoxification of metals in crayfish, at least in the short term (Almar *et al*. 1987; Almar *et al*. 1988). Mammalian evidence suggests that although glutathione might bind Cd or other metals, certain toxic effects may be associated with the depletion of available gluathione as it is bound up. Glutathione can serve as a chaperone molecule to allow Cd to be transported between organ systems in the blood. It can be secreted by the liver, along with its bound Cd, into the bile for elimination from the body. Glutathione bound Cd may also be excreted by the kidneys and eliminated in the urine. It may even serve as a means of metal transport into the cell via cysteine or glutathione transporters though cellular membranes (Zalups and Ahamd 2003) .

However, the metallothioneins (MT) are the primary molecules that bind Cd in most animals, including crayfish. Cd binds strongly to MTs, making it metabolically unavailable in the cytosol, thus detoxifying it. MTs have several other metabolic functions besides heavy metal detoxification including Zn and Cu storage/regulation, and oxygen radical scavenging (Del Ramo *et al*. 1989a; Del Ramo *et al*. 1989b; Martinez *et al*. 1993; Palmiter 1998; Ahearn *et al*. 2004). Zinc is often displaced from MT in the process of Cd binding. This makes it free to bind to various proteins, including the proteins that regulate MT transcription. The crustacean MT molecules that have been assessed all belong to group I MTs (Pedersen *et al*. 1996; Geidroc *et al*. 2001). These are the MTs that resemble the MT that was first isolated from horse kidney (Margoshes and Vallee 1957). Only mollusk and crustacean MTs belong to this group among the invertebrates (Pedersen *et al*. 1996). Crustacean MTs are small (approximately 6 kDa),

usually possessing approximately 57-58 amino acids, of which 18 are cysteines arranged in highly conserved configurations. Typically, mammalian MTs possesses 20 cysteine residues and are slightly larger (6-7 kDa) than crustacean MTs (Pedersen *et al*. 1996). While most crustacean MTs are smaller than vertebrate MTs, other forms exist. In the crab *Callinectes sapidus*, a Cu inducible form of MT exists that possesses 61 amino acids of which 21 are cysteine residues. While still a group I MT, it is more closely related to molluscan MTs than other crustacean MTs (Syring *et al*. 2000). Group II MTs are those that are still transcriptionally produced, but do not closely resemble group I MTs. They include many invertebrate MTs. Group III MTs includes metal binding proteins that are nontranslationally synthesized and include various plant and fungi MTs. They are also called phytochelatins or phytometallothioneins (Roesijadi 1992).

Group I MTs typically possess two metal binding domains. The α domain typically is arranged to bind up to 4 divalent metal ions while the β domain is arranged to bind only 3. While many vertebrate MTs contain both an α and a β domain, most crustacean MTs only contain β domains (Roesijadi 1992). However, some evidence suggests that crustacean MT can possess an additional divalent ion binding site, although with a much lower binding affinity than the other six (Overnell *et al*. 1988; Sparla and Overnell 1990). Generally, β domains tend to have a higher affinity for Cu than α domains (Nielson 1985; Li and Otvos 1996a; Li and Otvos 1996b). The reliance of crustaceans on the Cu containing respiratory pigment hemocyanin may explain the difference between vertebrate and crustacean group I MTs. The order of binding affinity for several important toxic and nutritional metals has been established for mammalian MT: Hg>Ag≈Cu>Cd>Zn>Ni≈Co (Nielson 1985; Li and Otvos 1996a; Li and Otvos 1996b).

In mammalian systems, metallothionein is known to be induced by the binding of a MRE-binding transcription factor (MTF-1) binds to a metal response element (MRE) in the promoter region of MT DNA. Only Zn is known to activate MTF-1 which then translocates into the nucleus and is able to bind to a MRE. The induction of MT by other metals is likely an indirect effect caused by the dislocation of Zn from other molecules, such as glutathione or MT, which is then free to bind to MTF-1 (Giedroc *et al*. 2001).

Most metallothionein in crustaceans seems to be localized in the hepatopancreas (approximately 0.2 mg MT/g wet tissue mass), with kidney and gill tissues containing about 25% as much. All types of hepatopancreatic cells are able to take in heavy metals. However, E cells within the hepatopancreas seem to be more capable of binding metals because they have a significantly higher MT content than other cell types (over 30mg MT/g cell protein). This is twice as high as R and F cells and ten times greater than B cells (Chavez-Crooker *et al*. 2003). However, R cells tended to accumulate more Zn and Cu, and R and F cells tended to accumulate more Fe than other cell types as insoluble granules (Lyon and Simkiss 1984; Roldan and Shivers 1987). Perhaps E cells accumulate metals initially, then store them, as these stem cells differentiate into other cell types. However the overall mechanism is still quite poorly understood.

For longer-term storage, heavy metals may be deposited into lysosomes, possibly still attached to metallothionein. The metal can also be precipitated into granules of sulfide or phosphate minerals that are insoluble, thus removing them from the biologically active ion pool (Ahearn *et al.* 2004). Metals can also be stored in insoluble granules in mitochondria (Chavez-Crooker *et al*. 2002). Either the lysosomes or granules may be excreted in the feces by the hepatopancreas or in the urine by the green glands to remove the metals from the crayfish's body. It is not known whether granules that may

be formed in the gills are excreted or not (Ahearn *et al.* 2004). However, crayfish and other decapods seem to be able to excrete other foreign objects, such as injected ink granules. The particles get sequestered in the gills and shed with the old exoskeleton when the individual molts (Martin *et al*. 2000).

1.5 Review of previous research in the toxicology of cadmium to crayfish

1.5.1 Acute toxicity

Several crayfish species have been tested for their acute, 96h toxicity values (*e.g.* LC10, LC50). The methods have varied considerably from researcher to researcher. The crayfish *O. virilis* was found to have a 96h LC50 of 6.1 mg Cd/L, a 168h LC50 of 1.8mg Cd/L, a 10d LC50 of 1.0 mg Cd/L, and a 14d LC50 of 0.70 mg Cd/L at a hardness of 26 mg $CaCO_3$/L using a flow through exposure system (Mirenda 1986). *O. limosus* was reported to have a 96h LC50 of 0.400mg Cd/L (Boutet and Chaisemartin 1973). *O. immunis* has been investigated by two set of researchers. Phipps and Holcombe (1985) used a flow through method that allowed for the testing of up to 7 species simultaneously in glass tanks divided by stainless steel screens. In this case, the 96h LC50 value was calculated to be >10.20 mg Cd/L at 44.4 mg $CaCO_3$ hardness. Thorp and Gloss (1986) attempted to compared laboratory and field toxicity values by comparing three laboratory 96h static toxicity tests using three water mixtures to a test run in a series of experimental ponds treated with Cd. However, mortality in these tests were inconsistent and the LC50 value for this species was estimated to be >10 mg Cd/L (Hardness = 141 mg $CaCO_3$/L). Comparisons to the field test were difficult to make because the Cd concentration in the water tended to decrease by 85% to 95% during the first 24h of the experiment. *P. clarkii* has also been investigated by multiple researchers. Del Ramo *et al*. (1987) reported a much higher 96h LC50 value for this species than any other tested, 34.8 mg Cd/L at

24°C, but in a static test with very hard water (180-300 mg $CaCO_3$/L). The wide variation in water hardness makes comparisons with other toxicity tests difficult. Naqvi and Howell (1993) also investigated the effects of Cd on *P. clarkii*, however, their assay was on juveniles 1.0-1.5 cm in length. The 96h LC50 was 1.04 mg Cd/L at a hardness of 30.32 mg $CaCO_3$/L. Fennikoh *et al*. (1978) reported a 96h LC50 value of 5.0 mg Cd/L for an unknown *Procambarus* species (Hardness = 20 mg $CaCO_3$), but with a very small number of individuals tested (n=2, no replication of treatments).

1.5.2 Physiological effects

Besides mortality, Cd has been shown to have many effects on different crayfish species. Effects included reduced amylase activity because of a reduction in stomach acidity after a 96h exposure at 5.0 mg Cd/L to *P. clarkii* (Reddy and Fingerman 1994) and an inhibition of acetylcholinesterase activity (Devi and Fingerman 1995). It was found that a 72h exposure to 5.0mg Cd/L increased hemolymph glucose levels in *P. clarkii* (Reddy *et al*. 1994). In 0.010mg Cd/L and 0.30mg Cd/L exposures, the adenylate energy charge in the tail muscle of the crayfish *Procambarus pubescens* was found to be significantly reduced, indicating that this may serve as a sensitive measure of Cd stress (Duke *et al*. 1978). Glutahione and glutathione s-transferase activity in *P. clarkii* were both found to be reduced by a 96h Cd exposure (Almar *et al*. 1987; Almar *et al*. 1988). Cadmium injections were found to impair ovarian maturation in *P. clarkii* (Reddy *et al.* 1997). After 96h Cd exposures of 0.0032 mg Cd/L to 1.0 mg Cd/L the excised gills of *P. clarkii* showed some trends toward increased oxygen uptake at lower Cd exposure levels (Diaz-Mayans *et al*. 1986a). In the same species, a 96h exposure to 1.0mg Cd/L did not alter Na/K ATPase activity or Mg ATPase activity in the gills (Torreblanca *et al*. 1989). However, sublethal exposures to Pb and Cd in *Astacus astacus* caused the activity of

succinic dehydrogenase and NADPH-cytochrome P450 reductase to decrease in gill and hepatropancreatic tissues (Meyer *et al*. 1991). A variety of changes were measured in the composition and energetics of *P. clarkii* gill and hepatopancreas tissues, including protein, lipid, glucose, and caloric content after a 96h exposure to 10 mg Cd/L (Torreblanca *et al*. 1991).

1.5.3 Tissue residue

After a 14d exposure to 0.4 to 7.2 mg Cd/L, *O. virilis* showed consistent trends to accumulate more Cd in the gills than the hepatopancreas. Tissue content was followed in descending order by green glands, carapace, and abdominal muscle. The maximum whole body bioconcentration factor (BCF- the tissue concentration divided by the exposure water concentration) was 71 at 0.4 mg Cd/L exposure (Mirenda 1986). Lower level accumulations patterns were assessed in *P. clarkii* after 96h exposures of 0.0032 mg Cd/L to 0.100 mg Cd/L. At the lowest exposure concentration and in the controls, most Cd was localized in the green glands, while at all higher levels, the gills had higher concentrations, followed by hepatopancreas, then muscle tissues (Diaz-Mayans *et al*. 1986b). Gillespie *et al*. (1977) found that *O. propinquus* accumulated a whole body Cd BCF of 380 at an exposure level of 1.0 mg Cd/L in 94.5h and a BCF of 530 after 190.5h at the same Cd concentration. At their lowest exposure level, 0.010 mg Cd/L, the whole body BCF was 1840 after 190.5h. Maranhao *et al*. (1999) found that while *P. clarkii* accumulated Cd at all 96h exposure levels (0.025 to 0.100mg Cd/L), only at the highest level did the crayfish contain enough Cd to be considered dangerous to human health (> 1.0mg Cd/kg in Portugal). The crayfish *P. clarkii* showed significant accumulation of Cd from toxicant laden food (*Lemna gibba*). Hepatopancreas concentrations were 26 times higher than crayfish fed uncontaminated *Lemna*. A 55% reduction in

acetylecholinesterase activity in the central nervous system and impacts on ovarian condition were also observed (Devi *et al*. 1996). Similarly, Simon *et al*. (2000) found a considerable buildup of Cd in the hepatopancreas, stomach and gills of the crayfish *A. astacus* when fed Cd tainted Asiatic clams (*Corbicula fluminea*).

1.5.4 Chronic exposures and effects

Only a few long term Cd exposures have been conducted with crayfish. After a 5 month exposure, to 0.005 mg Cd/L and 0.010 mg Cd/L, overall mortality in the crayfish *Cambarus latimanus* was found to be 4% and 16% respectively, compared to 0% in the controls. Cadmium burden was significantly different between controls and treated animals and increased with exposure concentration. No effects on growth or temperature tolerance were noted (Thorp *et al*. 1979). In a 55 day exposure to a combined solution of both Cd and Zn, Cd uptake from both test water and from Cd loaded earthworms used as food were found to be significant avenues of exposure (Giesy *et al*. 1980). Cadmium was found to reduce the number of eggs laid from an average of 203 to 48 and reduce the success of egg hatching from 95% to 17% in *P. clarkii* when mated females were exposed to 0.5 mg Cd/L for five months (Naqvi and Howell 1993). A long-term study of Cd exposure in the field indicated that the aqueous route of exposure was very important to the accumulation of Cd in crayfish and that nutrient enrichment may have reduced the tendency of crayfish to accumulate Cd. This may have been because, while Cd was cycled from sediment into lake water more strongly in nutrient enriched conditions, much of it was particle bound (Currie *et al*. 1998).

1.5.5 Behavioral effects

Only a small number of studies have been completed on the effects of Cd exposure on crayfish behavior, despite this group's wide use in other types of behavioral

research. Maciorowski *et al.* (1980) found that the crayfish *Cambarus acuminatus* was attracted to Cd at low and moderate levels, 0.0058 to 0.0185mg Cd/L, but was repelled at a higher level (0.1483mg Cd/L). This study reported that crayfish were able to sense low levels of Cd, but that the toxicant may have disturbed the crayfish's chemosensory aparatus, hence the increased amount of time spent in the highest level of Cd treated water compared to clean water. Juvenile *O. rusticus* showed an increase in activity levels at exposure levels of 1-3mg Cd/L (nominal concentrations, Hardness = 226 mg $CaCO_3$/L) resulting in reduced shelter use relative to controls (Alberstadt *et al.* 1999). Juvenile red swamp *P. clarkii* showed similar, although weaker, trends (Misra *et al.* 1996).

1.5.6 Crayfish as monitors of environmental cadmium

Crayfish have been used as monitors of environmental contamination by a number of researchers, probably their most consistent application in the environmental field. Finerty *et al*. (1990) collected the crayfish *P. clarkii* and *P. zonangulus* in a broad range of locations in Louisiana to determine if seasonality and whether being from aquaculture sources or wild caught caused differences in various metal burdens in commercially harvested crayfish. No differences were noted and Cd concentrations were generally low. Similarly, Santerre *et al*. (2001) found that Cd levels in commercial pond raised crayfish to be below U.S. EPA human consumption limits. Stinson and Eaton (1983) measured levels of several metals, including Cd, accumulated in *P. leniusculus* placed in a lake receiving urban runoff. No differences were found between reference crayfish and those held in cages for 14d in experimental locations. However, Anderson and Brower (1978) found that the crayfish *O. virilis* accumulated more Cd from a site receiving outfall from urban industrial sources than from less impacted sites in the area. Three species of crayfish were collected by Bendell-Young and Harvey (1991), *C.*

bartoni, *C. robustus*, and *O. virilis*, from various Canadian lakes of varying pH and levels of metal contamination. Canonical analysis separated lakes into two groups based on increased Cd content in gills of crayfish from low pH lakes. In contrast however, Bagatto and Alikhan (1987b) did not find significant differences between Cd content in the crayfish *C. bartoni* in circumneutral and acidic lakes. These same authors (1987a) found an inverse relationship between crayfish (*O. virilis* and *C. bartoni*) Cd content and distance down stream from a copper-nickel smelting site in Canada.

Dickson *et al*. (1979) measured metal concentrations in crayfish inhabiting a cave, the troglobitic *Orconectes australis australis* and troglophilic *Cambarus tenebrosus*. Cd and Pb concentrations were higher in the obligate cave dweller than in *C. tenebrosus*, probably because the former is a longer lived species. Khan *et al*. (1995) measured various heavy metal concentrations in wild collected crayfish exoskeleton and muscle samples and compared them to environmental sediment samples. These were collected to establish a baseline for sediment and crayfish tissue concentrations for two locations in Alabama. *P. clarkii* collected from road side ditches had accumulated enough Cd (Whole body BCF= 5.1) that the authors consider roadside crayfish to be a potential health hazard (Naqvi *et al*. 1993).

In a study of many fish species and crayfish in a Spanish park, Rico *et al* (1987) found that the introduced crayfish *P. clarkii* always showed a higher whole body Cd concentration than fish species. However, Anderson (1977) found no differences between crayfish Cd accumulation and that of other invertebrates, except, possibly, for two mayfly genera. Mason *et al*. (2000) found that crayfish buildup more Cd from the Appalachian stream environment they inhabit, than any of the fish species sampled.

However, crayfish were intermediate in their tendency to accumulate Cd among invertebrates.

Rincon-Leon *et al*. (1988) found no statistically significant differences between the tissue burden of different size crayfish (*P. clarkii*) in a Spanish river with low levels of Cd pollution. Additionally, Cd levels were higher in exoskeleton than abdominal muscle. In a nearby river, Sanchez-Lopez *et al*. (2003) found elevated Cd tissue residues in the same species after a large (5×10^6 m^3) accidental discharge from a mine tailing pool. However, the increases for other metals were considerably greater. Using Cd and other metals (Pb, Cu, Zn), crayfish samples were broken into groups based on concentrations that related to their distance from the spill (Sanchez-Lopez *et al*. 2004). Crayfish (*P. acutus*) collected from an area contaminated by coal ash showed in increase in Cd tissue residue and also an increase in standard metabolic rate compared to individuals from a reference site (Rowe *et al*. 2001). A crayfish species introduced into Europe from the United States for human consumption has been used as an environmental monitor as well. *O. limosus* was found to accumulate varying levels of Cd in the hepatopancreas along a reach of the river Meuse in the Netherlands (Schilderman *et al*. 1999).

Chapter 2: The toxicity of cadmium to six crayfish species in two genera and the effect of cadmium on molting.

2.1 Summary

A series of nine acute (96h) toxicity tests were conducted on six species of crayfish in the family Cambaridae. Six of the tests focused on adults while three examined juveniles. LC50 and LC10 values for the adults of individual test species were as follows: *Orconectes juvenilis*, 2.44 and 0.623 mg Cd/L; *Orconectes placidus*, 0.487 and 0.092 mg Cd/L; *Orconectes virilis*, 3.30 and 0.947 mg Cd/L; *Procambarus acutus*, 0.368 and 0.048 mg Cd/L; *Procambarus alleni*, 3.07 mg Cd/L; *Procambarus clarkii*, 2.66 and 0.486 mg Cd/L. The above toxicity data were used to calculate Genus Mean Acute Values (GMAV) for these two genera. The *Orconectes* LC50 GMAV was 1.57 mg Cd/L, while the LC50 GMAV for *Procambarus* was 1.44 mg Cd/L. The LC10 GMAVs were 0.379 mg Cd/L and 0.208 mg Cd/L, respectively. Family Mean Acute Values (FMAVs) were also calculated for the Cambaridae using this data (LC50 = 1.51 mg Cd/L; LC10 = 0.281 mg Cd/L). For tests with juvenile crayfish, the LC50 and LC10 values were as follows: *Orconectes juvenilis*, 0.060 and 0.014 mg Cd/L; *Orconcetes placidus*, 0.037 and 0.002 mg Cd/L; *Procambarus clarkii*, 0.624 and 0.283 mg Cd/L. GMAVs were calculated for juvenile *Orconectes* (LC50 = 0.047 mg Cd/L; LC10 = 0.005 mg Cd/L). Additionally, FMAVs were calculated for juvenile crayfish (LC50 = 0.111 mg Cd/L; LC10 = 0.020 mg Cd/L). Molting was found to be a very sensitive life stage for crayfish as most that molted shortly before or during exposure to Cd died, while all controls that molted in the six adult toxicity tests survived. Given that molting is a sensitive, recurring life cycle event, and that molting frequencies seen in toxicity tests can be found in natural populations, it seems appropriate to include molting individuals in toxicological analysis.

2.2 Introduction

North America has a wide variety of crayfish species. The United States alone possesses more than 320 species, which accounts for 61% of the worldwide total of approximately 520 recognized species. An estimated 50% of crayfish species in the United States are imperiled or vulnerable to extinction (Taylor *et al.* 1996; Stein *et al.* 2000). In contrast, only four species of crayfish are currently listed as endangered or threatened under the U.S. Endangered Species Act (U.S. Fish and Wildlife Service 2005). Most states contain multiple crayfish species and some possess more than 50. Some species, such as *Orconectes virilis*, have a very wide distribution encompassing many states while others have very limited ranges, sometimes as small as a single cave or stream (Hobbs 1976; Stein *et al.* 2000). Crayfish are considered to be omnivores (Nyström 2002). Their dietary habits tend towards predation under favorable conditions (Momot 1995; Whitledge and Rabeni 1997). Even when the animal engages in predation, some plant material is still consumed possibly for its micronutrient content, as well as incidental material taken along with prey items (Goddard 1988; Momot 1995). However herbivory and detritivory can be more important energetically under some environmental conditions (France 1996). All species are benthic, thus placing them in close proximity to sediments and many species also burrow to some extent (Hobbs 1976).Consequently, crayfish have broad access to a variety of avenues of exposure, including several potential dietary uptake pathways as well as respiratory uptake. Adults are typically nocturnal while juveniles can be active at any time (Pennak 1991). Crayfish can provide a large component of the animal biomass in an aquatic ecosystem, sometimes exceeding 50% of the macroinvertebrate biomass in lakes or streams (Momot 1995). Crayfish also serve as important prey items to a variety of aquatic and terrestrial predators. These

include aquatic insects, other crayfish, many fish species, some amphibians and reptiles, and a variety of birds and mammals (Huner and Barr 1991).

Crayfish can serve as an excellent model species to increase the knowledge-base for invertebrate ecotoxicology. Their active gill ventilation (up to 156 mL/kgh for *Orconectes virilis*) could make dissolved toxicants highly available via respiratory exposure (Burggren and McMahon 1983). The broad diet of crayfish could make them useful for investigating oral uptake of toxicants, resulting in dietary exposure which has been neglected in metal criterion development (Birge *et al.* 1998; U.S. EPA 2001). Their central location in many aquatic food webs make them a species of special interest, possibly allowing them to transfer toxicants between trophic levels. They are large invertebrates and easily reared in a laboratory setting. This makes them useful not only for toxicity testing, but as an invertebrate model for a variety of physiological experiments. The eggs are very large for a crustacean and can easily be counted and assessed for fertilization status, fungal infection, mortality, etc., thus making them potentially useful for early life stage studies. Depending greatly upon species and size, a single female may carry from 10 to >700 eggs. Many crayfish species are easy to collect or obtain from aquaculture sources (Pennak 1991; Holdich 2002b).

Cadmium is a common, widespread toxicant and is found in approximately half of hazardous waste sites on past and present U.S. EPA National Priorities Lists. It finds its way into the environment primarily through mining activities and fossil fuel combustion (Taylor *et al.* 1999). The Cd^{2+} ion is the most bioavailable form and the most toxic to aquatic organisms. It is thought to be taken up through the gills by the same mechanism as Ca and Zn. It is a nonessential metal and can cause a wide range of effects in aquatic organisms, including slowed growth, reduced reproductive performance, and

impaired Ca metabolism, in addition to mortality (Albert *et al.* 1992). These factors, especially the impact of Cd upon Ca metabolism, make Cd a toxicant of special interest for crustaceans because of its implications for the molting process, a time of greatly increased Ca metabolism (Wheatly and Ayers 1995).

Despite their importance to aquatic ecosystems, acute toxicity tests, which are important for determining toxicant criteria, have been conducted on only a few crayfish species. Only four species have been included in the United States Environmental Protection Agency's (U.S. EPA) ambient water quality criterion document for Cd (2001). All of these species are widely distributed and very common. It would be beneficial to test some less common species with more limited distributions, from different regions, and varied lifestyles to determine the variation in Cd tolerance across these different groups. By comparison, twenty-four species of fish were tested, many several times, and included in this criterion document. The work included in this manuscript presents toxicology data for six species, five of which have not been tested previously. Three of these species have large ranges encompassing many states (*O. virilis*, *Procambarus acutus*, *Procambarus clarkii*). Two have smaller ranges. That for *Procambarus alleni* encompasses more than half the state of Florida and Orconectes placidus is found in portions of Tennessee and Kentucky. *Orconectes juvenilis* has the smallest home range, less than a third of Kentucky. Between all six species, a wide geographic range is encompassed and several different lifestyles represented. This study uses a consistent methodology so that the interspecific variability of Cd tolerance can be assessed. It also presents new data on the toxicity of Cd to the juveniles of two species of crayfish. Two toxicity tests, one on adults and one on juveniles, were conducted to allow results from this methodology to be compared to values reported by other authors.

2.3 Methods

Nine independent toxicity tests were conducted over the course of 4 years, six with adult crayfish and three with juveniles. The species assayed in the tests with adults included *O. juvenilis* (Hagen 1870), *O. placidus* (Hagen 1870), *O. virilis* (Hagen 1870), *P. acutus* (Girard 1852), *P. alleni* (Faxon 1884), and *P. clarkii* (Girard 1852). The species *O. juvenilis* (Average mass = 4.58g) and *O. placidus* (7.06g) were collected from Wolf Run in Lexington, KY and Blackburn Fork near Cookeville, TN, respectively. Water samples were taken at the time of collections to determine if any environmental contaminants were present that might complicate toxicity testing. *O. virilis* (12.8g) was obtained from Clear Creek Fisheries, Martinsville, IN. Individuals of the species *P. acutus* (15.5 g) were purchased from Blue Spruce Biological Supply Company, Boulder CO; *P. alleni* (5.14g) from Fish2U.com, Gibsonton, FL; and *P. clarkii* (18.5g) from Louisiana Seafood Exchange, New Orleans LA. The species included in juvenile testing were *O. juvenilis*, *O. placidus*, and *P. clarkii*. The individuals for these three test were obtained from collecting ovigerous ("in berry") female crayfish from the same source streams listed above or using ovigerous females obtained from laboratory populations originally collected or purchased for the adult tests. Mass for juveniles was approximately 0.02g for the 3rd to 5th instar individuals. Toxicity test and water quality analysis methods were based on standard methods provided by U.S. EPA and other sources (U.S. EPA 1993; U.S. EPA 1994a; U.S. EPA 1994b; APHA 1995; ASTM 1996). All toxicity tests were conducted for 96h in a temperature controlled environmental room at 25°C ± 1°C and a 16h light/ 8h dark photoperiod. Only one species was tested at a given time. Cadmium chloride ($CdCl_2$) was used to make all exposure solutions.

In assays with adult crayfish, individuals were housed in 11.3L polypropylene tubs. Aeration was provided to each tub to ensure that dissolved oxygen levels remained above 4.0 mg/L, as stipulated by U.S. EPA (1993). Except the test with *O. juvenilis*, all crayfish were isolated in 800mL polypropylene beakers within the tub to prevent injury or mortality from fighting. Beakers were well perforated to allow water flow within the test chamber. Individuals were assigned randomly to test chambers 3d prior to the start of the test to allow them to acclimate. Number of replications, number of organisms tested, water hardness, and toxicant exposure levels (mg of the Cd ion per L), are summarized in Table 2.1. In all tests, except the test with *O. juvenilis*, 4.0L of each test or control solution was used per treatment. For the *O. juvenilis* test, 3.5L was used. All solutions were renewed daily. In all tests, except those with *P. alleni*, and *O. virilis*, crayfish were checked every 6h to allow acute lethal time (LT) values to be calculated. In the remaining tests, the animals were checked every 24h. At each check, animals were examined for responsiveness. Complete lack of response to visual stimulation (*i.e.* from reaching over the tubs) or five taps on the abdomen with a glass rod was counted as mortality.

In tests with juvenile crayfish, individuals were housed in 6-well cell culture plates and each well was perforated to allow water exchange. Lids were held on with rubber bands. The culture plates were placed in 1.0L polypropylene beakers. In each case, 950mL of control or exposure solution was used per beaker. Again, complete lack of response to visual or physical stimulation was counted as mortality. Assay design and exposure conditions are summarized in Table 2.1.

Concentrations lethal to 50% and 10% of individuals (LC50; LC10) and times required to kill a percentage of individuals at a given exposure concentration (LT50;

LT10) were calculated using the probit method with software provided by U.S. EPA (1992). In each case, a chi-square test for heterogeneity was conducted to ensure that the data fit the probit model adequately. The geometric mean of the three sets of adult toxicity values from species in the genus *Orconectes* was used to calculate Genus Mean Acute Values (GMAV) for that taxon using standard U.S. EPA procedures (1986). This was done for adults of the genus *Procambarus* as well. All six sets of toxicity data were used to calculate Family Mean Acute Values (FMAV) for the Cambaridae using the same procedures. GMAVs also were calculated using the two sets of juvenile *Orconectes* data. All three juvenile tests were used to calculate juvenile FMAVs.

Water samples were collected daily from at least two replicates of each exposure concentration. The sampling scheme was designed so that some samples were taken immediately before and after a water change to ensure that Cd levels remained constant. These samples were analyzed on either inductively-coupled plasma optical emission spectrophotometry (ICP OES- Varian Vista MPX) or graphite furnace atomic absorption spectrophotometry (GFAAS- Varian SpectrAA-20 with GTA-96 Graphite Tube Analyzer) to determine actual Cd exposure concentrations. Either a Denver Instruments Model 215 or Orion Research Expandable Ion Analyzer EA-960 was used for pH measurements. A YSI Model 51A oxygen meter was used to detemine dissolved oxygen. Water hardness was measured using the EDTA titrimetric method, then confirmed using ICP-OES (APHA 1995). Temperatures were checked daily.

2.4 Results

All quality control measurements were within U.S. EPA acceptability guidelines (1993). Toxicity values for the six experiments with adults are given in Table 2.2. *O. juvenilis* adults proved to be quite tolerant to Cd with a median toxicity value (LC50) of

2.44 mg Cd/L and a threshold value (LC10) of 0.623 mg Cd/L. *O. placidus* proved less tolerant. Adults had a LC50 value of 0.487 mg Cd/L and a LC10 value of 0.092 mg Cd/L. *O. virilis* possessed the greatest tolerance to Cd among the six species tested. Its LC50 value was 3.30 mg Cd/L and LC10 value was 0.947 mg Cd/L. For *P. acutus*, the LC50 value was 0.368 mg Cd/L and the LC10 value was 0.048 mg Cd/L, making it the most sensitive crayfish species tested. *P. alleni* proved to be tolerant with a LC50 value of 3.07 mg Cd/L and a LC10 value of 0.386 mg Cd/L. Lastly, *P. clarkii* was nearly as tolerant, with a median toxicity value of 2.66 mg Cd/L and a threshold value of 0.486 mg Cd/L. Species ranked from most tolerant to least tolerant based on LC50 values from these tests with adults were *O. virilis* > *P. alleni* > *P. clarkii* > *O. juvenilis* > *O. placidus* > *P. acutus*.

Results from the juvenile *O. juvenilis* experiment indicated that immature crayfish were much more sensitive than adults, as can be seen from their Cd LC50 value (0.060 mg Cd/L) and LC10 value (0.014 mg Cd/L; Table 2.2). For juvenile *O. placidus*, a LC50 value of 0.037 mg Cd/L and a LC10 value of 0.002 mg Cd/L were calculated (Table 2.2). As with adults of this species, juvenile *P. clarkii* proved to be quite tolerant with a LC50 value of 0.624 mg Cd/L and a LC10 value of 0.283 mg Cd/L (Table 2.2). Species ranked from most tolerant to least tolerant based on LC50 values in this set of juvenile tests were *P. clarkii* > *O. juvenilis* > *O. placidus*.

The GMAVs for the two genera of crayfish assayed were generally similar, with *Orconectes* having a GMAV LC50 of 1.57 mg Cd/L and a LC10 of 0.379 mg Cd/L. Results for *Procambarus* were slightly lower with that genus having a GMAV LC50 of 1.44 mg Cd/L and a LC10 of 0.208 mg Cd/L (Table 2.2). The FMAVs calculated for the Cambaridae were LC50 = 1.51 mg Cd/L and LC10 = 0.281 mg Cd/L (Table 2.2).

GMAVs were calculated for juvenile *Orconectes* using the two available sets of data, yielding a LC50 of 0.047 mg Cd/L and LC10 of 0.005 mg Cd/L (Table 2.2). Juvenile FMAVs were calculated for the Cambaridae with a LC50 value of 0.111 mg Cd/L and a LC10 value of 0.020 mg Cd/L.

Additionally, for tests with *O. juvenilis*, *O. placidus*, *P. acutus*, and *P. clarkii*, the LT50 and LT10 values were calculated using probit analysis (Table 2.3). Treatment levels between 1.65 and 3.50 mg Cd/L were selected for comparison. *O. juvenilis* and *P. clarkii* both required more than 100 hours of exposure to cause 50% mortality. However, mortality occurred in less than 32.7 hours for the more sensitive *O. placidus* and *P. acutus*. To cause 10% mortality, the two more tolerant species required more than 37.6 hours, while this level of mortality occurred in just over 7 hours for the two sensitive species (Table 2.3). Ranked in order from slowest to fastest response, based on LC50 values, were: *P. clarkii* >*O. juvenilis* >*P. acutus* > *O. placidus*.

2.5 Discussion and conclusions

Toxicity values for *O. juvenilis*, *O. placidus*, *P. alleni, P. acutus,* and *P. clarkii* adults corresponded well to toxicity values given for crayfish species by other researchers (U.S. EPA 2001). All values, except those for *P. acutus,* were between those for *Orconectes virilis* (96h LC50 = 6.100 mg Cd/L, 26 mg $CaCO_3$/L; Mirenda 1986) and *Orconectes limosus* (96h LC50 = 0.400mg Cd/L, Boutet and Chaisemartin 1973) in tolerance. Toxicity values for *P. acutus* were very close to *O. limosus.* The results in this study with *O. virilis* were lower than those reported by Mirenda (1986). However, the variation between these two tests was smaller than what can be seen in tests on other species, such as *D. magna* (U.S. EPA 2001). All six species were much less tolerant than

Orconectes immunis (96h LC50 >10.20 mg Cd/L, 44.4 mg $CaCO_3$ hardness; Phipps and Holcombe 1985).

Results from the juvenile *O. juvenilis* experiment indicated, as noted above, that immature crayfish were much more sensitive than the adults. The LC10 value (0.623 mg Cd/L) of the adults was more than 10 times greater than the LC50 value (0.060 mg Cd/L) of the juvenile. Again, early life stage *O. placidus* proved to be much more sensitive than the adult. The juvenile LC50 value (0.037 mg Cd/L) was slightly more than one third of the adult LC10 value (0.092 mg Cd/L). Juvenile *P. clarkii* proved to be more tolerant, with a LC50 value of 0.624 mg Cd/L, similar to the LC10 value of the adult (0.624 mg Cd/L). The literature value for *P. clarkii* corresponded well to the current toxicity test with juveniles of that species (96h LC50 = 1.050 mg Cd/L, 30.3 mg $CaCO_3$ hardness; Naqvi and Howell 1993). This was especially true given that the individuals in the current test were smaller than in Naqvi and Howell's test (0.7-1.1cm vs. 1.0-1.5 cm; 1993).

O. juvenilis adults (LC50 = 2.44 mg Cd/L) proved to have a similar level of Cd tolerance to the goldfish (*Carassius auratus*), a commonly used tolerant test species (96h LC50 = 2.130 mg Cd/L, 20 mg $CaCO_3$/L hardness; McCarty *et al.* 1978). However, these numbers are much higher than those for some sensitive freshwater fish species, such as *Oncorhynchus mykiss*, the rainbow trout (96h LC50 = 0.003 mg Cd/L, 44.4 mg $CaCO_3$/L; Phipps and Holcombe 1985). The median lethal toxicity value for *O. placidus* (LC50 = 0.487 mg Cd/L) was less than that for the fathead minnow (*Pimephales promelas,* 96h LC50 = 1.280 mg Cd/L, 43.5 mg $CaCO_3$/L hardness; Spehar and Carlson 1984) but still higher than for the rainbow trout. *O. virilis* possessed the greatest tolerance for Cd among the six species tested. Its LC50 value of 3.30 mg Cd/L was the highest calculated in this

series of experiments, making this organism nearly as resistant to Cd as the channel catfish (*Ictalurus punctatus*) which has an 96h LC50 of 4.480 mg Cd/L at a hardness of 44.4 mg $CaCO_3$/L (Phipps and Holcombe 1985).

Results from the two tests with juvenile *Orconectes* spp. corresponded closely to toxicity values for adult *D. magna* (96h LC50 = 0.030-0.122 mg Cd/L; 46.1 mg $CaCO_3$ hardness; Barata *et al.* 1998) and adult *D. pulex* (96h LC50 = 0.0324 mg Cd/L; 53.5 mg $CaCO_3$ hardness; Stackhouse and Benson 1988), two of the more sensitive invertebrate species to Cd. Juvenile *P. clarkii* proved to be more tolerant than adults of some crayfish species tested in the current study (*O. placidus*, *P. acutus*) and elsewhere (*O. limosus*; 96h LC50 = 0.400mg Cd/L, Boutet and Chaisemartin 1973).

P. clarkii was the species that showed the slowest onset of mortality in the fours tests in which LT50 values were calculated, followed by *O. juvenilis*. *P. acutus* responded slightly more rapidly than *O. placidus*. This pattern corresponds to the pattern of LC50 values except that the species with the lowest toxicity values, *P. acutus* (LT 50 = 32.7h), was slightly slower to respond than *O. placidus* (LT= 28.1h). However, that difference is not significant (Table 2.3). On average, the sensitive species experienced 50% mortality over three times more rapidly than the tolerant species and 10% mortality nearly six times more rapidly.

During this series of nine toxicity tests, molting effects on crayfish tolerance to Cd became of considerable interest. In each of the toxicity tests, a certain proportion of individuals would undergo a molt. In the adult crayfish test series, molting rates varied from a minimum of 0.556% for *O. juvenilis* to a maximum of 41.7% for *P. acutus* (Figure 2.1). The other four tests varied between 8.33% and 13.3%. Molting rates of 13.3% and below are not unusual for a natural population, especially during the months

of active growth (Wetzel 2002; Taylor and Schuster 2004). It also has been reported that in the spring, the majority of the crayfish from a particular stream sector will sometimes molt in rapid succession. Therefore, the very high level of molting that occurred with *P. acutus* was not unreasonable (Taylor and Schuster 2004). Juvenile *Orconectes* spp. showed a considerable number of molts as well. In the juvenile *O. juvenilis* test, 16.0% of crayfish molted while 16.7% molted in the test with *O. placidus*. However, no molting was observed in the juvenile test with *P. clarkii*. As the intermolt period can vary from 6-30 days at 20-22°C for juveniles of this species, it is possible that the test occurred between molting events (Huner 2002).

It was observed that for crayfish molting during the 3day acclimation period or during a toxicity test, Cd exposure often resulted in mortality. Molts that occurred in concurrent controls had high survival rates. When data for all six adult toxicity tests was summed into four exposure classes (control organisms, treatments between 0.020 and 0.100 mg Cd/L, treatments between 0.101 and 2.00 mg Cd/L, and treatments above 2.01 mg Cd/L), it was found that all molts that occurred in control treatments were successful, but that as the concentration of Cd increased, the proportion of molts that resulted in mortality increased. At the higher concentrations all molts resulted in death (Figure 2.2). If the tolerant species (*O. juvenilis, O. virilis, P. alleni,* and *P. clarkii*) and sensitive species (*O. placidus* and *P. acutus*) are separated, it can be seen that the tolerant species only had mortalities in the middle and higher exposure classes. However, for sensitive species, about 18% died while molting in the lowest exposure groups and fully 100% died in the middle group.

Again, for tests with adults, mortalities associated with molting accounted for the majority of deaths in the lowest exposure class and nearly half of those in the middle

exposure class (Figure 2.3). Mortality not linked with molting dominated in the high exposure concentrations. Molting mortality was, in general, a less important factor for tolerant crayfish, however, this may be an artifact of the lower overall molting rate in assays with those species. For sensitive crayfish species, molting mortalities accounted for all deaths in the lowest exposure class and a majority in the middle group (Figure 2.3).

In the juvenile toxicity test with *O. placidus*, one control individual died while molting, but no *O. juvenilis* controls died in ecdysis. In both tests, about 50% or more molting individuals died in the lowest exposure concentration while all molting individuals died in the higher concentrations (Figure 2.4). Given that 1) crayfish experience greatly increased Ca metabolism, including Ca uptake, when they molt (Wheatly and Ayers 1995); 2) Cd uptake can be increased along with Ca uptake in molting individuals (Bondgaard and Bjerregaard 2005); and 3) Cd can interfere with Ca metabolism (Albert *et al.* 1992), it is possible that this interference is at least part of the cause of the increased mortality observed in conjunction with molting.

Other researchers have suggested omitting individuals that molt from toxicity value calculations because those crayfish are at a different life stage and undergoing considerably different physiological processes than nonmolting crayfish (Mirenda 1986). If toxicity values are recalculated while omitting individuals that molted, it can be seen that for most tests there was insignificant change in the LC50 values (Table 2.4). While the percent change was as high as 42% for the *O. placidus* test, the "no molt" toxicity value was within the 95% confidence interval of the LC50 value with all individuals included (Table 2.2). There was greater change in the LC10 values. The *O. placidus* test showing an increase of some 109%. However, the *P. acutus* test showed significant

changes for both the LC50 and LC10 values with increases of 208% and 496%, respectively. Although for most tests, the change was not significant, it would be prudent to calculate toxicity values with molting individuals included, to ensure that risk to this important life stage has been accounted for. This is especially important during times of the year when crayfish are most likely to molt, the spring and fall (Wetzel 2002). Given the very high level of molting seen in the *P. acutus* test with molting individuals included, it may be overly protective of that species, except during times of high natural molting rates in the spring. For the juvenile toxicity tests, if molting juveniles are excluded from analysis, toxicity values could not be calculated because the chi-square test for heterogeneity failed, indicating that the probit model may not have been appropriate for these data. Given that, under natural conditions, the juvenile life stages molt very rapidly during the first few months of life, up to 14 times during the first summer (Taylor and Schuster 2004), it seems appropriate to include molting individuals in the analysis because it represents an important, sensitive, and recurring life cycle event.

When overall molting frequencies for each adult test were regressed against their respective LC50 values, a weak trend was measured ($r=0.618$, $p=0.191$). However the trend vanished ($r=0.053$, $p=0.932$) when the results from the *P. acutus* test were exclude. Thus, that trend was being driven by a single data point. Regressions of molting frequencies versus the LC10 values showed similar results. Regression analysis of average crayfish mass per test versus LC50 and LC10 values also failed to find any significant relationships, indicating that differences in crayfish toxicity values were not a function of organism mass, but intrinsic to the given species' ability to detoxify Cd, a condition possibly affected by environmental conditions prior to collection.

Juvenile crayfish in general tended to be considerably more sensitive than adults as juvenile *O. placidus* had LC50 values 13 times lower than the adults, while juvenile *O. juvenilis* had LC50 values more than 40 times lower (Table 2.2). LC10 values for both species were more than 40 times lower than corresponding values for adults. Juvenile *P. clarkii* were considerably more tolerant and only showed a reduction of about 4.3 times in LC50 value (Table 2.2). Since the juvenile stages are so much more sensitive than adults, they are certainly the stages most likely to be affected by pollutants such as Cd. Amphibians, as a group, can show a 2 to 3 order of magnitude variation in response to particular toxicants (Birge *et al.* 2000a). If the same were to hold true for crayfish, then the most sensitive crayfish species could be considerably more sensitive to Cd than those tested here. Additional testing should be conducted to include a broader range of juvenile crayfish species from less commonly tested species, with smaller ranges and differing lifestyles. Chronic tests should also be conducted on both adult and juveniles, especially embryo-larval type tests (Birge *et al.* 1985). This would ensure that risk to crayfish populations can be adequately assessed. Embryo-larval tests are well established (U.S. EPA 1994a) and have been used to provide risk assessment tools for other important, threatened groups, such as amphibians (Westerman *et al.* 2003).

Certain aspects of the crayfish's lifestyles may have affected their susceptibility to Cd. Species adapted to different water temperatures may find themselves at different stress levels at a uniform temperature. A cool water species may be under more temperature stress than a warm water species at 25° C. This may enhance the toxicity of Cd to cool water species relative to others. A similar issue may exist with regard to water hardness. Species adapted to hard water conditions may find themselves more stressed at a hardness of 48±5 mg $CaCO_3$/L, potentially increasing toxicity to such species.

No discernable patterns were observed between the range, habitat, or lifestyle preferences of the crayfish species tested in this study and their toxicity values. The two sensitive species do not seem to show any ecological characteristics between them that might be diagnostic for predicting species sensitivity. *O. juvenilis* had the smallest home range, but a median LC50 value in adult tests. However, none of the crayfish in the current study are considered imperiled or vulnerable to extinction (Taylor *et al.* 1996; Stein *et al.* 2000). Since no research has been conducted on such species, the assays in the current study may be used to estimate the tolerance of Cd to species that are threatened in some way (*e.g.* GMAV, FMAV). This is especially true of the sensitive early life stages which have received almost no attention until now.

Table 2.1. Adult and juvenile crayfish toxicity tests. Water hardness (mg $CaCO_3$/L), and cadmium exposure concentrations.

Species	Replicates	n	Hardness	Cd concentrations (mg Cd/L)				
Toxicity tests with adults								
P. acutus	4	5	44.5	0.049	0.303	1.65	8.01	45.2
P. alleni	4	5	45.8	0.066	0.312	1.85	9.83	48.8
P. clarkii	4	5	52.9	0.099	0.631	3.50	17.4	91.2
O. juvenilis	3	10	44.1	0.141	0.476	0.826	1.94	5.78
O. placidus	4	5	44.1	0.022	0.128	0.617	2.56	11.0
O. virilis	4	5	42.5	0.063	0.306	1.61	8.98	46.5
Toxicity tests with juveniles								
P. clarkii	4	6	42.1	0.007	0.028	0.112	0.436	1.76
O. juvenilis	4	6	44.0	0.102	0.400	0.783	1.13	4.17
O. placidus	3	6	54.6	0.080	0.340	0.875	1.74	3.53

Table 2.2. Cadmium lethal concentrations (mg Cd/L) for adults of six crayfish species and juveniles of three species with 95% confidence intervals (CI), Genus Mean Acute Values (GMAV), and Family Mean Acute Values (FMAV).

Species	LC50	LC50 95% CI	LC10	LC10 95% CI
Toxicity tests with adult crayfish				
Orconectes juvenilis	2.44	1.84-3.491	0.623	0.364-0.876
Orconectes placidus	0.487	0.295-0.785	0.092	0.033-0.168
Orconectes virilis	3.30	2.10-5.20	0.947	0.370-1.57
Orconectes GMAV	1.57	0-3.21	0.379	0-0.867
Procambarus acutus	0.368	0.196-0.646	0.048	0.012-0.102
Procamabrus alleni	3.07	1.74-5.45	0.386	0.122-0.771
Procambarus clarkii	2.66	1.57-4.44	0.486	0.164-0.906
Procamabrus GMAV	1.44	0-3.09	0.208	0-0.468
Cambaridae FMAV	1.51	0.472-2.55	0.281	0.011-0.551
Toxicity tests with juvenile crayfish				
Procambarus clarkii	0.624	0.399-0.894	0.283	0.098-0.432
Orconectes juvenilis	0.060	0.011-0.109	0.014	0.000-0.039
Orconectes placidus	0.037	0.000-0.115	0.002	0.000-0.020
Orconectes GMAV	0.047	0.025-0.070	0.005	0-0.017
Cambaridae FMAV	0.111	0-0.488	0.020	0-0.200

Table 2.3. Lethal time (LT) values (h) for four crayfish species with 95% confidence intervals (CI). Exposure concentrations are given in mg Cd/L.

Species	Exposure Conc.	LT50	LT50 95% CI	LT10	LT10 95% CI
Orconectes juvenilis	1.94	103	90.7-125	48.9	41.3-54.5
Orconectes placidus	2.56	28.1	22.9-33.0	7.80	4.62-10.1
Procambarus acutus	1.65	32.7	26.3-39.1	7.02	3.59-10.5
Procambarus clarkii	3.50	110	89.9-159	37.6	27.2-45.0

Table 2.4. Toxicity values for nonmolting adults of six crayfish species with 95% confidence intervals (CI), Genus Mean Acute Values (GMAV), and Family Mean Acute Values (FMAV). All values are given in mg Cd/L.

Species	LC50	LC50 95% CI	LC10	LC10 95% CI
Orconectes juvenilis	2.49	1.89-3.49	0.687	0.411-0.953
Orconectes placidus	0.692	0.428-1.11	0.169	0.062-0.293
Orconectes virilis	4.29	2.86-6.31	1.98	0.925-2.95
Orconectes GMAV	1.95	0-3.98	0.612	0-1.67
Procambarus acutus	1.19	0.585-2.33	0.286	0.061-0.581
Procamabrus alleni	3.27	1.73-6.19	0.381	0.106-0.810
Procambarus clarkii	3.53	2.12-5.61	0.944	0.301-1.66
Procambarus GMAV	2.39	0.937-3.84	0.469	0.066-0.871
Cambaridae FMAV	2.16	1.04-3.28	0.536	0.0003-1.07

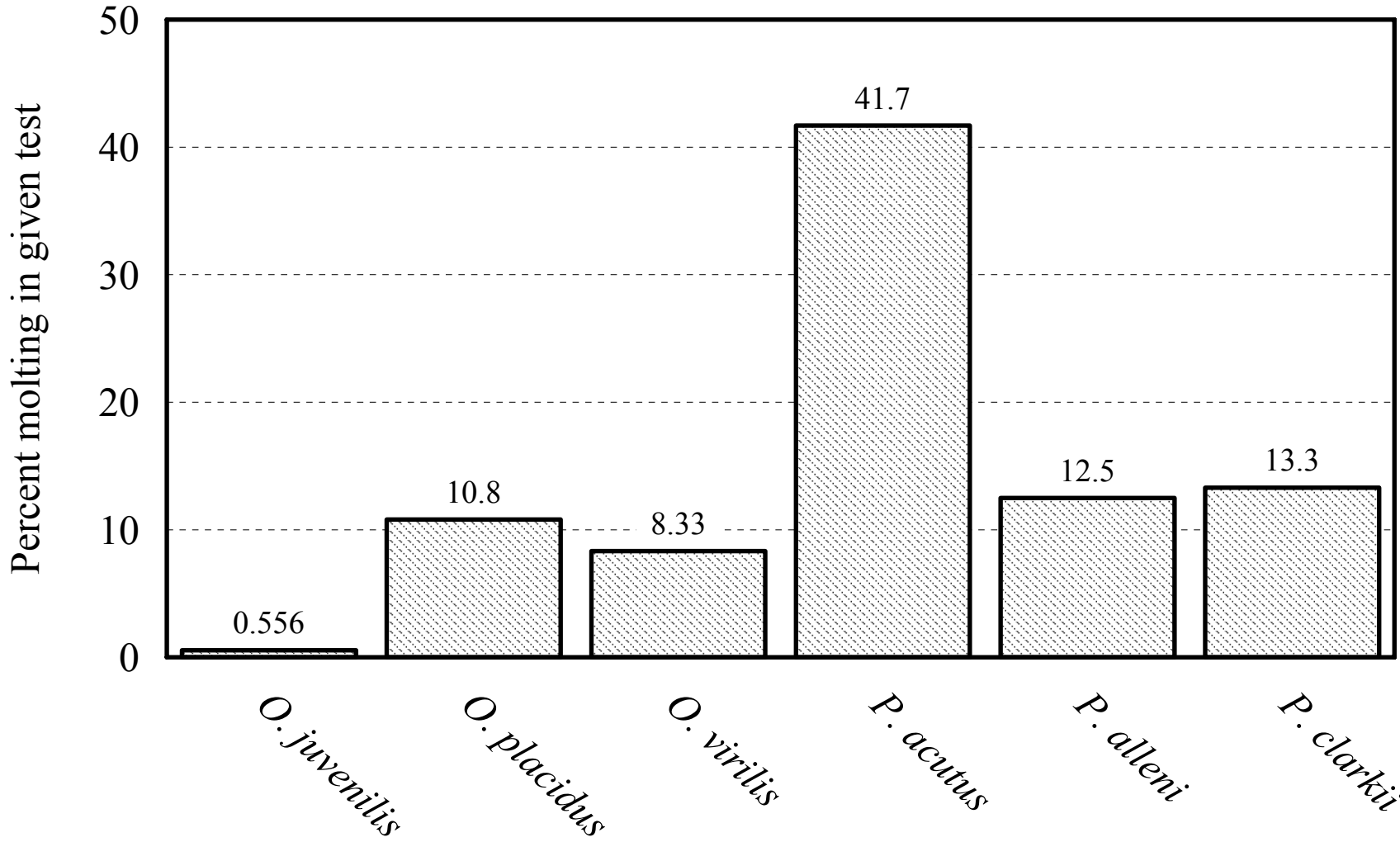


Figure 2.1. Percentage of adult crayfish experiencing a molt either during or within three days prior to a toxicity test. Values are based on all individual involved in a given test, n=120 in each case except *O. juvenilis* (n=180).

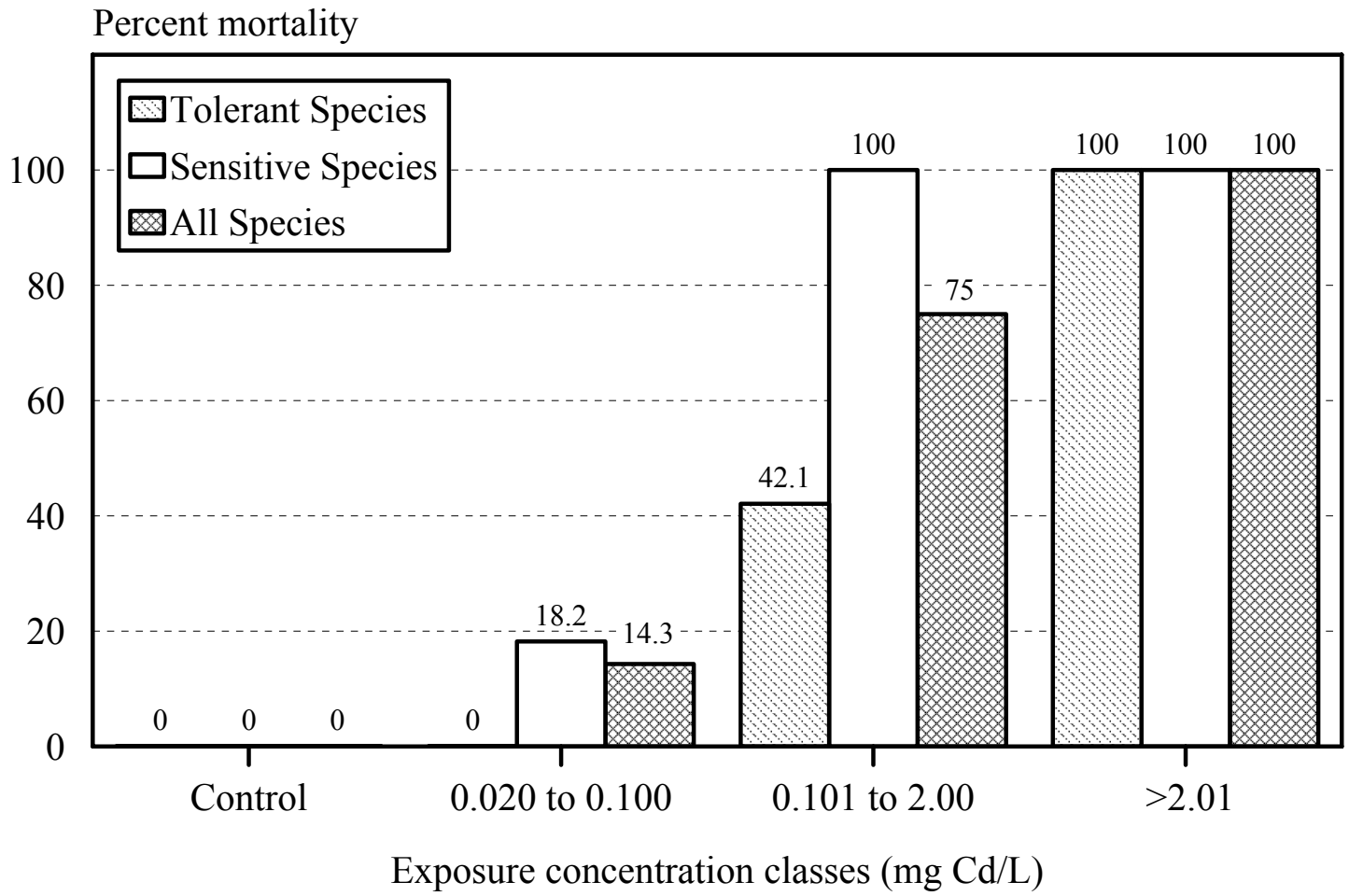


Figure 2.2. Percentage of mortalities of individuals undergoing a molt during or within three days prior to a toxicity test. For the different tests, data for similar exposure concentrations are grouped into exposure classes (mg Cd/L). Tolerant species include *O. juvenilis, O. virilis, P. alleni*, and *P. clarkii* and sensitive species include *O. placidus* and *P. acutus*.

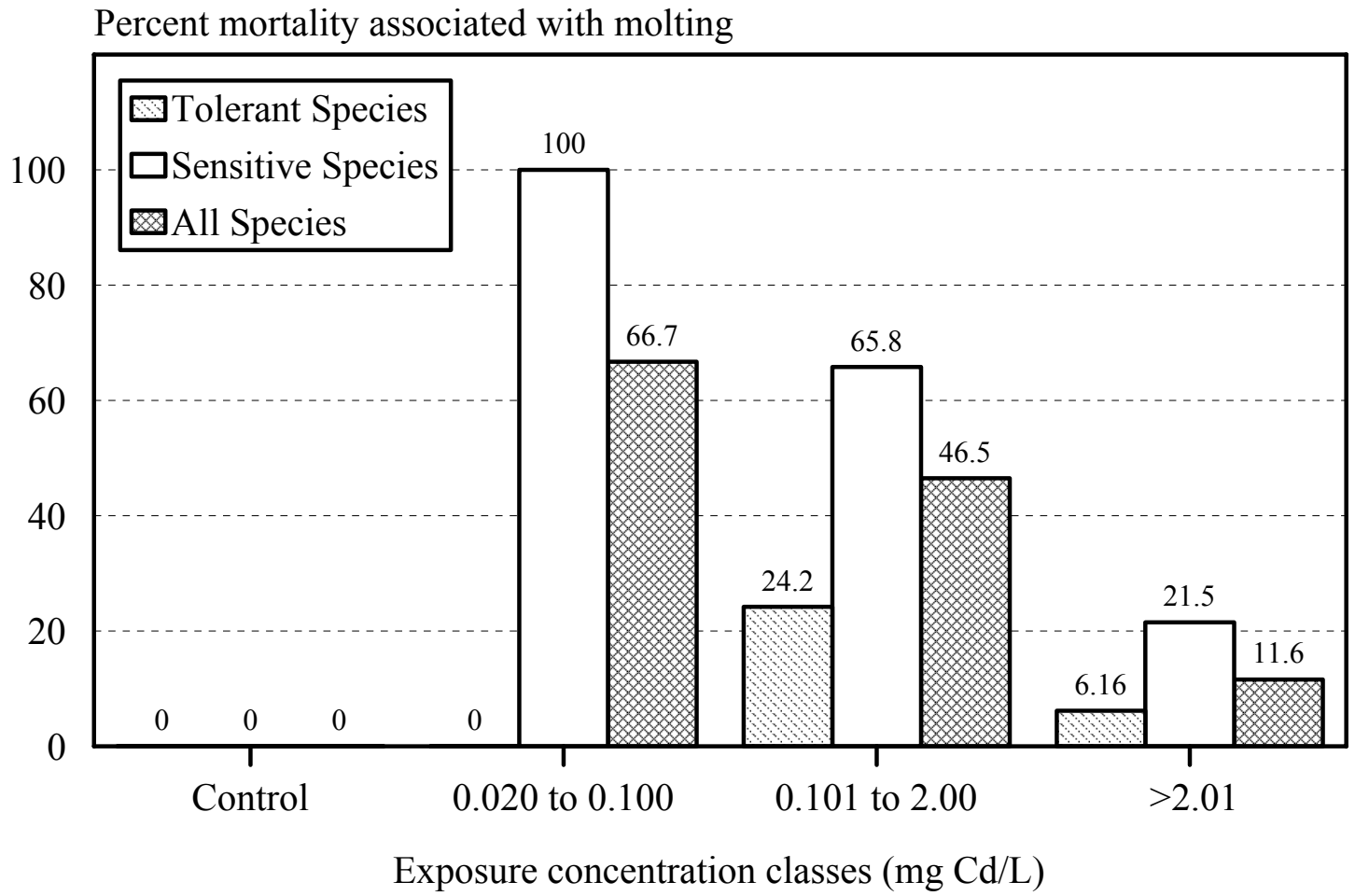


Figure 2.3. Percentage of the total number of mortalities that occurred after the individual molted. For the different tests, data for similar exposure concentrations are grouped into exposure classes (mg Cd/L). Tolerant species include *O. juvenilis, O. virilis, P. alleni*, and *P. clarkii* and sensitive species include *O. placidus* and *P. acutus*.

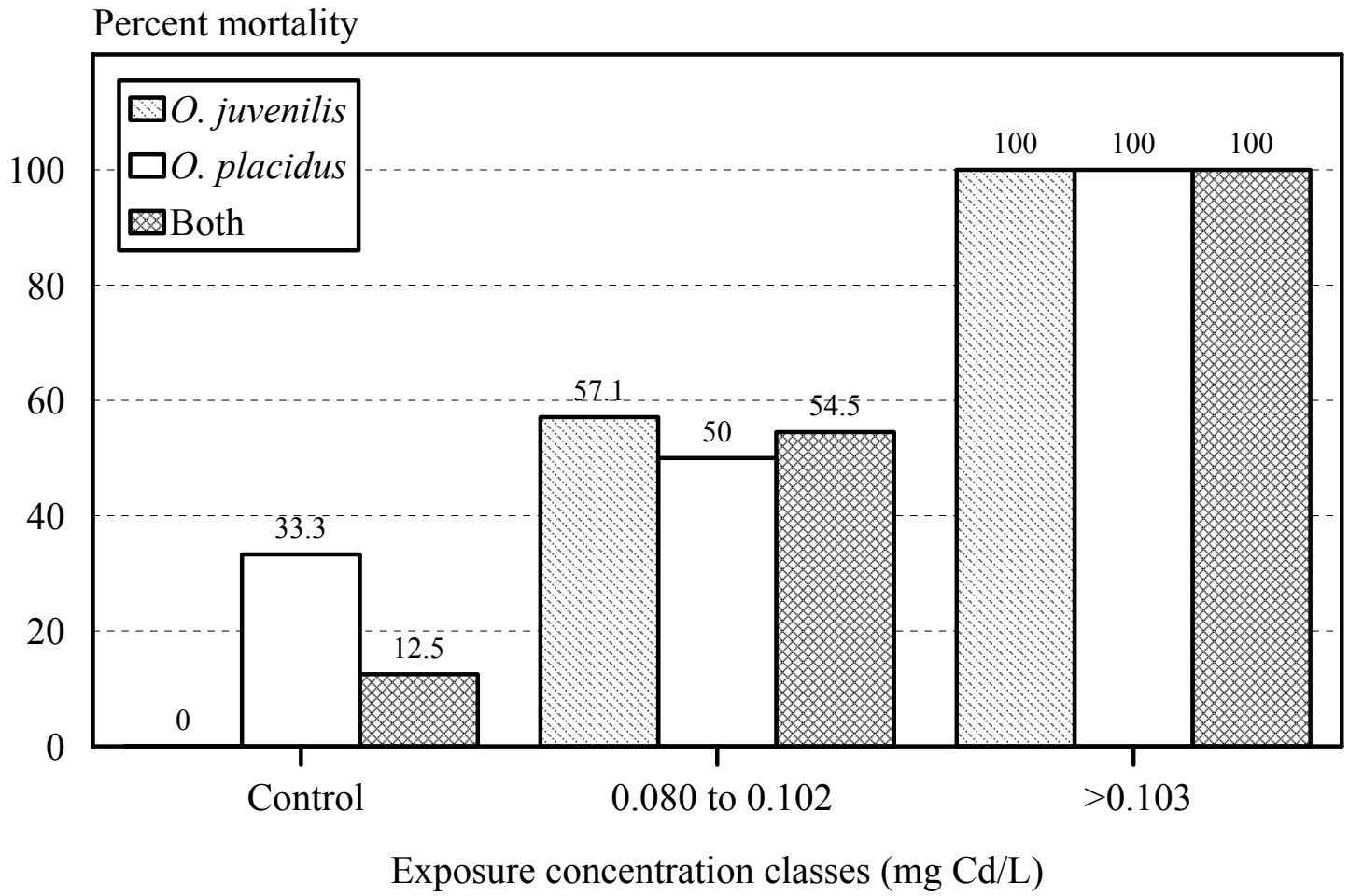


Figure 2.4. Percent mortality of juvenile crayfish undergoing a molt during or within three days prior to a toxicity test. For the two tests, data for similar exposure concentrations are grouped into exposure classes (mg Cd/L). Both indicates the summation of each assay's mortality data.

Chapter 3: The effects of cadmium exposure on the metal tissue concentrations of two tolerant and two sensitive crayfish species in the family Cambaridae.

3.1 Summary

Four 96h acute toxicity tests were performed with the crayfish *O. juvenilis*, *O. placidus*, *P. acutus*, and *P. clarkii* with various concentrations of Cd and control groups. *O. placidus* and *P. acutus* were more sensitive with LC50 values of 0.487 and 0.368 mg Cd/L respectively, while *O. juvenilis* and *P. clarkii* were more tolerant with LC50 values of 2.44 and 2.66 mg Cd/L, respectively. Tissue samples, including gills, green glands, hemolymph, hepatopancreas, muscles, ovaries, exoskeletal and remaining tissues, and testes, were dissected from survivors and analyzed for their content of Ca, Cd, Cu, Fe, Mg, P, Sr, Zn and various other elements. Whole body element content values were derived from the preceding. In most tissues, Cd tissue content correlated strongly with Cd exposure. Cd tissue residues were compared to exposure concentrations to calculate 96h BCFs. Regression analysis was used to derive equations estimating the curve of 96h BCFs to allow for better comparisons between species. Cd accumulated more strongly in the hepatopancreas, gills and green glands of sensitive species than in tolerant species. These data seem to indicate that a fundamental difference exists between how sensitive and tolerant crayfish species either take in or excrete heavy metals. Zn tended to show negative correlations with Cd exposure in the hepatopancreas. Cu showed several strong trends versus Cd tissue concentrations. Cu increased in green glands, gills, and hemolymph and, in some cases, decreased in the hepatopancreas. Ca, Fe, Mg, and Zn also showed significant trends. These data indicate that Cd may displace Cu and/or Zn in the hepatopancreas and the displaced metal then may move into other tissues, especially the gills and green glands, possibly to be excreted.

3.2 Introduction

Cadmium is a common, widespread toxicant and is found in 776 of 1,476 hazardous waste sites on past and present U.S. EPA National Priorities Lists (Taylor *et al.* 1999). It finds its way into the environment primarily through mining activities and fossil fuel combustion. It is typically recovered as a byproduct from zinc, copper, and lead mining. Globally, 25,000 to 30,000 tons of Cd are released into the environment annually, with perhaps 4,000 to 13,000 tons of that total coming from anthropogenic sources (Taylor *et al.* 1999). The Cd^{2+} ion is the most bioavailable form and the most toxic to aquatic organisms. It is thought to be taken up through the gills by the same mechanism as Ca and Zn. It is a nonessential metal that can cause a wide range of effects in aquatic organisms, including slowed growth, reduced reproductive performance, and impaired Ca metabolism, in addition to outright mortality (Albert *et al.* 1992).

North America has the greatest diversity of crayfish species in the world. In the United States alone there are more than 320 described species. This accounts for about 61% of the world's total of approximately 520 species. It is estimated that about 50% of United States crayfish species are imperiled or vulnerable to extinction (Taylor *et al.* 1996; Stein *et al.* 2000). However, only four species of crayfish currently receive protection under the U.S. Endangered Species Act, and no more are currently proposed or candidates for inclusion (U.S. Fish and Wildlife Service 2005). Most states possess multiple species of crayfish and some, especially those in the southeastern U.S., contain more than 50. Some species, such as *Cambarus diogenes*, have a very wide distribution encompassing many states while others have very limited ranges, sometimes as small as a single cave or stream (Hobbs 1976; Jezerinac *et al.* 1995; Pflieger 1996; Stein *et al.* 2000).

Crayfish are considered to be omnivores (Nyström 2002). While they engage in a predatory lifestyle under favorable conditions (Momot 1995; Whitledge and Rabeni 1997), herbivory and detritivory can be more important energetically under some environmental conditions (France 1996). Even when the animal engages in predation, some plant material is consumed, possibly for its micronutrient content, as well as incidental material taken along with prey items (Goddard 1988; Momot 1995). All species are benthic and many also burrow to some extent placing them in close proximity to sediments (Hobbs 1976). Adults are typically nocturnal while juveniles can be active at any time (Pennak 1991). Crayfish can comprise a large fraction of the animal biomass in an aquatic ecosystem, sometimes exceeding 50% of the macroinvertebrate biomass in lakes or streams (Momot 1995). Crayfish also serve as important prey items to a variety of aquatic and terrestrial predators. These include aquatic insects, other crayfish, many fish species, some amphibians and reptiles, and a variety of birds and mammals (Huner and Barr 1991). Their central location in many aquatic food webs may allow them to transfer toxicants between trophic levels (Bendell Young and Harvey 1991).

Crayfish typically take in metal toxicants through their digestive system or through their gills. Uptake through the integument is relatively minor for crayfish because of their impermeable, hardened exoskeleton. Cadmium in particular enters the gills through the apical calcium channel (Verbost *et al.* 1989). Transport through the basolateral membrane can occur through the Ca^{2+} ATPase ion pump or by facilitated diffusion, probably using the Na^{+}/Ca^{2+} ion exchanger (Verbost *et al.* 1989; Rainbow 1995; Silvestre *et al.* 2004). Heavy metals are then transported through the crayfish's body bound to hemolymph molecules, including the respiratory pigment hemocyanin (Roesijadi and Robinson 1994), and also in hemocytes (Ahearn *et al.* 2004). When it

reaches a target tissue, Cd in particular may cause various negative effects by binding to different proteins and altering their functional efficiency, changing the induction of various genes, or by promoting reactive oxygen species formation (Verbost *et al.* 1989; Almeida *et al.* 2002; Hartwig *et al.* 2002). In addition to direct mortality, Cd has been shown to reduce amylase activity by reducing stomach acidity (Reddy and Fingerman 1994), inhibit acetylcholinesterase activty (Devi and Fingerman 1995), reduce molting success (Chapter 2) decrease fecundity (Naqvi and Howell 1993), impair ovarian maturation (Reddy *et al.* 1997), and alter behavior (Maciorowski *et al.* 1980; Misra *et al.* 1996; Alberstadt *et al.* 1999; ibid.).

Crayfish possess two organs that are known to be involved in heavy metal detoxification and depuration: the hepatopancreas and the green glands. There seems to be a general tendency to partition biologically important heavy metals, such as Fe, into the hepatopancreas and ones with no known biological function, like Pb, into the green glands (Roldan and Shivers 1987). The gills may function in detoxification and depuration, but insufficient evidence has been collected to make a determination at this point (Handy 1996; Ahearn *et al.* 2004). In the hepatopancreas and green glands, the detoxification of metals can proceed along several pathways. Metals may be bound up by certain proteins, especially metallothionein (MT), that remove them from the biologically available pool (Ahearn *et al.* 2004). After this, the metal may be deposited into lysosomes for storage, possibly still attached to MT. The metal also can be precipitated into granules of sulfide or phosphate minerals that are insoluble, rendering them biologically inactive. Metals can also be stored in insoluble granules in mitochondria. Either the lysosomes or granules may be excreted in the feces by the hepatopancreas or in

the urine by the green glands to remove the metal from the crayfish's body (Ahearn *et al.* 2004).

Crayfish have been used as environmental monitors of heavy metal pollution by a number of researchers (*e.g.* Stinson and Eaton 1983; Allard and Stokes 1989; Bendell Young and Harvey 1991; Parks *et al.* 1991; Anderson *et al.* 1997). These authors noted the usefulness of crayfish as biomonitors of water and/or sediment heavy metal contamination. Concern also was raised about the role crayfish may play in distributing contaminants to higher trophic levels (Bendell Young and Harvey 1991). Several studies have been conducted that measure the accumulation of Cd in crayfish under laboratory conditions under short term exposure (Mirenda 1986) and long term, chronic exposure (Thorp *et al.* 1979; Giesy *et al.* 1980). Cadmium tissue concentrations tended to increase in a time and dose dependent fashion and depuration seemed slow (Thorp *et al.* 1979; Giesy *et al.* 1980).

However, this study is the first to compare how Cd accumulation may differ between species, especially those that may be more or less tolerant to Cd toxicity. It is also the first to assess the impact of Cd on the partitioning of other, biologically important metals such as Cu, Ca and Fe in various tissues and the whole body of the crayfish. This provides information about some of the 'whole animal' responses to Cd insult.

3.3 Methods

Four 96h acute toxicity tests were conducted with four species of crayfish in an environmentally controlled room at 25º ± 1º C and a 16h light, 8h dark photoperiod. Toxicity test and water quality analysis methods were based on standard methods provided by U.S. EPA and other sources (U.S. EPA 1993; U.S. EPA 1994a; U.S. EPA

1994b; APHA 1995; ASTM 1996). Carbon filtered tap water was used for housing crayfish before the experiments. Exposure water consisted of carbon filtered tap water standardized to 48 ± 5 mg Ca/L hardness for each test as measured using the EDTA titrimetric method, then confirmed using inductively coupled plasma optical emission spectrophotometry (ICP-OES) (APHA 1995). In each test, water was aerated to ensure continuously high dissolved oxygen levels (*i.e.* above 4.0 mg O_2/L; U.S. EPA 1993). Cadmium chloride ($CdCl_2$)was used to make all exposure solutions. Concentrations of Cd are expressed as the Cd ion and either graphite furnace atomic absorption spectrophotometry (GF-AAS) or ICP-OES was used to confirm water concentrations. The sampling scheme of water samples used to confirm exposure concentrations and hardness was designed to determine if these parameters changed over the course of the experiment. Accordingly, some samples were taken just before the renewal of test solutions, and some after 24h exposure.

In the toxicity tests, individuals were housed in 11.3 L polypropylene tubs. Crayfish were allowed to acclimate under test conditions 3 days prior to the start of Cd exposure. Solutions were changed daily during the 96h exposures. Animals were checked every 6 hours, and any dead crayfish were removed and examined to determine if they were in the process of molting when they died based on whether the outer exoskeleton was soft and had begun to separate from the inner, new exoskeleton, or the presence of shed exoskeleton. Mortality was used to calculate toxicity values using probit analysis (U.S. EPA 1992).

The experiment with *Orconectes juvenilis* adults was a triplicate design (n=10) with five test concentrations (0.141, 0.476, 0.826, 1.94, and 5.78 mg Cd/L). Each tub contained 3.5 L of treatment water. The assays with adult *Orconectes placidus*,

Procambarus clarkii, and *Procambarus acutus* were generally similar to the first, but incorporated several changes. These toxicity tests were run with four replicates (n=5) and five exposure concentrations in addition to controls. Exposure concentrations were set at 0.022, 0.128, 0.617, 2.56, and 11.0 mg Cd/L for the test with *O. placidus* and 0.099, 0.631, 3.50, 17.4, and 91.2 mg Cd/L for the test with *P. clarkii*. For *P. acutus,* the exposure concentrations were 0.049, 0.303, 1.652, 8.01, and 45.2 mg Cd/L. Again, 11.3 L polypropylene tubs were used, but this time each individual was housed in an 800 mL polypropylene beaker within the tub. Each beaker was well perforated to allow water flow This eliminated the possibility of agonistic interactions between the crayfish and any consequent mortality. Each tub was filled with 4.0 L of test solution.

Animals that survived the full 96 hours were subsampled to provide at minimum of five individuals per test concentration. These were then dissected for several tissues including hepatopancreas, green gland, muscle tissue, gills, gonads, hemolymph, and shell and remaining tissues. These samples were digested in nitric acid and peroxide using methods modified from Hogstrand *et al.* 1996. Briefly, 3.0ml/g concentrated trace-metal grade HNO_3 was added to each sample and left to dissolve for 24h. Samples were then heated to 95°C for 10m. Then, 1.0ml/g certified ACS grade 30% H_2O_2 was added to each sample, which was then heated briefly to 95°C . This step was repeated until no bubbles were evolved from the mixture or 10ml 30% H_2O_2 had been added. Samples were subsequently heated at 95°C until dry then redissolved in 0.5% HNO_3 (trace metal grade acid diluted with >17.6 MΩ high purity water). Samples were then filtered to remove any insoluble matter with prewashed glass fiber filters. The resulting sample was brought to a known volume with 0.5% HNO_3. Tissue residues reported were based on wet tissue mass. Silver (Ag), aluminum (Al), arsenic (As), barium (Ba), beryllium (Be),

calcium (Ca), cadmium (Cd), cobalt (Co), iron (Fe), potassium (K), lithium (Li), magnesium (Mg), molybdenum (Mo), manganese (Mn), sodium (Na), nickel (Ni), phosphorus (P), lead (Pb), antimony (Sb), selenium (Se), tin (Sn), strontium (Sr), titanium (Ti), tellurium (Tl), vanadium (V), and zinc (Zn) tissue residues were measured using ICP-OES (Varian Vista MPX) and/or GF-AAS (Varian SpectrAA-20 with GTA-96 Graphite Tube Analyzer). Cd tissue residues were used to calculate 96 hour bioconcentration factor (96h BCF) values. These were defined as the wet tissue concentration divided by the water concentration. This gives an index of how much more concentrated tissue samples were compared to their exposure water concentrations. ANOVA was conducted to investigate whether the treatment was affecting the means of the tissue residues for the array of elements. Ca, Cd, Cu, Fe, Mg, P, Sr, and Zn were selected for further analysis using linear regression with F-tests to test for significance of regression. Thus Pearson correlation coefficients (r) and p values were calculated for each metal in each tissue from each test. A standard spreadsheet program, Quattro Pro version 11, by Corel Corp., and Statistica version 5.5, Student edition, by Statsoft Inc., were used for this analysis. Additionally, Statistica was used to conduct natural logarithm (ln) transformations on all data to determine if the data might fit a curvilinear model more closely than a simple linear model. The model providing the tightest fit as defined by the highest significant ($p<0.05$) Pearson correlation coefficient was reported.

3.4 Results

Cadmium tended to accumulate in tissues with concentrations increasing as aqueous concentrations of dissolved Cd increased. The highest whole body tissue burdens occurred in *P. clarkii* and *O. juvenilis* (48.8mg Cd/kg and 49.6mg Cd/kg at exposure concentrations of 5.78 mg Cd/L and 17.4 mg Cd/L, respectively). The highest

96h BCF values were from tests with *O. placidus* and *P. acutus* (136 and 59 at exposure levels of 0.022 mg Cd/L and 0.049 mg Cd/L, respectively). The whole body 96h BCF values between species indicate that all four species accumulate similar burdens of Cd under similar exposure conditions.

In *O. placidus* and *P. acutus*, the highest concentrations of Cd were accumulated in the hepatopancreas while for *O. juvenilis* and *P. clarkii*, the highest concentrations were found in the gills (Table 3.1). The tissues that had the lowest Cd concentrations were the muscle, hemolymph, and ovary tissues (Table 3.1).

In general, the 96h BCFs showed a decreasing trend as aqueous concentrations increased, thus the highest 96h BCFs occurred in the lowest Cd exposure concentrations (Table 3.2). *O. juvenilis* showed its highest BCFs in its gills then its hepatopancreas with values of 76.2 and 59.6 respectively (Table 3.2).The highest 96h BCF value of any species tested was measured in *O. placidus* with 96h BCFs of 1230 in the hepatopancreas and 266 in the gills (Table 3.2). In *P. acutus*, similar high 96h BCF values were measured. The hepatopancreas and green glands showed 96h BCFs of 931 and 288 respectively. The highest 96h BCFs in *P. clarkii* were measured in the hepatopancreas (110) and the gills (60.6). In *O. juvenilis*, *O. placidus*, and *P. clarkii*, the ovary and muscle showed the lowest 96h BCFs and for *P. acutus*, the lowest were the hemolymph and muscle tissues (Table 3.2). The orders of tissue 96h BCF values from highest to lowest for each species are given in Table 3.3.

Natural logarithmic regression models were developed to allow the 96h BCF patterns between species to be compared more clearly. They allowed the application of consistent, hypothetical exposure concentrations between species. Whole body estimated 96h BCF values were similar between species. The estimated 96h BCF values derived

from them showed that the hepatopancreas usually had the highest 96h BCF at lower hypothetical exposure levels (Table 3.4). Usually gills possessed the second highest, at these concentrations. The two sensitive species *O. placidus* and *P. acutus* had much higher hepatopancreas, gill, and green gland 96h BCF values compared to the two tolerant species, *O. juvenilis* and *P. clarkii*. At the 0.141 mg Cd/L exposure level, the estimated hepatopancreas 96h BCFs for *O. placidus* and *P. acutus* were 749 and 453 respectively, compared to 47.4 and 44.4 for *O. juvenilis* and *P. clarkii* respectively. At the same exposure level, the gill 96h BCFs were 136 and 92.6 for *O. placidus* and *P. acutus* respectively, compared to 33.3 and 55.1 for *O. juvenilis* and *P. clarkii* respectively.

As the exposure levels increased, the differences in estimated 96h BCF values between species decreased. Estimates at the 0.631mg Cd/L exposure concentration for gill and green gland 96h BCF values showed almost no differences between species (Table 3.4). At this exposure concentration, the interspecific difference between hepatopancreas 96h BCF estimates was reduced to a factor of 3 to 7, compared to a 10 to 17 fold difference at lower concentrations of Cd. Also, at higher theoretical exposure concentrations, the hepatopancreas in the two sensitive species continued have the highest 96h BCFs. However, the gills were higher in the more tolerant species, although the difference between the two tissues was small (Table 3.4). When the estimated 96h BCF values from the two sensitive species and the two tolerant species were averaged, these relationships can be seen more clearly (Table 3.5). At an exposure level of 0.141 mg Cd/L, the estimated hepatopancreas 96h BCF was only 45.9 for tolerant species, while for sensitive species it was 601, a 13 fold increase. Green gland and gill estimated

96h BCFs were also much higher for sensitive species than for tolerant species (Table 3.5).

Cadmium tissue burden tended to show moderately weak to strong correlations with aqueous Cd exposure (r= 0.491 to 0.909, average significant r =0.732 , $p \leq 0.025$; Table 3.6). Ovary tissues did not evince strong correlations in general as only *P. clarkii* showed a statistically significant trend. The green glands and hepatopancreas of *P. acutus* also failed to reveal significant trends ($p>0.05$).

In regression analysis of crayfish tissue concentration versus aqueous Cd exposure, Zn showed the most demonstrative patterns (Table 3.6). In three of four species, Zn decreased significantly (r=-0.520 to -0.672, $p<0.04$) in the hepatopancreas. In the fourth species, *O. juvenilis*, a weak negative trend was also evident (r=-0.208, p=0.143). Thus, crayfish may loose Zn from their hepatopancreas as Cd exposure increases. Some crayfish seemed to have accumulated Zn in their testes under Cd exposure. *O. placidus* showed a strong increasing relationship (r=0.941 p=0.002) while *P. acutus* showed a moderate trend (r=0.639, p=0.088). Significant trends were measured in Zn residues in muscle tissue under Cd exposure. *O. juvenilis* and *O. placidus* showed significant decreasing relationships (r=-0.273 to -0.524, $p<0.05$) while *P. acutus* showed a trend of increasing Zn concentrations (r=0.525, p=0.045).

Copper tissue residues may be increasing in the gills under Cd exposure (Table 3.6). In *O. placidus* and *P. clarkii*, significant increasing trends were measured (r= 0.557 and 0.370, p= 0.004 and 0.04). In *P. acutus*, a weaker trend was recorded (r= 0.437, p= 0.054).

A wider variety of significant correlations were measured between various metal tissue concentrations and Cd accumulation (Table 3.7). Copper showed several strong

trends as Cd tissue concentration increased. Copper concentrations in the green glands increased in all four species tested ($r=0.328$ to 0.854, $p<0.02$). There were similar increasing trends in all four species for gill tissue samples ($r=0.290$ to 0.695, $p<0.04$). Hemolymph samples also showed trends of increasing Cu concentrations as Cd tissue burden increased in both species tested ($r=0.575$ to 0.669, $p<0.001$). Muscle tissues showed some trends towards the accumulation of Cu (Table 3.7). A positive trend existed in *O. placidus* and *P. acutus* ($r=0.477$ to 0.511, $p< 0.03$) and a weak trend existed for *O. juvenilis* ($r=0.255$, $p<0.065$). In hepatopancreas samples, three of the four species showed very weak trends of decreasing Cu concentrations, but none were significant (Table 3.7). However, Analysis of Variance (ANOVA) with Least Significant Difference (LSD) post hoc comparison was used to test whether Cd exposure caused any differences in hepatopancreatic Cu residue. The 0.022 and 0.617 mg Cd/L exposure levels for *O. placidus* and the 0.303 and 1.65 mg Cd/L concentrations for *P. acutus* had Cu burdens that were significantly lower than controls ($p<0.05$).

There were several positive trends measured between increasing Cd tissue accumulation and tissue Fe concentrations (Table 3.7). All species except *O. placidus* showed a significant increasing trend in gill tissues ($r=0.328$ to 0.681, $p<0.02$). In both *P. acutus* and *P. clarkii*, Fe concentrations in hemolymph increased ($r= 0.614$ to 0.639, $p<0.005$). There were some moderate to strong correlations between Fe content in the shell and remnant samples and Cd tissue residues ($r=0.574$ to 0.981, $p<0.005$). Whole body Fe levels showed the same pattern, although more weakly (0.509 to 0.865, $p<0.03$).

Calcium content in muscle increased as Cd tissue burden increased. *P. acutus* and *P. clarkii* show significant trends ($r= 0.782$ and $r=0.366$, $p<0.05$) and *O. juvenilis* shows a weak trend ($r=0.257$, $p=0.063$; Table 3.7). A stronger relationship existed between Ca

and Cd tissue residues in the hemolymph. Both *P. acutus* and *P. clarkii* show strong positive trends (r= 0.779 and 0.837 respectively, $p<0.001$) for an increase in Ca as Cd built up. Magnesium showed weak increasing trends in green gland tissues as Cd concentrations increased in all species except *P. acutus* (r=0.358 to 0.474, $p<0.05$; Table 3.7). A somewhat stronger set of increasing trends existed for gill tissue Mg content (r= 0.530 to 0.597, $p<0.01$) in *O. placidus*, *P. acutus*, and *P. clarkii*. Both species tested for hemolymph tissue concentrations showed significant positive trends for Mg concentrations (r=0.500 to 0.627, $p<0.03$). In both *Procambarus* species, there was a weak increase in Mg in the hepatopancreas (r=0.381 to 0.474, $p<0.04$).

Zinc tissue concentrations showed only a few trends as Cd tissue burden increased (Table 3.7). While all crayfish species showed weak increasing trends for Zn in green gland tissues, only the trend for *P. clarkii* was significant (r= 0.453, $p<0.02$) while *O. placidus* showed a weak trend (r=0.336, $p<0.100$). In gill samples, only *P. clarkii* showed a weak increasing trend (r=0.355, p=0.054). Zinc seemed to show an increasing trend in the ovaries of the two *Orconectes* species as Cd tissue residues increased (r= 0.389 to 0.829, $p<0.05$).

3.5 Discussion

The similarities in whole body burden of Cd between species taken by itself is misleading. In isolation, it might indicate that there were no differences in Cd accumulation between species. However, the differing patterns of Cd partitioning within tissues alludes to some important differences between species, even if the burdens in those tissues sum to similar totals.

During this series of assays, two groups of crayfish species were identified based on their 96h LC50 scores, a sensitive group containing *O. placidus* and *P. acutus*, and a

tolerant group containing *O. juvenilis* and *P. clarkii*. These two groups showed somewhat dissimilar sets of responses to Cd exposure.

Mirenda (1986) reported a pattern of Cd accumulation for *O. virilis*, a tolerant crayfish species (LC50 = 6.1 mg Cd/L), that was similar to tolerant species in this study. Tolerant crayfish tended to have a somewhat different Cd accumulation pattern compared to sensitive crayfish species. This was seen in both the tissue element concentrations and the calculated and estimated 96h BCF values (Table 3.1 - 3.5). The pattern, whereby the gills accumulated more Cd than the hepatopancreas of tolerant species than sensitive species, may indicate that tolerant species have a more efficient depuration mechanism centered on the gills. Since crayfish excrete a great deal of Ca during molting (Huner *et al*. 1978; Wheatly and Ayers 1995), a mechanism may be in place to facilitate this. It may also be that the since the gills are a site of metallothionein induction (Chavez-Crooker *et al.* 2003), more metal is simply being sequestered there. Unfortunately the role of the gills in heavy metal detoxification and depuration is too poorly understood at the present time to evaluate this possibility fully (Ahearn *et al.* 2004).

Also, typically the green glands showed a higher 96h BCF in sensitive species than in tolerant ones. The green glands are a major site for the detoxification and depuration of Pb (Roldan and Shivers 1987) so it is possible that more tolerant species might clear the Cd from their green glands more quickly. In some aquatic insects, it seems that the tendency to accumulate heavy metals can mark an intolerant species while more tolerant species have a greater ability to either avoid the uptake of pollutants or to detoxify and/or depurate metal pollutants (Birge *et al.* 2000b). This tendency may be related to the number of receptor sites for metal uptake (*i.e.* ionocytes) found in different species (Buchwalter and Luoma 2005). The more tolerant species also showed a

tendency to accumulate more Cd in their exoskeleton, another possible means of removing Cd from the biologically active metal ion pool in the body. During molting, it may be possible that the Cd sequestered in the exoskeleton might be remobilized however, or that the crayfish may regain a portion of this Cd when it eats the old exoskeleton after finishing its molt. However, it is also possible that these values simply represented a greater tendency to adsorb Cd onto the exterior surface of the exoskeleton (Mirenda 1986).

When regression was used to model tissue accumulation at consistent levels between species, the differences between the two more sensitive species and two more tolerant species were clearly seen. At an exposure level of 0.141 mg Cd/L, while whole body 96h BCF values were fairly similar across species, the tissue specific 96h BCFs showed some striking differences. Gill, hepatopancreas, and green gland tissues all showed higher BCFs in the sensitive species than in the tolerant species. These three tissues represent the primary uptake surface under aqueous exposure, and the two primary detoxification and depuration organs respectively. As previously mentioned, this indicates that there may be a fundamental difference between how sensitive and tolerant species of crayfish either take in Cd, how they depurate or otherwise detoxify Cd, or both. Since total Cd burdens seem to be similar between tolerant and sensitive species, and several internal organs accumulate more Cd in the sensitive species, the exoskeletons are making up the difference in total body burden for tolerant species.

The pattern of decreased 96h BCF values as exposure concentration increased could have had several sources (Table 3.2, Table 3.4) and has been noted by Mirenda (1986) as well. Several potential explanations exist. First, the uptake pathway could have been saturated. The uptake curve resembles the upper portion of a Michaelis-Menten

enzyme activity curve. This may be because the uptake of Cd is driven by the Ca/divalent ion uptake pathway, a transport enzyme mediated process. Second, ligands such as metallothionein and glutathione in the target tissues may have been saturated, reducing the tendency of Cd to accumulate in particular tissues relative to others. Third, the animals had achieved an equilibrium between uptake and depuration, although given the short duration of these tests, this is unlikely (Mirenda 1986).

Correlation coefficients for Cd accumulation in tissues during this set of experiments showed an average value of r=0.732 when statistically significant values were averaged (Table 3.6). This reflects a considerable amount of inter-individual variation in response to Cd. Giesy *et al.* (1980) found similar levels of inter-individual variation in a longer term study of the accumulation of Cd in the crayfish *P. acutus*. In that study, correlation coefficients ranged from r=0.566 to 0.686 for regression estimates of the accumulation rate of Cd over the first reported time segment of their experiment, a time frame similar to this experiment.

While most tissues showed significant trends to accumulate more Cd as toxicant water concentrations increased, the hepatopancreas and green gland tissues of *P. acutus* failed to show an increase for Cd tissue burden under aqueous Cd exposure (Table 3.6). While average Cd values for exposed individuals for both tissues were much higher than controls, the variation was sufficiently high to prevent statistical significance (Table 3.1). Also, the highest exposure concentration for that group actually showed slightly lower average Cd tissue concentration (not statistically different) than the next lower aqueous exposure concentration. These unexpected finding may have been caused by the unusually high rate of molting found in that assay. Molting rates with *O. juvenilis*, *O. placidus*, and *P. clarkii* ranged from 0.556% to 13.3% during the exposure or within 3d

prior. These rates were consistent with what might be found under natural conditions (Wetzel 2002; Taylor and Schuster 2004). However, *P. acutus* had a molting rate over the same time period of 41.7%. While much higher, it was still an environmentally feasible molting rate because some crayfish populations may molt nearly simultaneously in the spring (Taylor and Schuster 2004). However, this could help to explain why the response of *P. acutus* was not as clearly modeled by regression analysis as the other three species.

Zn concentrations in the hepatopancreas show a decreasing trend in all species under Cd exposure via the water. Three of four were significant trends indicating that perhaps Zn is being lost by Zn binding proteins in favor of Cd. Whether this response is an induced mechanism of detoxification or simply the displacement of one ion for another with a higher binding affinity for the target ligands, especially metallothionein, cannot be determined from these data. Zn can often be displaced from metallothionein in favor of Cd (Leber and Miya 1976; Funk *et al.* 1987; Roesijadi 1992). However, this has not been tested specifically in crayfish. This idea's feasibility would be related to the relative binding affinities for Zn and Cd in crayfish metallothioneins. Some additional observations about these toxicity tests support the possibility that Zn was being removed from the body under Cd exposure. In some cases, water samples taken after 24h of exposure showed elevated levels of Zn compared to newly changed exposure water. In the *P. clarkii* toxicity test, exposure levels of 0.099 and 0.631 mg Cd/L showed an increased level of Zn when all 24h water samples were compared with all samples of fresh exposure water (t-test, $p<0.004$). The 3.50 mg Cd/L exposure level showed a similar, nearly significant ($p=0.058$) pattern. The controls did not show this pattern ($p>0.05$). The test with *O. placidus* showed a similar trend in which the Zn water

concentration was elevated ($p<0.05$) after 24h in the 0.022, 0.128, and 10.8 mg Cd/L exposure concentrations. A similar, although non-significant, trend was observed in the test with *P. acutus*. The relative lack of an accumulation trend for Zn in green glands or gills could indicate that an efficient mechanism for Zn elimination exists.

Copper showed a number of significant trends in relation to Cd tissue burden. Copper showed increasing trends in the gills and green glands of all four of the species tested, and both of the species tested for hemolymph metal residues. This indicates several possibilities. One, crayfish have increased Cu uptake relative to controls. Two, crayfish are losing Cu from some tissue(s) and the excess Cu is moving towards the ionoregulatory organs for elimination. Since there is build up in the green glands, an increase in the excretion of Cu seems more likely. There is also evidence from ANOVA analysis with LSD post hoc comparisons that Cu is being lost in some cases from the hepatopancreas, possibly from metallothionein. Typically, Cd is not considered to displace Cu in metallothionein (Funk *et al.* 1987; Li and Otvos 1996b), however, at least some evidence suggests that Cd may replace Cu in some cases (Satarug *et al.* 2000). As with Zn, this ultimately depends on the relative binding affinities for Cu and Cd in crayfish metallothioneins, which have not been measured. Whether or not Cd was displacing Cu from metallothionein, other Cu binding proteins were likely losing their Cu as Cd accumulated. Copper concentrations in exposure water were much lower than Zn concentrations, preventing statistical analysis of changes in aqueous Cu concentrations. However, it is worth noting that the only water samples in either the *P. acutus* or *P. clarkii* test to show levels of Cu above the detection limit of the ICP-OES were a few samples of Cd exposure water taken either 12h or 24h after the daily water change.

Fe, Ca, and Mg showed similar, though weaker, trends to Cu, which perhaps indicated that some of these metals were being displaced from their transport proteins. The strong correlations between increased Fe content of the shell and remnant samples and Cd concentrations possibly indicates that this metal was being sequestered in the exoskeleton instead of being lost. Negative trends existed for Fe in the hepatopancras in three species, but only one was a significant decreasing trend. Thus it was not clear in this case if metal was being lost from the hepatopancreas or being taken up by the gills, and, in either case, being subsequently redistributed.

3.6 Conclusions

Differences between the whole body burden for Cd between crayfish species were slight. Even at the lowest exposure concentrations, a considerable amount of Cd was accumulated. However, this should not be taken as evidence that the partitioning and metabolism of Cd is similar in all species.

Crayfish are capable of accumulating relatively high levels of Cd in several tissues. However, the extent a given species tends to accumulate Cd in various tissues depends upon the crayfish's overall tolerance to Cd. Sensitive species tend to accumulate more Cd in the hepatopancreas than any other tissue, up to 1,230 times the exposure concentration. More tolerant species of crayfish tend to accumulate generally lower levels of Cd in the gills, green glands and hepatopancreas. The concentration of Cd accumulated in the gills and hepatopancreas similar in tolerant species, although often slightly higher in the gills. This indicates a possibly fundamental difference between tolerant and sensitive crayfish. It is probably indicative of differences in the capacity of the detoxification apparatus, which is centered on the hepatopancreas, green glands and probably the gills, between the two groups. Since the exoskeleton tends to accumulate

more Cd in tolerant species, it may help to detoxify this metal as well. Tolerant species may be able to excrete Cd more effectively or may be able to avoid taking in as much Cd, a relationship noted elsewhere (Birge *et al.* 2000b). Since whole body Cd burdens are similar between the groups, the status of Cd in or on the exoskeleton would determine which scenario is more likely. If Cd is being adsorbed onto the surface of the exoskeleton in tolerant species, then the lower 96h BCFs of their internal organs indicates that they take in less Cd. If that metal is being sequestered within the exoskeleton, then sensitive and tolerant species take in similar quantities of Cd, but have a more efficient detoxification mechanism. It may be that tolerant crayfish use an intermediate strategy that incorporates both of these possibilities.

Cadmium exposure seems to cause considerable alteration to the metabolism of several biologically important metals, especially Ca, Cu, Fe, Mg, and Zn. However these differences did not seem to be dependent upon species tolerance, so perhaps the pattern of response to Cd is similar between species and dependent upon Cd accumulated, which did vary between species.Correlations were much more plentiful between Cd tissue burden and other metal tissue burden as compared to correlations with Cd water concentrations.

Some metals are perhaps being displaced from proteins that store, detoxify, and help regulate free metal ion levels in cells, especially metallothionein. The evidence suggests that some of these metals, especially Cu and Zn, may be transported out of the animal and into the aqueous exposure media. It may involve the gills and/or green glands since Cu accumulates strongly in both tissues. Since Zn did not accumulate in either the green glands or gills, an efficient mechanism for depurating this metal may exist in either or both tissues. The latter are known to be involved in the detoxification of heavy metals

(Vogt 2002), however the precise mechanism of action for either tissue is not well understood (Ahearn *et al.* 2004).

It has been proposed that crayfish may not be suitable for monitoring metal pollutant concentrations over long periods of time because the cells in the hepatopancreas that are responsible for a considerable amount of the animal's detoxification only have a one to two week life span (Vogt 2002). After that time, they are shed into the lumen of the gastrointestinal tract and the sequestered metal is lost in the feces. Similarly, crayfish may be able to depurate heavy metals from the green glands by the exocytosis of metal containing lysosomes (Ahearn *et al.* 2004). However, Giesy *et al.* (1980) reported that crayfish failed to excrete any significant amount of Cd during the 22d depuration period after the termination of a 55d Cd exposure to Cd in water and food. Several other researchers (*e.g*. Stinson and Eaton 1983; Allard and Stokes 1989; Bendell Young and Harvey 1991; Parks *et al.* 1991; Anderson *et al.* 1997) have used or recommended the use of crayfish in biomonitoring. In any case, the ability of sensitive crayfish species to accumulate high concentrations of Cd over a short period of time indicates that they could be useful as short term monitors of metal exposure. Although some questions remain about their utility as long term monitors, perhaps their rapid uptake allows them to reach equilibrium between excretion and accumulation relatively quicky, thus allowing them to serve as dynamic biomonitors with tissue residues rising and falling with recent exposure. Thus, wild caught crayfish would be at steady state conditions effectively integrating periods of exposure and depuration over time. However, exposing caged, naive crayfish to contaminated waters could provide information about the bioavailable metal in the environment at that moment.

Table 3.1. Chemical residues of various crayfish tissues (mg/kg). Cd concentrations are given in mg Cd/L and n= number of tissue samples dissected for a particular tissue and species.

	Cd Conc.	n=	Avg. Mass	Std. Error	Ca	Std. Error	Cd	Std. Error	Cu	Std. Error	Fe	Std. Error
Calcium stone tissue residue												
O. juvenilis	0.476	1	0.008	-	62300	-	3.78	-	13.9	-	32.7	-
	0.826	1	0.001	-	150000	-	33.8	-	49.5	-	337	-
O. placidus	0.022	1	0.007	-	218000	-	0.262	-	12.6	-	16.7	-
P. clarkii	0.099	1	0.022	-	197000	-	0.398	-	2.88	-	18.5	-
	0.631	1	0.318	-	33000	-	0.623	-	1.09	-	2.28	-
Green gland tissue residue												
O. juvenilis	0.141	13	0.011	0.002	1770	582	3.94	0.626	34.3	5.92	53.6	18.1
	0.476	12	0.012	0.001	2430	834	11.9	3.33	45.1	9.03	43.0	5.14
	0.826	13	0.014	0.002	1370	559	7.86	1.26	23.5	1.47	68.0	27.8
	1.94	9	0.019	0.002	739	61.9	11.5	1.47	28.2	4.60	28.6	2.81
	5.78	5	0.014	0.003	1150	383	34.0	12.4	38.7	7.74	75.6	31.0
O. placidus	0.000	7	0.022	0.006	262	33.6	0.343	0.230	3.34	0.494	24.8	5.53
	0.022	6	0.025	0.004	455	110	3.50	1.52	4.87	0.381	20.6	1.43
	0.128	6	0.023	0.006	504	152	12.5	6.00	13.5	7.18	29.3	9.63
	0.617	6	0.023	0.002	553	60.8	14.3	3.67	9.52	2.05	21.3	1.51
P. acutus	0.000	5	0.037	0.003	238	25.6	0.244	0.108	2.80	0.214	18.7	0.590
	0.049	5	0.069	0.005	249	34.3	14.1	6.36	3.57	0.354	15.9	1.88
	0.303	5	0.074	0.008	309	22.4	18.4	6.96	4.31	0.674	23.0	2.61
	1.65	5	0.060	0.008	251	49.1	9.76	3.63	3.59	0.419	29.6	5.94
P. clarkii	0.000	10	0.064	0.006	226	26.5	0.482	0.232	3.23	0.225	22.9	2.22
	0.099	6	0.054	0.004	369	106	1.15	0.901	4.45	0.529	20.1	1.13
	0.631	6	0.050	0.003	318	52.7	4.96	3.59	5.47	0.976	27.7	7.10
	3.50	6	0.054	0.010	1470	1020	24.2	8.49	11.4	4.93	34.4	10.6
	17.4	2	0.070	0.014	261	11.2	38.0	25.5	10.1	7.02	20.6	0.116

Table 3.1 (continued). Chemical residues of various crayfish tissues (mg/kg). Cd concentrations are given in mg Cd/L and n= number of tissue samples dissected for a particular tissue and species.

	Cd Conc.	n=	Avg. Mass	Std. Error	Ca	Std. Error	Cd	Std. Error	Cu	Std. Error	Fe	Std. Error
Gill tissue residue												
O. juvenilis	0.141	13	0.123	0.018	9050	7630	10.7	1.04	46.6	3.90	29.3	6.14
	0.476	13	0.128	0.018	2300	608	27.9	6.03	50.4	7.38	29.5	9.39
	0.826	13	0.132	0.022	1470	153	22.3	2.15	57.9	4.83	27.6	4.97
	1.94	9	0.151	0.020	1150	78.7	33.9	4.14	51.4	3.31	29.2	6.62
	5.78	5	0.157	0.047	1270	65.0	92.1	26.1	52.9	6.68	39.2	15.6
O. placidus	0.000	7	0.111	0.015	1460	263	0.044	0.020	20.0	4.34	9.45	3.96
	0.022	6	0.102	0.030	1260	147	5.84	0.927	35.8	3.59	8.87	1.16
	0.128	6	0.097	0.020	1370	264	18.1	3.58	32.1	3.38	7.30	0.636
	0.617	6	0.081	0.010	2070	215	30.1	2.15	44.9	4.77	14.3	2.40
P. acutus	0.000	5	0.341	0.034	867	88.6	0.019	0.011	8.76	1.77	3.17	0.151
	0.049	5	0.427	0.032	982	107	5.44	1.61	13.3	1.85	7.76	3.03
	0.303	5	0.429	0.016	933	98.2	17.5	3.44	15.8	1.18	9.53	4.76
	1.65	5	0.398	0.021	992	108	33.4	6.94	16.2	1.42	45.6	39.7
P. clarkii	0.000	10	0.707	0.132	817	67.7	0.014	0.003	15.5	1.94	13.9	3.36
	0.099	6	0.507	0.139	842	102	6.00	1.77	18.4	1.34	15.8	4.13
	0.631	6	0.348	0.042	887	32.0	21.9	6.54	19.9	1.38	148	117
	3.50	7	0.479	0.118	1300	283	61.8	13.6	21.4	2.06	37.0	6.83
	17.4	2	0.496	0.063	1060	103	210	139	24.4	3.26	22.4	13.6

Table 3.1 (continued). Chemical residues of various crayfish tissues (mg/kg). Cd concentrations are given in mg Cd/L and n= number of tissue samples dissected for a particular tissue and species.

	Cd Conc.	n=	Avg. Mass	Std. Error	Mg	Std. Error	P	Std. Error	Sr	Std. Error	Zn	Std. Error
Hemolymph tissue residue												
P. acutus	0.000	5	1.43	0.141	378	14.0	0.006	0.001	19.4	4.64	0.354	0.097
	0.049	5	1.37	0.181	568	57.2	0.334	0.190	26.0	4.02	0.663	0.113
	0.303	5	1.30	0.096	600	47.9	0.532	0.243	38.2	4.29	0.791	0.150
	1.65	5	1.12	0.098	560	39.9	0.771	0.077	38.5	4.29	1.11	0.255
P. clarkii	0.000	10	1.24	0.136	323	16.5	0.024	0.010	33.4	3.86	0.427	0.103
	0.099	5	0.975	0.060	464	46.2	0.310	0.216	39.7	4.85	0.648	0.107
	0.631	6	0.902	0.176	496	46.6	0.541	0.255	44.0	4.50	1.20	0.370
	3.50	5	0.735	0.077	735	65.6	16.6	11.8	49.7	6.29	1.36	0.291
	17.4	2	0.862	0.004	662	56.0	21.4	15.3	56.3	14.1	0.834	0.240

Table 3.1 (continued). Chemical residues of various crayfish tissues (mg/kg). Cd concentrations are given in mg Cd/L and n= number of tissue samples dissected for a particular tissue and species.

	Cd Conc.	n=	Avg. Mass	Std. Error	Ca	Std. Error	Cd	Std. Error	Cu	Std. Error	Fe	Std. Error
Hepatopancreas tissue residue												
O. juvenilis	0.141	13	0.218	0.036	449	81.7	8.40	1.28	445	71.4	62.2	10.3
	0.476	12	0.247	0.023	1840	825	26.3	9.94	228	60.2	49.2	12.2
	0.826	12	0.235	0.030	434	70.5	24.0	6.42	321	53.1	70.9	16.8
	1.94	9	0.303	0.050	383	53.1	49.3	9.16	370	70.7	56.5	17.2
	5.78	5	0.233	0.031	606	169	61.9	18.5	244	52.1	59.8	10.3
O. placidus	0.000	7	0.100	0.024	299	18.3	0.874	0.103	180	38.8	59.7	9.89
	0.022	6	0.121	0.022	623	310	27.1	10.5	57.6	25.4	58.3	10.4
	0.128	6	0.102	0.028	401	45.8	91.5	34.8	115	54.4	42.8	7.61
	0.617	6	0.091	0.011	427	63.0	177	50.3	70.0	20.2	41.1	11.8
P. acutus	0.000	5	0.308	0.051	148	14.5	2.60	1.18	26.1	8.13	125	17.7
	0.049	5	0.302	0.045	217	34.9	45.6	17.6	19.3	8.15	95.1	22.1
	0.303	5	0.359	0.020	188	30.8	88.5	22.3	6.91	2.07	99.0	15.8
	1.65	5	0.368	0.049	199	20.3	78.1	42.4	3.16	0.313	74.3	19.3
P. clarkii	0.000	10	0.801	0.102	298	154	0.299	0.046	22.4	16.9	46.6	8.35
	0.099	6	0.684	0.277	431	250	10.9	8.50	5.24	2.25	46.0	9.06
	0.631	6	0.491	0.101	145	20.7	8.88	4.30	32.5	30.2	95.3	38.7
	3.50	7	0.621	0.236	1220	848	46.5	11.9	7.11	1.78	80.7	23.7
	17.4	2	0.897	0.197	136	2.11	73.8	34.5	5.10	2.76	47.3	10.4

Table 3.1 (continued). Chemical residues of various crayfish tissues (mg/kg). Cd concentrations are given in mg Cd/L and n= number of tissue samples dissected for a particular tissue and species.

	Cd Conc.	n=	Avg. Mass	Std. Error	Ca	Std. Error	Cd	Std. Error	Cu	Std. Error	Fe	Std. Error
Muscle tissue residue												
O. juvenilis	0.141	13	0.327	0.046	580	100	0.610	0.078	13.4	1.07	3.77	0.533
	0.476	13	0.505	0.072	1850	714	1.36	0.435	15.7	3.54	3.70	0.780
	0.826	13	0.415	0.071	559	176	0.774	0.102	11.9	0.731	2.89	0.237
	1.94	9	0.409	0.049	443	49.1	2.05	0.494	14.7	1.49	3.70	0.804
	5.78	5	0.309	0.048	428	53.3	3.91	1.83	10.6	1.40	6.60	3.10
O. placidus	0.000	7	0.484	0.067	222	40.8	0.026	0.005	3.03	0.561	2.71	0.272
	0.022	6	0.627	0.098	225	34.7	0.208	0.043	5.16	0.590	2.79	0.317
	0.128	6	0.649	0.090	236	33.9	0.452	0.117	4.79	1.05	3.76	1.27
	0.617	6	0.597	0.041	399	129	0.690	0.088	6.62	1.11	2.28	0.313
P. acutus	0.000	4	1.95	0.311	126	6.10	0.004	0.001	1.37	0.199	1.41	0.135
	0.049	5	2.45	0.126	198	6.48	0.127	0.062	1.38	0.182	1.40	0.045
	0.303	5	2.25	0.125	171	7.57	0.179	0.046	1.94	0.173	1.50	0.173
	1.65	5	2.29	0.221	186	11.3	0.272	0.043	2.13	0.289	1.58	0.247
P. clarkii	0.000	10	2.36	0.175	141	9.81	0.017	0.011	2.21	0.286	1.30	0.058
	0.099	6	1.91	0.267	196	59.6	0.108	0.050	2.03	0.307	1.50	0.126
	0.631	6	1.53	0.146	140	7.83	0.129	0.036	2.34	0.419	1.77	0.221
	3.50	7	1.93	0.195	601	413	1.47	0.600	2.29	0.244	1.75	0.214
	17.4	2	2.47	0.135	152	12.4	1.86	1.38	2.41	0.798	1.29	0.255

Table 3.1 (continued). Chemical residues of various crayfish tissues (mg/kg). Cd concentrations are given in mg Cd/L and n= number of tissue samples dissected for a particular tissue and species.

	Cd Conc.	n=	Avg. Mass	Std. Error	Ca	Std. Error	Cd	Std. Error	Cu	Std. Error	Fe	Std. Error
Ovary tissue residue												
O. juvenilis	0.141	6	0.184	0.057	1140	538	2.30	1.63	56.5	30.2	25.1	11.9
	0.476	7	0.096	0.025	2610	1090	4.70	1.74	40.5	6.88	18.6	3.83
	0.826	9	0.206	0.043	690	142	1.12	0.347	27.7	3.50	15.7	5.62
	1.94	4	0.271	0.149	563	72.7	1.43	0.420	31.3	6.97	10.4	1.94
	5.78	2	0.375	0.126	536	4.13	1.79	1.20	27.0	0.573	12.4	0.897
O. placidus	0.000	1	0.034	-	550	-	0.157	-	4.99	-	14.2	-
	0.022	4	0.046	0.008	228	40.9	0.557	0.647	5.84	0.555	13.3	1.90
	0.128	4	0.054	0.024	525	228	1.64	1.21	6.91	2.76	17.1	1.52
	0.617	4	0.044	0.012	251	30.7	1.03	0.263	8.10	1.32	12.3	1.60
P. acutus	0.000	2	0.008	0.002	321	69.8	0.225	0.259	3.13	1.05	33.9	6.40
	0.049	2	0.008	0.003	392	66.6	4.77	0.873	5.00	3.39	57.9	8.70
	0.303	2	0.021	0.011	414	24.5	6.65	5.23	4.94	2.16	45.9	23.9
	1.65	2	0.008	0.002	472	77.2	2.04	1.54	6.74	1.06	89.0	33.7
P. clarkii	0.000	6	0.396	0.260	324	64.9	0.094	0.061	17.2	10.7	11.7	2.12
	0.099	1	0.089	-	243	-	0.119	-	7.86	-	10.5	-
	0.631	3	0.412	0.280	392	81.3	1.27	1.05	11.6	5.66	11.8	1.85
	3.50	3	0.307	0.084	325	126	2.61	1.11	5.63	2.54	9.47	2.16
	17.4	2	0.100	0.029	373	81.0	13.8	4.86	7.36	2.42	12.8	1.01

Table 3.1 (continued). Chemical residues of various crayfish tissues (mg/kg). Cd concentrations are given in mg Cd/L and n= number of tissue samples dissected for a particular tissue and species.

	Cd Conc.	n=	Avg. Mass	Std. Error	Ca	Std. Error	Cd	Std. Error	Cu	Std. Error	Fe	Std. Error
Shell and remnant tissue residue												
O. juvenilis	0.141	13	3.50	0.487	62800	3190	5.66	1.86	15.7	1.14	19.1	8.07
	0.476	13	3.60	0.383	58300	3700	6.97	0.899	20.2	2.45	12.0	2.43
	0.826	13	3.52	0.496	61900	2190	9.76	2.31	17.0	0.687	12.9	2.58
	1.94	9	4.98	0.548	62000	2270	13.5	2.95	15.6	1.49	14.0	2.56
	5.78	5	3.61	0.898	67900	3200	52.6	28.5	17.9	1.46	61.0	45.6
O. placidus	0.000	7	4.77	0.962	60200	4330	0.009	0.004	5.49	1.07	2.73	0.949
	0.022	6	4.11	0.730	58400	1740	1.82	1.12	8.71	0.842	3.75	0.831
	0.128	6	4.88	1.12	60100	1740	2.08	0.439	8.46	1.05	3.32	0.944
	0.617	6	3.76	0.194	54600	1450	7.20	2.03	11.9	1.30	8.81	2.40
P. acutus	0.000	5	6.63	0.949	33700	5210	0.007	0.003	2.85	0.450	1.77	0.104
	0.049	5	8.17	0.734	29300	4510	0.849	0.334	4.57	0.723	2.11	0.177
	0.303	5	8.32	0.520	36000	2340	1.76	0.191	5.57	0.482	3.88	1.52
	1.65	5	8.27	0.226	44100	3930	5.36	3.12	5.44	0.432	35.2	33.0
P. clarkii	0.000	6	12.1	1.37	52600	4600	0.003	0.002	4.90	0.468	10.9	5.28
	0.099	5	11.4	2.02	56600	9520	5.51	0.874	5.52	0.677	9.60	2.14
	0.631	6	10.8	1.37	48800	9010	7.78	2.61	6.25	0.616	28.7	16.6
	3.50	5	12.3	1.15	63700	3850	22.4	6.55	6.09	0.614	44.1	15.8
	17.4	2	14.9	1.21	53600	510	48.9	31.9	7.47	2.84	47.6	40.2

Table 3.1 (continued). Chemical residues of various crayfish tissues (mg/kg). Cd concentrations are given in mg Cd/L and n= number of tissue samples dissected for a particular tissue and species.

	Cd Conc.	n=	Avg. Mass	Std. Error	Ca	Std. Error	Cd	Std. Error	Cu	Std. Error	Fe	Std. Error
Testes tissue residue												
O. placidus	0.000	5	0.013	0.003	344	97.9	0.494	0.251	4.15	1.06	25.4	5.06
	0.128	1	0.022	-	221	-	7.02	-	3.40	-	10.1	-
	0.617	1	0.003	-	1990	-	8.03	-	6.63	-	62.5	-
P. acutus	0.000	2	0.008	0.003	296	66.6	0.994	0.556	2.38	0.870	53.8	2.36
	0.049	3	0.013	0.004	462	205	1.75	0.692	4.66	0.949	123	98.8
	0.303	3	0.010	0.001	270	18.0	4.76	1.52	6.18	0.840	61.0	23.0
	1.65	2	0.008	0.002	575	341	10.5	2.21	10.4	5.39	85.3	4.66
P. clarkii	0.000	4	0.012	0.002	351	83.6	0.413	0.132	3.99	1.61	89.9	60.4
	0.099	5	0.015	0.004	670	251	3.99	3.08	11.0	4.37	42.1	14.8
	0.631	3	0.007	0.001	823	247	3.49	1.69	11.6	2.49	81.2	15.2
	3.50	3	0.013	0.004	1030	554	30.6	10.6	11.3	0.873	62.3	27.4

Table 3.1 (continued). Chemical residues of various crayfish tissues (mg/kg). Cd concentrations are given in mg Cd/L and n= number of tissue samples dissected for a particular tissue and species.

	Cd Conc.	n=	Avg. Mass	Std. Error	Ca	Std. Error	Cd	Std. Error	Cu	Std. Error	Fe	Std. Error
Whole body residue												
O. juvenilis	0.000	6	3.86	0.373	54800	4080	0.018	0.001	30.6	1.94	5.95	0.660
	0.141	13	4.26	0.591	51700	2640	5.42	1.51	36.4	3.29	20.3	6.63
	0.476	12	4.66	0.500	44700	2420	7.34	1.03	29.1	2.72	12.2	1.74
	0.826	12	4.19	0.582	48900	2010	9.51	1.93	33.9	2.47	16.0	3.02
	1.94	9	5.98	0.677	52000	2580	14.7	2.74	32.7	2.35	15.6	2.50
	5.78	5	4.47	1.05	54400	2710	49.6	22.1	30.7	2.82	54.7	36.7
O. placidus	0.000	7	5.78	1.11	49600	3910	0.041	0.006	11.3	1.03	5.03	0.787
	0.022	6	5.24	0.912	45800	1350	2.98	0.716	11.0	1.20	6.64	0.550
	0.128	6	5.99	1.29	48300	1230	4.96	0.881	11.4	1.38	5.01	0.702
	0.617	6	4.75	0.235	43300	1290	12.9	1.37	14.2	1.47	9.32	1.59
P. acutus	0.000	4	12.0	1.69	22000	2520	0.090	0.057	7.17	0.420	8.89	1.03
	0.049	5	13.5	1.07	17800	2600	2.89	1.05	7.22	1.05	6.72	1.12
	0.303	5	13.5	0.689	22300	1590	7.14	1.53	8.80	0.526	8.78	1.92
	1.65	5	13.3	0.456	28100	3600	9.74	2.60	8.07	0.572	31.5	26.1
P. clarkii	0.000	6	18.5	2.55	34900	5160	0.026	0.005	7.74	1.06	10.5	3.22
	0.099	5	16.0	2.48	40300	7170	4.58	0.493	7.62	1.18	11.0	2.20
	0.631	6	15.2	1.73	35500	6750	5.56	0.807	9.05	0.996	36.1	19.1
	3.50	5	16.8	1.64	47400	3890	21.9	4.96	7.71	1.20	39.5	12.3
	17.4	2	22.1	0.628	36100	1550	48.8	7.25	9.24	3.26	35.3	23.8

Table 3.1 (continued). Chemical residues of various crayfish tissues (mg/kg). Cd concentrations are given in mg Cd/L and n= number of tissue samples dissected for a particular tissue and species.

	Cd Conc.	n=	Avg. Mass	Std. Error	Mg	Std. Error	P	Std. Error	Sr	Std. Error	Zn	Std. Error
Calcium stone tissue residue												
O. juvenilis	0.476	1	0.008	-	4740	-	13000	-	98.9	-	21.9	-
	0.826	1	0.001	-	3760	-	10900	-	443	-	97.7	-
O. placidus	0.022	1	0.007	-	4850	-	13100	-	466	-	17.8	-
P. clarkii	0.099	1	0.022	-	1830	-	11700	-	1410	-	57.3	-
	0.631	1	0.318	-	1520	-	7850	-	1490	-	13.8	-
Green gland tissue residue												
O. juvenilis	0.141	13	0.011	0.002	240	30.0	3030	397	1.78	0.585	44.3	14.6
	0.476	12	0.012	0.001	300	45.5	2550	182	2.69	0.841	34.7	3.87
	0.826	13	0.014	0.002	244	36.5	2540	165	1.23	0.404	33.2	7.20
	1.94	9	0.019	0.002	212	11.7	1910	75.5	1.05	0.169	20.7	1.67
	5.78	5	0.014	0.003	302	120	2540	395	1.57	0.558	39.9	10.1
O. placidus	0.000	7	0.022	0.006	183	25.4	2010	107	1.26	0.195	20.3	3.55
	0.022	6	0.025	0.004	175	11.9	1980	89.0	1.13	0.149	30.7	6.29
	0.128	6	0.023	0.006	231	44.8	2540	340	1.89	0.640	24.5	3.93
	0.617	6	0.023	0.002	240	18.9	2240	86.1	1.11	0.187	23.5	3.22
P. acutus	0.000	5	0.037	0.003	129	7.14	1850	68.8	0.660	0.080	14.2	0.578
	0.049	5	0.069	0.005	130	3.86	1760	61.0	0.718	0.113	14.9	1.11
	0.303	5	0.074	0.008	126	4.67	1780	92.0	0.706	0.042	15.7	1.77
	1.65	5	0.060	0.008	130	10.5	1790	145	0.700	0.073	15.2	1.45
P. clarkii	0.000	10	0.064	0.006	144	10.2	1830	96.1	1.27	0.122	12.8	1.03
	0.099	6	0.054	0.004	166	18.3	2010	48.9	1.74	0.421	20.9	4.04
	0.631	6	0.050	0.003	195	19.0	2080	65.3	2.11	0.356	14.3	0.851
	3.50	6	0.054	0.010	270	98.3	1950	163	8.44	6.01	19.1	5.33
	17.4	2	0.070	0.014	135	4.67	1680	62.0	1.61	0.047	18.7	0.399

Table 3.1 (continued). Chemical residues of various crayfish tissues (mg/kg). Cd concentrations are given in mg Cd/L and n= number of tissue samples dissected for a particular tissue and species.

	Cd Conc.	n=	Avg. Mass	Std. Error	Mg	Std. Error	P	Std. Error	Sr	Std. Error	Zn	Std. Error
Gill tissue residue												
O. juvenilis	0.141	13	0.123	0.018	120	8.21	926	66.1	1.93	0.198	12.0	0.879
	0.476	13	0.128	0.018	188	42.0	1040	95.0	2.96	0.559	17.6	3.15
	0.826	13	0.132	0.022	144	9.26	1060	96.3	2.13	0.339	13.2	1.90
	1.94	9	0.151	0.020	116	6.92	973	49.2	1.49	0.109	8.97	0.856
	5.78	5	0.157	0.047	90.0	10.3	815	54.1	2.57	0.483	10.7	1.62
O. placidus	0.000	7	0.111	0.015	124	6.60	784	52.5	4.55	0.593	9.56	0.797
	0.022	6	0.102	0.030	126	15.6	1020	117	4.06	0.939	9.11	1.05
	0.128	6	0.097	0.020	154	25.3	958	84.4	4.04	0.869	12.5	2.25
	0.617	6	0.081	0.010	190	22.2	1080	73.7	4.25	0.962	9.26	1.07
P. acutus	0.000	5	0.341	0.034	73.5	3.27	606	49.1	2.62	0.369	6.90	0.751
	0.049	5	0.427	0.032	84.1	2.35	651	28.3	3.05	0.348	8.80	0.932
	0.303	5	0.429	0.016	82.3	4.22	647	37.7	3.10	0.230	8.11	1.12
	1.65	5	0.398	0.021	85.7	3.35	729	44.9	3.37	0.382	7.99	0.645
P. clarkii	0.000	10	0.707	0.132	64.9	4.41	686	46.3	5.83	0.651	5.83	0.593
	0.099	6	0.507	0.139	85.3	12.8	689	41.4	5.95	0.571	8.68	1.42
	0.631	6	0.348	0.042	107	11.5	767	44.3	7.96	0.474	18.2	9.79
	3.50	7	0.479	0.118	105	12.7	758	47.0	9.49	1.33	7.58	0.674
	17.4	2	0.496	0.063	92.6	15.5	793	41.1	7.41	0.809	4.58	0.335

Table 3.1 (continued). Chemical residues of various crayfish tissues (mg/kg). Cd concentrations are given in mg Cd/L and n= number of tissue samples dissected for a particular tissue and species.

	Cd Conc.	n=	Avg. Mass	Std. Error	Mg	Std. Error	P	Std. Error	Sr	Std. Error	Zn	Std. Error
Hemolymph tissue residue												
P. acutus	0.000	5	1.43	0.141	33.9	3.39	37.0	6.45	1.26	0.143	0.930	0.151
	0.049	5	1.37	0.181	70.6	3.21	54.8	9.71	1.71	0.105	1.96	0.337
	0.303	5	1.30	0.096	60.5	11.8	53.4	6.98	1.86	0.210	1.21	0.150
	1.65	5	1.12	0.098	46.3	3.44	63.4	3.93	1.70	0.167	0.958	0.103
P. clarkii	0.000	10	1.24	0.136	13.4	3.38	52.6	5.44	1.98	0.148	1.42	0.148
	0.099	5	0.975	0.060	35.4	8.59	43.9	5.95	2.65	0.097	1.94	0.670
	0.631	6	0.902	0.176	46.1	3.94	68.8	12.0	2.80	0.412	1.97	0.233
	3.50	5	0.735	0.077	43.2	6.98	55.9	7.04	3.93	0.394	1.85	0.603
	17.4	2	0.862	0.004	36.4	5.89	56.2	15.6	3.39	0.172	1.75	0.614

Table 3.1 (continued). Chemical residues of various crayfish tissues (mg/kg). Cd concentrations are given in mg Cd/L and n= number of tissue samples dissected for a particular tissue and species.

	Cd Conc.	n=	Avg. Mass	Std. Error	Mg	Std. Error	P	Std. Error	Sr	Std. Error	Zn	Std. Error
Hepatopancreas tissue residue												
O. juvenilis	0.141	13	0.218	0.036	88.5	5.24	1300	64.9	0.785	0.160	29.2	3.96
	0.476	12	0.247	0.023	113	16.3	1190	125	2.37	0.706	26.7	4.38
	0.826	12	0.235	0.030	101	8.70	1330	164	0.898	0.162	29.3	3.40
	1.94	9	0.303	0.050	88.9	5.94	1130	44.4	0.708	0.147	25.5	6.18
	5.78	5	0.233	0.031	77.8	5.40	1090	62.7	1.22	0.357	19.3	3.53
O. placidus	0.000	7	0.100	0.024	154	19.1	1840	173	1.62	0.222	286	56.6
	0.022	6	0.121	0.022	183	27.2	2180	118	1.84	0.499	151	43.9
	0.128	6	0.102	0.028	204	38.9	2340	257	1.68	0.304	160	70.5
	0.617	6	0.091	0.011	181	8.38	2440	80.6	1.90	0.316	82.7	43.5
P. acutus	0.000	5	0.308	0.051	78.0	5.34	1330	94.3	0.607	0.058	59.0	12.7
	0.049	5	0.302	0.045	95.3	3.58	1550	80.6	0.789	0.154	61.1	10.7
	0.303	5	0.359	0.020	84.8	7.48	1390	160	0.676	0.135	61.2	9.58
	1.65	5	0.368	0.049	112	7.49	1670	82.7	0.732	0.105	32.7	7.94
P. clarkii	0.000	10	0.801	0.102	51.7	5.35	949	102	1.45	0.611	54.7	18.2
	0.099	6	0.684	0.277	85.4	20.1	1070	225	1.47	0.317	38.9	2.43
	0.631	6	0.491	0.101	53.6	7.72	835	99.9	0.775	0.069	26.8	6.89
	3.50	7	0.621	0.236	94.7	30.9	967	113	6.27	3.87	21.6	3.88
	17.4	2	0.897	0.197	53.3	1.02	792	49.7	0.875	0.061	22.2	4.41

Table 3.1 (continued). Chemical residues of various crayfish tissues (mg/kg). Cd concentrations are given in mg Cd/L and n= number of tissue samples dissected for a particular tissue and species.

	Cd Conc.	n=	Avg. Mass	Std. Error	Mg	Std. Error	P	Std. Error	Sr	Std. Error	Zn	Std. Error
Muscle tissue residue												
O. juvenilis	0.141	13	0.327	0.046	281	10.0	2480	123	0.605	0.123	12.8	0.525
	0.476	13	0.505	0.072	310	25.6	2290	143	2.13	0.790	13.0	1.32
	0.826	13	0.415	0.071	281	10.3	2790	133	0.704	0.194	11.2	0.545
	1.94	9	0.409	0.049	252	8.21	2470	106	0.413	0.039	10.8	0.564
	5.78	5	0.309	0.048	266	13.2	2520	154	0.510	0.122	11.4	0.691
O. placidus	0.000	7	0.484	0.067	301	23.4	2450	124	0.598	0.112	10.4	0.381
	0.022	6	0.627	0.098	345	41.0	2490	218	0.555	0.164	11.9	1.04
	0.128	6	0.649	0.090	376	28.4	2600	66.7	0.432	0.030	10.3	0.269
	0.617	6	0.597	0.041	400	19.4	2710	86.8	0.466	0.145	9.92	0.268
P. acutus	0.000	4	1.95	0.311	411	14.8	2490	43.9	0.190	0.008	8.80	0.233
	0.049	5	2.45	0.126	280	7.93	2460	34.9	0.267	0.042	8.80	0.282
	0.303	5	2.25	0.125	315	30.0	2500	117	0.226	0.017	9.39	0.200
	1.65	5	2.29	0.221	390	42.7	2670	81.1	0.257	0.022	9.54	0.244
P. clarkii	0.000	10	2.36	0.175	243	18.2	2330	94.0	0.597	0.111	9.34	0.402
	0.099	6	1.91	0.267	363	39.5	1880	339	0.714	0.371	9.91	0.913
	0.631	6	1.53	0.146	238	19.3	2210	189	0.476	0.064	9.14	0.400
	3.50	7	1.93	0.195	267	25.1	1980	125	3.37	2.63	9.50	0.869
	17.4	2	2.47	0.135	241	4.33	2440	75.3	0.647	0.038	9.85	0.639

Table 3.1 (continued). Chemical residues of various crayfish tissues (mg/kg). Cd concentrations are given in mg Cd/L and n= number of tissue samples dissected for a particular tissue and species.

	Cd Conc.	n=	Avg. Mass	Std. Error	Mg	Std. Error	P	Std. Error	Sr	Std. Error	Zn	Std. Error
Ovary tissue residue												
O. juvenilis	0.141	6	0.184	0.057	1130	560	9350	4610	1.35	0.415	79.0	39.6
	0.476	7	0.096	0.025	260	58.6	2450	610	3.02	0.796	22.1	3.55
	0.826	9	0.206	0.043	457	74.7	3670	561	0.829	0.216	31.1	4.59
	1.94	4	0.271	0.149	373	100	3380	718	0.760	0.084	24.7	4.68
	5.78	2	0.375	0.126	634	31.3	5170	276	0.789	0.300	38.4	1.17
O. placidus	0.000	1	0.034	-	246	-	2970	-	2.00	-	16.5	-
	0.022	4	0.046	0.008	200	30.2	3020	344	0.904	0.260	11.4	1.71
	0.128	4	0.054	0.024	309	37.4	3430	369	1.24	0.106	30.1	12.5
	0.617	4	0.044	0.012	233	32.4	2850	149	0.584	0.111	10.9	2.89
P. acutus	0.000	2	0.008	0.002	165	1.55	2660	31.0	0.490	0.047	24.1	4.37
	0.049	2	0.008	0.003	208	9.12	3420	163	0.993	0.397	35.9	0.279
	0.303	2	0.021	0.011	230	3.89	3090	510	0.646	0.039	21.3	7.39
	1.65	2	0.008	0.002	220	32.8	2900	14.3	0.926	0.364	27.8	2.86
P. clarkii	0.000	6	0.396	0.260	315	127	2880	447	2.09	0.345	18.3	2.79
	0.099	1	0.089	-	164	-	2500	-	1.54	-	14.1	-
	0.631	3	0.412	0.280	321	88.5	3290	377	2.38	0.634	23.9	2.18
	3.50	3	0.307	0.084	290	158	2440	1100	2.43	1.07	17.2	7.56
	17.4	2	0.100	0.029	269	108	3370	722	2.44	0.654	18.5	5.68

Table 3.1 (continued). Chemical residues of various crayfish tissues (mg/kg). Cd concentrations are given in mg Cd/L and n= number of tissue samples dissected for a particular tissue and species.

	Cd Conc.	n=	Avg. Mass	Std. Error	Mg	Std. Error	P	Std. Error	Sr	Std. Error	Zn	Std. Error
Shell and remnant tissue residue												
O. juvenilis	0.141	13	3.50	0.487	799	27.4	3510	97.6	75.1	17.5	25.6	1.16
	0.476	13	3.60	0.383	751	38.2	3630	223	84.2	19.0	22.6	0.882
	0.826	13	3.52	0.496	752	21.4	3550	115	59.0	12.3	24.6	1.10
	1.94	9	4.98	0.548	813	46.7	3880	107	68.6	13.4	24.7	1.40
	5.78	5	3.61	0.898	758	26.0	3450	151	88.5	22.1	24.6	1.57
O. placidus	0.000	7	4.77	0.962	621	49.0	2770	256	215	32.7	14.6	0.941
	0.022	6	4.11	0.730	673	61.6	2800	224	192	45.3	16.7	1.72
	0.128	6	4.88	1.12	734	86.7	3110	309	163	24.2	15.6	1.48
	0.617	6	3.76	0.194	736	20.3	3050	156	119	34.1	17.4	0.704
P. acutus	0.000	5	6.63	0.949	509	81.2	3410	373	113	26.9	14.8	0.953
	0.049	5	8.17	0.734	432	70.6	2860	256	91.0	21.1	15.6	0.768
	0.303	5	8.32	0.520	555	52.1	3140	219	126	18.6	18.9	0.708
	1.65	5	8.27	0.226	611	93.6	3630	209	112	2.53	18.4	1.06
P. clarkii	0.000	6	12.1	1.37	854	95.8	3840	113	330	34.8	22.2	2.57
	0.099	5	11.4	2.02	866	184	3120	373	362	66.0	25.8	6.02
	0.631	6	10.8	1.37	931	160	3390	409	320	60.0	22.1	1.52
	3.50	5	12.3	1.15	1050	89.3	3460	221	444	28.8	24.3	2.44
	17.4	2	14.9	1.21	848	60.7	3050	8.02	370	3.32	22.4	2.35

Table 3.1 (continued). Chemical residues of various crayfish tissues (mg/kg). Cd concentrations are given in mg Cd/L and n= number of tissue samples dissected for a particular tissue and species.

	Cd Conc.	n=	Avg. Mass	Std. Error	Mg	Std. Error	P	Std. Error	Sr	Std. Error	Zn	Std. Error
Testes tissue residue												
O. placidus	0.000	5	0.013	0.003	289	33.7	4000	246	1.11	0.353	26.9	2.52
	0.128	1	0.022	-	289	-	3460	-	1.10	-	19.1	-
	0.617	1	0.003	-	706	-	5800	-	1.41	-	102	-
P. acutus	0.000	2	0.008	0.003	228	39.0	4030	352	0.415	0.054	37.1	12.6
	0.049	3	0.013	0.004	301	108	3040	253	1.35	0.463	30.4	5.28
	0.303	3	0.010	0.001	208	11.1	3670	241	0.543	0.120	30.8	1.85
	1.65	2	0.008	0.002	234	63.0	2890	366	1.43	0.318	44.1	2.51
P. clarkii	0.000	4	0.012	0.002	221	14.5	3280	259	2.00	0.498	25.3	3.57
	0.099	5	0.015	0.004	215	22.6	2920	493	3.86	1.84	35.3	5.31
	0.631	3	0.007	0.001	323	18.7	4150	938	3.02	0.397	73.6	34.2
	3.50	3	0.013	0.004	241	39.4	3150	247	6.04	2.80	27.6	3.58

Table 3.1 (continued). Chemical residues of various crayfish tissues (mg/kg). Cd concentrations are given in mg Cd/L and n= number of tissue samples dissected for a particular tissue and species.

	Cd Conc.	n=	Avg. Mass	Std. Error	Mg	Std. Error	P	Std. Error	Sr	Std. Error	Zn	Std. Error
Whole body residue												
O. juvenilis	0.000	6	3.86	0.373	634	34.6	3160	161	47.0	4.95	19.5	1.25
	0.141	13	4.26	0.591	700	21.8	3300	89.5	60.7	13.6	24.9	1.08
	0.476	12	4.66	0.500	631	32.3	3230	180	68.0	16.2	21.0	0.548
	0.826	12	4.19	0.582	643	22.2	3310	102	48.1	10.5	23.8	1.12
	1.94	9	5.98	0.677	714	43.4	3570	106	57.4	11.3	23.3	1.24
	5.78	5	4.47	1.05	652	25.1	3200	133	70.5	17.5	23.2	1.66
O. placidus	0.000	7	5.78	1.11	553	43.9	2640	218	177	27.2	23.1	1.77
	0.022	6	5.24	0.912	586	54.2	2660	176	151	35.9	22.7	2.68
	0.128	6	5.99	1.29	646	67.8	2950	251	130	18.7	19.7	2.95
	0.617	6	4.75	0.235	647	13.2	2910	126	95.7	28.0	18.4	1.40
P. acutus	0.000	4	12.0	1.69	410	54.3	2710	198	75.8	15.5	13.5	0.827
	0.049	5	13.5	1.07	328	39.9	2290	129	55.1	12.5	14.5	0.620
	0.303	5	13.5	0.689	409	33.7	2480	155	77.8	11.7	17.2	0.588
	1.65	5	13.3	0.456	473	68.4	2890	183	70.4	3.08	15.6	0.802
P. clarkii	0.000	6	18.5	2.55	614	89.6	3000	350	220	34.0	18.8	2.38
	0.099	5	16.0	2.48	672	132	2510	324	258	48.9	22.9	4.80
	0.631	6	15.2	1.73	722	115	2840	275	239	37.7	19.5	1.53
	3.50	5	16.8	1.64	830	80.7	2920	184	330	28.8	21.4	1.71
	17.4	2	22.1	0.628	620	27.9	2580	28.1	249	15.3	18.3	0.953

Table 3.2. Cadmium 96h Bioconcentration Factors (96h BCF) for various tissues and whole body. Exposure Cd concentrations are given in mg/L and tissue Cd concentrations are given in mg/kg.

Tissue	*O. juvenilis*			*O. placidus*			*P. acutus*			*P. clarkii*		
	Exposure Cd Conc.	Tissue Cd Conc.	96h BCF	Exposure Cd Conc.	Tissue Cd Conc.	96h BCF	Exposure Cd Conc.	Tissue Cd Conc.	96h BCF	Exposure Cd Conc.	Tissue Cd Conc.	96h BCF
Ca stones	0.476	3.78	7.94	0.022	0.262	11.9	0			0.099	0.398	4.02
	0.826	33.8	40.9							0.631	0.623	0.987
Green Glands	0.141	3.94	28.0	0.000	0.343	-	0.000	0.244	-	0.000	0.482	-
	0.476	11.9	25.0	0.022	3.50	159	0.049	14.1	288	0.099	1.15	11.6
	0.826	7.86	9.51	0.128	12.5	97.4	0.303	18.4	60.7	0.631	4.96	7.85
	1.94	11.5	5.94	0.617	14.3	23.2	1.65	9.76	5.91	3.50	24.2	6.91
	5.78	34.0	5.89							17.4	38.0	2.19
Gills	0.141	10.7	76.2	0.000	0.044	-	0.000	0.019	-	0.000	0.014	-
	0.476	27.9	58.7	0.022	5.84	266	0.049	5.44	111	0.099	6.00	60.6
	0.826	22.3	27.0	0.128	18.1	141	0.303	17.5	57.8	0.631	21.9	34.6
	1.94	33.9	17.5	0.617	30.1	48.8	1.65	33.4	20.2	3.50	61.8	17.7
	5.78	92.1	15.9							17.4	210	12.1
Hemolymph	*			*			0.000	0.006	-	0.000	0.024	-
							0.049	0.334	6.81	0.099	0.310	3.13
							0.303	0.532	1.76	0.631	0.541	0.858
							1.65	0.771	0.467	3.50	16.6	4.74
										17.4	21.4	1.23
Hepato-pancreas	0.141	8.40	59.6	0.000	0.874	-	0.000	2.60	-	0.000	0.299	-
	0.476	26.3	55.3	0.022	27.1	1230	0.049	45.6	931	0.099	10.9	110
	0.826	24.0	29.0	0.128	91.5	715	0.303	88.5	292	0.631	8.88	14.1
	1.94	49.3	25.4	0.617	177	287	1.65	78.1	47.3	3.50	46.5	13.3
	5.78	61.9	10.7							17.4	73.8	4.25

* Tissue not tested in this species

Table 3.2 (Continued).Cadmium 96h Bioconcentration Factors (96h BCF) for various tissues and whole body. Exposure Cd concentrations are given in mg/L and tissue Cd concentrations are given in mg/kg.

Tissue	*O. juvenilis*			*O. placidus*			*P. acutus*			*P. clarkii*		
	Exposure Cd Conc.	Tissue Cd Conc.	96h BCF	Exposure Cd Conc.	Tissue Cd Conc.	96h BCF	Exposure Cd Conc.	Tissue Cd Conc.	96h BCF	Exposure Cd Conc.	Tissue Cd Conc.	96h BCF
Muscle	0.141	0.610	4.32	0.000	0.026	-	0.000	0.004	-	0.000	0.017	-
	0.476	1.36	2.86	0.022	0.208	9.45	0.049	0.127	2.60	0.099	0.108	1.09
	0.826	0.774	0.937	0.128	0.452	3.53	0.303	0.179	0.590	0.631	0.129	0.205
	1.94	2.05	1.06	0.617	0.690	1.12	1.65	0.272	0.164	3.50	1.47	0.422
	5.78	3.91	0.677							17.4	1.86	0.107
Ovary	0.141	2.30	16.3	0.000	0.157	-	0.000	0.225	-	0.000	0.094	-
	0.476	4.70	9.88	0.022	0.557	25.3	0.049	4.77	97.3	0.099	0.119	1.20
	0.826	1.12	1.35	0.128	1.64	12.8	0.303	6.65	21.9	0.631	1.27	2.02
	1.94	1.43	0.736	0.617	1.03	1.67	1.65	2.04	1.24	3.50	2.61	0.748
	5.78	1.79	0.311							17.4	13.8	0.792
Shell & remnant	0.141	5.66	40.1	0.000	0.009	-	0.000	0.007	-	0.000	0.003	-
	0.476	6.97	14.6	0.022	1.82	82.9	0.049	0.849	17.3	0.099	5.51	55.7
	0.826	9.76	11.8	0.128	2.08	16.3	0.303	1.76	5.81	0.631	7.78	12.3
	1.94	13.5	6.98	0.617	7.20	11.7	1.65	5.36	3.24	3.50	22.4	6.42
	5.78	52.6	9.10							17.4	48.9	2.81
Testes	*			0.000	0.494	-	0.000	0.994	-	0.000	0.413	-
				0.128	7.02	54.8	0.049	1.75	35.7	0.099	3.99	40.3
				0.617	8.03	13.0	0.303	4.76	15.7	0.631	3.49	5.54
							1.65	10.5	6.33	3.50	30.6	8.74

* Tissue not tested in this species

Table 3.2 (Continued).Cadmium 96h Bioconcentration Factors (96h BCF) for various tissues and whole body. Exposure Cd concentrations are given in mg/L and tissue Cd concentrations are given in mg/kg.

Tissue	*O. juvenilis*			*O. placidus*			*P. acutus*			*P. clarkii*		
	Exposure Cd Conc.	Tissue Cd Conc.	96h BCF	Exposure Cd Conc.	Tissue Cd Conc.	96h BCF	Exposure Cd Conc.	Tissue Cd Conc.	96h BCF	Exposure Cd Conc.	Tissue Cd Conc.	96h BCF
Whole Body	0.000	0.018	-	0.000	0.041	-	0.000	0.090	-	0.000	0.026	-
	0.141	5.42	38.4	0.022	2.98	136	0.049	2.89	59.0	0.099	4.58	46.3
	0.476	7.34	15.4	0.128	4.96	38.8	0.303	7.14	23.6	0.631	5.56	8.82
	0.826	9.51	11.5	0.617	12.9	20.8	1.65	9.74	5.90	3.50	21.9	6.27
	1.94	14.7	7.58							17.4	48.8	2.81
	5.78	49.6	8.58									

Table 3.3. Order of 96h BCF values for individual tissues listed from highest to lowest within species.

Species: Concentration examined	Order of 96h BCFs
O. juvenilis: 0.141 mg Cd/L	Gills > Hepatopancreas > Shell & remnant > Whole body > Green glands > Ovary > Muscle
O. placidus: 0.022 mg Cd/L	Hepatopancreas > Gills > Green glands > Whole body > Shell & remnant > Ovary > Muscle
P. acutus: 0.049 mg Cd/L	Hepatopancreas > Green glands > Gills > Ovary > Whole body > Testes > Shell & remnant > Hemolymph > Muscle
P. clarkii: 0.099 mg Cd/L	Hepatopancreas > Gills > Shell & remnant >Whole body > Testes > Green glands > Hemolymph > Ovary > Muscle

Table 3.4. 96h BCF values estimated using regression to model consistent concentrations of cadmium.

		Hypothetical Cd concentrations (mg Cd/L)				
	Model equation	0.141	0.303	0.476	0.617	0.631
Green Gland 96h BCF values						
O. juvenilis	y=10.2+2.60lnx	36.3	23.5	17.4	14.5	14.3
O. placidus	y=17.0+3.28lnx	75.3	43.3	30.7	25.0	24.6
P. acutus	y=12.6-1.20lnx	72.8	36.9	24.6	19.5	19.1
P. clarkii	y=5.90+2.05lnx	13.3	11.4	9.19	7.96	7.85
Gill 96h BCF values						
O. juvenilis	y=36.4+16.2lnx	33.3	56.3	51.2	46.3	45.9
O. placidus	y=33.4+7.28lnx	136	81.7	58.9	48.5	47.7
P. acutus	y=28.6+7.93lnx	92.6	63.1	47.7	40.1	39.5
P. clarkii	y=38.6+15.7lnx	55.1	65.3	56.5	50.2	49.7
Hemolymph 96h BCF values						
P. acutus	y=0.699+0.124lnx	3.23	1.82	1.27	1.04	1.02
P. clarkii	y=0.599+0.125lnx	2.51	1.48	1.06	0.873	0.858
Hepatopancreas 96h BCF values						
O. juvenilis	y=35.0+14.4lnx	47.4	58.5	50.9	45.4	44.9
O. placidus	y=193+44.8lnx	749	462	336	278	274
P. acutus	y=82.4+9.43lnx	453	235	158	126	124
P. clarkii	y=29.4+11.8lnx	44.4	50.5	43.3	38.4	38.0
Muscle 96h BCF values						
O. juvenilis	y=1.70+0.712lnx	2.14	2.79	2.45	2.19	2.17
O. placidus	y=0.756+0.144lnx	3.35	1.92	1.36	1.11	1.09
P. acutus	y=0.134+0.011lnx	1.16	0.641	0.447	0.362	0.355
P. clarkii	y=0.776+0.385lnx	0.794	0.399	0.265	0.209	0.205

Table 3.4 (Continued). 96h BCF values estimated using regression to model consistent concentrations of camdium.

		Hypothetical Cd concentrations (mg Cd/L)				
	Model equation	0.141	0.303	0.476	0.617	0.631
Ovary 96h BCF values						
O. juvenilis	y=1.78-0.347lnx	17.4	7.23	4.27	3.15	3.07
O. placidus	y=1.28+0.035lnx	8.57	4.08	2.63	2.04	2.00
P. acutus	y=7.88+1.03lnx	41.6	21.9	15.0	12.0	11.7
P. clarkii	y=1.68+0.719lnx	1.90	2.70	2.40	2.15	2.13
Shell and remnant 96h BCF values						
O. juvenilis	y=10.5+2.88lnx	34.4	23.3	17.6	14.8	14.5
O. placidus	y=7.44+1.96lnx	25.6	16.8	12.6	10.5	10.4
P. acutus	y=4.23+1.27lnx	12.3	8.94	6.90	5.86	5.77
P. clarkii	y=16.7+7.17lnx	19.0	27.0	24.0	21.5	21.3
Testes 96h BCF values						
O. placidus	y=8.35+0.645lnx	50.2	25.0	16.5	13.0	12.8
P. acutus	y=1.31+5.83x	15.1	10.1	8.57	7.94	7.89
P. clarkii	y=0.816+8.40x	14.2	11.1	10.1	9.72	9.70
Whole body 96h BCF values						
O. juvenilis	y=11.2+3.44lnx	31.4	23.3	18.1	15.4	15.2
O. placidus	y=13.1+2.93lnx	52.5	31.8	23.0	19.0	18.7
P. acutus	y=9.01+1.95lnx	36.7	22.0	15.9	13.1	12.8
P. clarkii	y=13.1+4.80lnx	36.7	29.3	23.2	19.9	19.7

Table 3.5. Averaged 96h BCF estimates for sensitive species and tolerant species. 96h BCF estimates are derived from the regression equations in Table 3.4.

	Hypothetical Cd concentrations (mg Cd/L)				
Tissue	0.141	0.303	0.476	0.617	0.631
Green Gland					
Sensitive Species	74.0	40.1	27.7	22.3	21.9
Tolerant Species	24.8	17.4	13.3	11.2	11.1
Gills					
Sensitive Species	114	72.4	53.3	44.3	43.6
Tolerant Species	44.2	60.8	53.9	48.3	47.8
Hemolymph					
Sensitive Species *	3.23	1.82	1.27	1.04	1.02
Tolerant Species *	2.51	1.48	1.06	0.873	0.858
Hepatopancreas					
Sensitive Species	601	348	247	202	199
Tolerant Species	45.9	54.5	47.1	41.9	41.4
Muscle					
Sensitive Species	2.25	1.28	0.905	0.737	0.724
Tolerant Species	1.47	1.60	1.36	1.20	1.19
Ovary					
Sensitive Species	25.1	13.0	8.79	7.01	6.87
Tolerant Species	9.66	4.97	3.34	2.65	2.60
Shell and remnant					
Sensitive Species	18.9	12.9	9.74	8.19	8.07
Tolerant Species	26.7	25.1	20.8	18.1	17.9
Testes					
Sensitive Species	32.7	17.6	12.5	10.5	10.3
Tolerant Species *	14.2	11.1	10.1	9.72	9.70
Whole Body					
Sensitive Species	44.6	26.9	19.5	16.0	15.8
Tolerant Species	34.0	26.3	20.6	17.7	17.4

* Represents only one species

Table 3.6. Pearson correlation coefficients and p values of various tissue metal residues regressed against Cd water concentration. Underline inidcates p<0.05. Double underline indicates 0.05< p <0.10.

	Ca	Ca		Cd	Cd		Cu	Cu		Fe	Fe	
	r =	p =		r =	p =		r =	p =		r =	p =	
Green Gland												
O. juvenilis	-0.145	0.304		0.600	<0.001		0.012	0.934		0.070	0.624	
O. placidus	0.317	0.123		0.591	0.010	*	0.390	0.110	*	-0.079	0.707	
P. acutus	-0.028	0.922	*	0.038	0.893	*	0.095	0.690		0.600	0.018	
P. clarkii	0.201	0.395	*	0.801	<0.001	*	0.312	0.093		0.228	0.333	*
Gills												
O. juvenilis	-0.234	0.092	*	0.742	<0.001	*	0.171	0.222	*	0.112	0.712	
O. placidus	0.497	0.036	*	0.907	<0.001	*	0.557	0.004		0.418	0.084	*
P. acutus	0.141	0.552		0.820	<0.001	*	0.437	0.054		0.387	0.092	
P. clarkii	0.424	0.055	*	0.882	<0.001	*	0.370	0.040		0.243	0.290	*
Hemolymph												
P. acutus	0.241	0.305		0.573	0.025	*	0.535	0.040	*	0.554	0.011	
P. clarkii	0.638	0.004	*	0.816	<0.001	*	0.402	0.034		0.342	0.164	*
Hepatopancreas												
O. juvenilis	-0.081	0.574		0.584	<0.001	*	-0.133	0.354		-0.004	0.979	
O. placidus	-0.042	0.868	*	0.682	0.002	*	-0.237	0.254		-0.345	0.161	*
P. acutus	0.124	0.602		0.307	0.188		-0.561	0.030	*	-0.351	0.129	
P. clarkii	0.093	0.689	*	0.718	<0.001	*	0.102	0.659	*	0.096	0.680	*
Muscle												
O. juvenilis	-0.142	0.310		0.491	<0.001	*	-0.120	0.391		0.287	0.037	
O. placidus	0.413	0.040		0.749	<0.001	*	0.460	0.021		-0.212	0.398	*
P. acutus	0.297	0.216		0.602	0.006		0.556	0.031	*	0.202	0.406	
P. clarkii	0.178	0.441	*	0.758	<0.001	*	0.170	0.461	*	-0.038	0.838	

*Data was transformed using the natural logarithm (ln) prior to regression analysis.

Table 3.6 (continued). Pearson correlation coefficients and p values of various tissue metal residues regressed against Cd water concentration. Underline inidcates p<0.05. Double underline indicates 0.05< p <0.10.

	Ca	Ca		Cd	Cd		Cu	Cu		Fe	Fe	
	r =	p =		r =	p =		r =	p =		r =	p =	
Ovary												
O. juvenilis	-0.250	0.199	*	-0.123	0.533		-0.192	0.327	*	-0.269	0.166	*
O. placidus	-0.258	0.419		0.452	0.162	*	0.271	0.393		-0.288	0.364	
P. acutus	0.550	0.158		-0.500	0.313	*	0.438	0.278		0.628	0.095	
P. clarkii	0.093	0.740		0.909	<0.001		-0.193	0.619	*	0.093	0.741	
Shell & remanent												
O. juvenilis	0.188	0.177		0.567	<0.001		0.080	0.568	*	0.349	0.010	
O. placidus	-0.344	0.162	*	0.723	0.001	*	0.600	0.002		0.587	0.002	
P. acutus	0.617	0.014	*	0.687	0.005	*	0.404	0.077		0.391	0.089	
P. clarkii	0.112	0.658	*	0.726	0.001	*	0.343	0.100		0.448	0.062	*
Testes												
O. placidus	0.925	0.003		0.828	0.022		0.414	0.356		0.728	0.064	
P. acutus	0.317	0.373		0.858	0.001		0.666	0.036		0.166	0.694	*
P. clarkii	0.355	0.194		0.818	<0.001		0.206	0.462		0.314	0.346	*
Whole Body												
O. juvenilis	0.141	0.296		0.659	<0.001	*	-0.081	0.570	*	0.378	0.004	
O. placidus	-0.319	0.121		0.902	<0.001		0.393	0.052		0.545	0.005	
P. acutus	0.623	0.013	*	0.624	0.004		0.156	0.525		0.354	0.137	
P. clarkii	0.124	0.505		0.886	<0.001		0.081	0.748	*	0.451	0.061	*

*Data was transformed using the natural logarithm (ln) prior to regression analysis.

Table 3.6 (continued). Pearson correlation coefficients and p values of various tissue metal residues regressed against Cd water concentration. Underline inidcates p<0.05. Double underline indicates 0.05< p <0.10.

	Mg	Mg		P	P		Sr	Sr		Zn	Zn	
	r =	p =		r =	p =		r =	p =		r =	p =	
Green Gland												
O. juvenilis	0.057	0.687		-0.305	0.028	*	-0.091	0.521		-0.145	0.307	*
O. placidus	0.449	0.062	*	0.279	0.263	*	-0.091	0.664		-0.228	0.362	*
P. acutus	0.039	0.871		0.025	0.928	*	0.023	0.923		0.068	0.777	
P. clarkii	0.078	0.744	*	-0.372	0.106	*	0.276	0.239	*	0.131	0.489	
Gills												
O. juvenilis	-0.216	0.120		-0.171	0.220		0.004	0.975		-0.213	0.126	*
O. placidus	0.517	0.008		0.359	0.078		0.061	0.810	*	-0.064	0.763	
P. acutus	0.339	0.143		0.450	0.046		0.292	0.212		-0.158	0.573	*
P. clarkii	0.223	0.330	*	0.282	0.215	*	0.468	0.032	*	-0.251	0.273	*
Hemolymph												
P. acutus	-0.523	0.046	*	0.416	0.068		0.173	0.467		-0.687	0.005	*
P. clarkii	0.194	0.323		0.226	0.368	*	0.483	0.042	*	0.035	0.861	
Hepatopancreas												
O. juvenilis	-0.190	0.181		-0.183	0.198	*	-0.051	0.722		-0.208	0.143	*
O. placidus	0.070	0.741		0.375	0.065		0.099	0.638		-0.672	0.004	
P. acutus	0.629	0.003		0.402	0.079		0.072	0.762		-0.540	0.038	*
P. clarkii	-0.054	0.816	*	-0.114	0.541		0.211	0.358	*	-0.520	0.016	*
Muscle												
O. juvenilis	-0.196	0.160	*	0.087	0.536	*	-0.143	0.305		-0.273	0.048	*
O. placidus	0.398	0.049		0.296	0.233	*	-0.144	0.568	*	-0.524	0.026	*
P. acutus	0.590	0.021	*	0.466	0.044		0.197	0.420		0.525	0.045	*
P. clarkii	-0.435	0.048	*	0.251	0.273	*	0.318	0.160	*	0.057	0.759	

*Data was transformed using the natural logarithm (ln) prior to regression analysis.

Table 3.6 (continued). Pearson correlation coefficients and p values of various tissue metal residues regressed against Cd water concentration. Underline inidcates p<0.05. Double underline indicates 0.05< p <0.10.

	Mg	Mg		P	P		Sr	Sr		Zn	Zn	
	r =	p =		r =	p =		r =	p =		r =	p =	
Ovary												
O. juvenilis	-0.172	0.381	*	-0.187	0.341	*	-0.271	0.163	*	-0.257	0.187	*
O. placidus	0.280	0.404	*	-0.172	0.592		-0.615	0.033		-0.226	0.481	
P. acutus	0.384	0.348		-0.149	0.725		0.266	0.524		-0.341	0.508	*
P. clarkii	-0.049	0.863		0.124	0.659		0.116	0.682		-0.108	0.783	*
Shell & remanent												
O. juvenilis	-0.033	0.814	*	0.090	0.519	*	0.057	0.687		-0.045	0.748	*
O. placidus	0.237	0.255		0.212	0.398	*	-0.385	0.058		0.257	0.214	
P. acutus	0.455	0.088	*	0.560	0.030	*	0.351	0.199	*	0.538	0.039	*
P. clarkii	0.181	0.472	*	-0.170	0.427		0.159	0.530	*	-0.026	0.905	
Testes												
O. placidus	0.911	0.004		0.757	0.049		0.169	0.717		0.941	0.002	
P. acutus	-0.233	0.579	*	-0.427	0.219		0.358	0.310		0.639	0.088	*
P. clarkii	0.269	0.423	*	0.211	0.534	*	0.384	0.157		-0.091	0.748	
Whole Body												
O. juvenilis	-0.051	0.724	*	0.074	0.604	*	0.077	0.572		-0.109	0.448	*
O. placidus	0.258	0.301	*	0.255	0.308	*	-0.385	0.057		-0.310	0.132	
P. acutus	0.532	0.041	*	0.606	0.017	*	0.378	0.165	*	0.273	0.324	*
P. clarkii	0.163	0.518	*	0.195	0.439	*	0.207	0.409	*	-0.105	0.679	*

*Data was transformed using the natural logarithm (ln) prior to regression analysis.

Table 3.7. Pearson correlation coefficients and p values for various tissue metal residues regressed against Cd tissue residue. Underline indicates p<0.05. Double underline indicates 0.05< p <0.10.

	Ca	Ca		Cu	Cu		Fe	Fe		Mg	Mg		P	P		Zn	Zn	
	r =	p =		r =	p =		r =	p =		r =	p =		r =	p =		r =	p =	
Green gland																		
O. juvenilis	0.206	0.144	*	0.328	0.018	*	0.336	0.015	*	0.358	0.009	*	0.095	0.501	*	0.143	0.313	*
O. placidus	0.715	<0.001	*	0.854	<0.001		0.105	0.617	*	0.474	0.017	*	0.469	0.018	*	0.336	0.100	*
P. acutus	0.298	0.201	*	0.577	0.008	*	0.231	0.326	*	0.225	0.340		-0.246	0.295		0.345	0.136	
P. clarkii	0.454	0.013	*	0.846	<0.001		0.209	0.276		0.408	0.028	*	0.216	0.261	*	0.453	0.014	*
Gills																		
O. juvenilis	-0.081	0.563		0.290	0.035	*	0.328	0.016	*	0.094	0.501	*	0.175	0.211	*	0.121	0.388	*
O. placidus	0.495	0.014		0.695	<0.001	*	0.331	0.114	*	0.530	0.008		0.460	0.024	*	0.271	0.200	
P. acutus	0.377	0.112		0.647	0.003	*	0.681	0.001		0.580	0.009	*	0.536	0.018		0.317	0.186	*
P. clarkii	0.327	0.078	*	0.448	0.013	*	0.400	0.029		0.597	<0.001	*	0.254	0.176	*	0.355	0.054	
Hemolymph																		
P. acutus	0.779	<0.001	*	0.669	0.001	*	0.639	0.002	*	0.500	0.025	*	0.545	0.013	*	0.259	0.270	*
P. clarkii	0.837	<0.001	*	0.575	0.001		0.614	0.001	*	0.627	<0.001	*	-0.079	0.689		0.517	0.005	
Hepatopancreas																		
O. juvenilis	-0.108	0.451		0.129	0.368	*	-0.132	0.356		-0.115	0.422		-0.122	0.394		0.087	0.543	
O. placidus	0.285	0.168	*	-0.198	0.342	*	-0.227	0.275	*	0.185	0.376	*	0.493	0.012	*	-0.435	0.030	*
P. acutus	0.564	0.010	*	-0.344	0.138	*	-0.493	0.027	*	0.474	0.035	*	0.237	0.314	*	0.268	0.254	
P. clarkii	0.242	0.190	*	-0.104	0.579		0.111	0.552	*	0.381	0.034	*	0.155	0.404	*	-0.312	0.088	*
Muscle																		
O. juvenilis	0.257	0.063		0.255	0.065		0.239	0.085	*	0.121	0.386		0.013	0.924	*	-0.092	0.514	*
O. placidus	0.328	0.109	*	0.477	0.016	*	-0.127	0.544		0.392	0.053		0.116	0.582	*	-0.193	0.355	
P. acutus	0.782	<0.001	*	0.511	0.025		0.151	0.536		-0.361	0.129	*	0.091	0.710	*	0.476	0.039	*
P. clarkii	0.366	0.043	*	0.169	0.363		0.452	0.011	*	-0.164	0.378		-0.212	0.252	*	0.118	0.528	*

*Data was transformed using the natural logarithm (ln) prior to regression analysis.

Table 3.7 (continued). Pearson correlation coefficients and p values for various tissue metal residues regressed against Cd tissue residue. Underline indicates p≤0.05. Double underline indicates 0.05< p ≤0.10.

	Ca	Ca		Cu	Cu		Fe	Fe		Mg	Mg		P	P		Zn	Zn	
	r =	p =		r =	p =		r =	p =		r =	p =		r =	p =		r =	p =	
Ovary																		
O. juvenilis	0.502	0.006	*	0.471	0.011		0.563	0.002		0.341	0.075		0.368	0.054		0.389	0.041	
O. placidus	0.872	<0.001		-0.277	0.410		0.298	0.374		-0.207	0.541		0.060	0.862	*	0.829	0.002	
P. acutus	-0.172	0.713		0.520	0.232		0.142	0.761	*	0.809	0.028	*	0.785	0.036		0.332	0.467	
P. clarkii	0.397	0.202	*	0.132	0.683	*	0.155	0.630	*	0.252	0.429	*	0.193	0.548	*	0.143	0.658	*
Shell & remnant																		
O. juvenilis	0.418	0.002	*	0.054	0.699		0.902	<0.001		0.140	0.316	*	0.084	0.549		0.362	0.008	*
O. placidus	-0.253	0.232		0.685	<0.001	*	0.801	<0.001		0.354	0.089	*	0.295	0.161		0.484	0.017	
P. acutus	0.633	0.011		0.398	0.142	*	0.983	<0.001		0.695	0.004		0.549	0.034		0.730	0.002	*
P. clarkii	0.185	0.398		0.326	0.129	*	0.574	0.004		0.136	0.535		-0.243	0.263	*	0.151	0.493	
Testes																		
O. placidus	0.627	0.132		0.150	0.748		0.331	0.468		0.650	0.114		0.369	0.415		0.606	0.149	
P. acutus	-0.071	0.867	*	0.234	0.576		0.314	0.449	*	-0.252	0.546		0.323	0.435	*	0.518	0.189	
P. clarkii	-0.307	0.388		-0.193	0.594	*	0.526	0.118	*	-0.480	0.160		0.389	0.267	*	-0.244	0.497	
Whole body																		
O. juvenilis	0.235	0.079		0.108	0.425	*	0.865	<0.001		0.133	0.323	*	0.137	0.308	*	0.399	0.002	*
O. placidus	-0.301	0.144		0.195	0.350		0.540	0.005		0.238	0.252	*	0.236	0.256		-0.287	0.164	*
P. acutus	0.455	0.050		0.275	0.254		0.519	0.023		0.419	0.074		0.220	0.366		0.647	0.003	
P. clarkii	0.253	0.234		0.163	0.446		0.509	0.011		0.244	0.251		-0.143	0.505	*	0.207	0.332	*

*Data was transformed using the natural logarithm (ln) prior to regression analysis.

Chapter 4: Effects of cadmium and body mass on the anti-predator behaviors of five species of crayfish and assessment of heart rate as a physiological marker of cadmium stress.

4.1 Summary

Five crayfish species (*Orconectes placidus*, *O. virilis*, *Procambarus acutus*, *P. alleni*, and *P. clarkii*) were subjected to Cd exposure in 96h acute tests. The tail-flip predator avoidance behavior was significantly affected by Cd exposure in each case (ANOVA $p<0.05$). At least one exposure concentration showed a significantly reduced response compared to controls in four of five species tested (Tukey-HSD $p<0.05$). In three of the five species tested, the claw raise defensive behavior also was significantly affected by Cd exposure (ANOVA $p<0.05$). In each of these three cases, at least one exposure concentration of Cd reduced the claw-raise behavior significantly compared to controls (Tukey-HSD $p<0.05$). When control groups were compared across species, a significant correlation was measured between body mass and both the tail flip and claw raise behaviors. Between species, as body mass increased, the tail flip response decreased in frequency ($r = -0.87$; $p<0.0001$) , and the claw raise response increased in frequency ($r= 0.68$; $p<0.0001$). *P. clarkii* was also analyzed for the effect of Cd exposure on heart rate and response to two stimuli. Some alteration was observed in the circadian rhythm of crayfish under Cd exposure. Heart rate decreased by 56% ($p<0.01$) relative to pretreatment levels as crayfish succumbed to Cd toxicity. In each control individual, heart rate slowed after a mild stimulus. After a stronger stimulus, heart rate increased in control animals. In contrast, no heart rate trends were evident in Cd exposed crayfish after stimulus. These data indicate that heart rate may be useful as a physiological marker of Cd toxicity.

4.2 Introduction

The United States contains within its borders more than 320 species of freshwater crayfish in two families, the Astacidae and Cambaridae. This accounts for 61% of the worldwide total of approximately 520 recognized species. It has been estimated that between 48% and 51% of crayfish species in this country are imperiled or vulnerable to extinction (Taylor *et al.* 1996; Stein *et al.* 2000). In contrast, only four species of crayfish are currently listed as endangered or threatened under the U.S. Endangered Species Act (U.S. Fish and Wildlife Service 2005). Most states contain multiple crayfish species and some possess more than 50. Some species, such as *Orconectes virilis*, have a very wide distribution encompassing many states while others have very limited ranges, sometimes as small as a single cave or stream (Hobbs 1976; Stein *et al.* 2000).

Crayfish can provide a large component of the animal biomass in an aquatic ecosystem, sometimes exceeding 50% of the macroinvertebrate biomass in a lake or stream (Momot 1995). Crayfish are also important food sources to a variety of aquatic and terrestrial predators (Rabeni 1983; Huner and Barr 1991). Their central location in many aquatic food webs make them a species of special interest, possibly allowing them to transfer toxicants between trophic levels.

Cadmium is a common, widespread toxicant and can be found in approximately half of hazardous waste sites on past and present U.S. EPA National Priorities Lists. It finds its way into the environment primarily through mining activities and fossil fuel combustion (Taylor *et al.* 1999). The Cd^{2+} ion is the most bioavailable form and the most toxic to aquatic organisms. It is thought to be taken up through the gills by the same mechanism as calcium and zinc. It is a nonessential metal and can cause a wide range of effects in aquatic organisms, including slowed growth, reduced reproductive output,

impaired Ca metabolism, and altered behavior, in addition to mortality (Nriagu and Sprague 1987; Albert *et al.* 1992). These factors, especially the impact of Cd on Ca metabolism, make Cd a toxicant of special interest to crustaceans because of its implications to the molting process, a time of greatly increased Ca metabolism (Wheatly and Ayers 1995).

The behavioral effects of heavy metals on fish has been widely documented. Black and Birge (1980) reported that three species of fish (*Oncorhynchus mykiss, Lepomis macrochirus, Micropterus salmoides*) and larvae of one amphibian species (*Bufo americana*) showed considerable interspecific and inter-toxicant variation in the extent of the avoidance or attraction response they exhibited. Scarfe *et al.* (1982) observed similar variability in teleost swimming behavior under aqueous Cu exposure. Behavioral responses to Cd include abnormalities in swimming behaviors at acute levels (*Ctenopharygodon idellus*; Yorulmazlar and Gul 2003), while reductions in competitiveness and decreased social dominance can occur at chronic levels of exposure in fish (*O. mykiss*; Sloman *et al.* 2003a; Sloman *et al.* 2003b).

Invertebrates have evinced a wide variety of responses to heavy metal insult as well. Scallops *(Argopecten purpuratus*) closed their shells in response to Cu exposure (Inda and Cuturrufo 1999). Behavioral responses to Cd included disruptions to feeding behaviors in the larvae of the chironomid *Glyptotendipes pallens* in 96 hour acute tests (Heinis *et al.* 1990). Lefcort *et al.* (2004) measured snail avoidance/attraction behavior. Snails (*Physella columbiana*) avoided both Cd and Zn at levels as low as 2.88 and 5.5 μg/L, respectively. Grass shrimp (*Palaemonetes pugio*) were less able to capture live prey after two weeks of being fed field collected Cd contaminated oligochaete prey (*Limnodrilus hoffmeisteri*) or lab contaminated *Artemia salina* (Wallace *et al.* 2000).

Only a little work has been done on the effects of heavy metal exposure on crayfish behavior, despite this group's wide use in behavioral research. Maciorowski *et al.* (1980) found that the crayfish *Cambarus acuminatus* was attracted to Cd at low and moderate levels, 5.8 to 18.5 μg/L, but was repelled at 148.3 μg/L. This variability of avoidance and attraction behavior is similar to findings by Black and Birge (1980) that American toad (*B. americana*) larvae and rainbow trout (*O. mykis*) were repelled at low levels of Cu exposure and attracted at higher levels. Exposure to Cu at levels as low as 0.02 mg/L caused the crayfish *Cambarus bartoni* to stop moving towards food sources and sometimes actively avoid food sources (Sherba *et al.* 2000). These results are similar to those for Steele *et al.* (1992) who tested *P. clarkii*, *C. bartoni*, and another crayfish species, *Orconectes rusticus*. They found that a sublethal mixture of Cu, Cr, As, and Se in proportions that simulated their proportions in coal fly ash slurry significantly reduced all three species' preference to move into areas with a feeding stimulant fluid relative to control areas with attractant. Juvenile rusty crayfish (*O. rusticus*) showed hyperactivity resulting in reduced shelter use relative to controls when exposed to sublethal concentrations of Cd (nominal concentrations of 1-3 mg/L; Hardness = 226 mg $CaCO_3$/L; (Alberstadt *et al.* 1999). Juvenile red swamp crayfish (*P. clarkii*) showed similar, although weaker, trends (Misra *et al.* 1996).

While avoidance/attraction, feeding, and shelter seeking behaviors have been investigated under heavy metal exposure, there has been no work assessing the potential effects of heavy metal pollution on anti-predator behaviors. Two basic anti-predator behaviors have been identified. One is the typical escape response involving backwards swimming using the uropod (also called the tail-flip behavior or simply escape behavior). The other is the predator response or defense behavior (also called the claw-raise

behavior or meral spread) (Hayes 1975; Hayes 1977; Bruski and Dunham 1987). Any impairment to the performance of these two behaviors could make crayfish more vulnerable to predation.

The heart of adult crayfish is neuorgenic and under the control of the cardiac ganglion (Maynard 1960; Yamagishi and Hirose 1997). Crayfish heart rate may serve as a good measure of the excitation state of an individual, especially since it may reveal internal reactions that may not have externally observable manifestations (Listerman *et al.* 2000). Schapker *et al.* (2002) noted that crayfish often react to a small stimulus, such an a drop of water falling into the holding tank, with a brief pause in heart beats. Sometimes there is no outward visible reaction of the crayfish to a stimulus of this sort, however physiological measures such as heart rate or ventilatory rate show pronounced changes. Lead is known to cause a reduction in heart rate in crayfish (Ahern and Morris 1999). Other heavy metals such as Cd may have similar effects.

In addition to possible direct effects of Cd on heart rate, Cd may affect a variety of behaviors so monitoring HR could provide an alternative, physiological measure for the reaction of crayfish to various stimuli. HR may reveal reactions or changes in reaction brought about by Cd that cannot be assessed by visual means.

4.3 Methods

Five toxicity tests were conducted with adult crayfish. The species included *Procambarus acutus* (Girard 1852), *P. alleni* (Faxon 1884*)*, *P. clarkii* (Girard 1852), *Orconectes placidus* (Hagen 1870), and *O. virilis* (Hagen 1870). Individuals of the species *P. acutus* (Average mass = 15.5 g) were purchased from Blue Spruce Biological Supply Company, Boulder CO; *P. alleni* (5.14g) from Fish2U.com, Gibsonton, FL; and *P. clarkii* (18.5g) from Louisiana Seafood Exchange, New Orleans LA. *O. placidus*

(7.06g) was collected from Blackburn Fork near Cookeville, TN. *O. virilis* (12.8g) was obtained from Clear Creek Fisheries, Martinsville, IN. Water samples were taken at the time of collection and analyzed to determine if there were any environmental contaminants that might complicate toxicity testing.

Toxicity tests were conducted for 96 h and are based on standard methods provided by U.S. EPA and other sources (U.S.EPA 1993; U.S.EPA 1994a; U.S.EPA 1994b; ASTM 1996; APHA 1995). All toxicity tests were conducted in a temperature controlled environmental room at 25˚C ± 1˚C and a 16h light/ 8h dark photoperiod.

Individuals were housed in 11.3L polypropylene tanks. Aeration was provided to each tank to ensure that dissolved oxygen levels remained above 4.0 mg/L, as stipulated by U.S. EPA (1993). In all tests, 800mL polypropylene beakers were used to isolate each crayfish within the tank to prevent injury or mortality from fighting. Each beaker was well perforated to allow water flow throughout the test chamber. Five crayfish were housed in each tank. Four replicates were used. Conditions of water hardness and toxicant exposure levels, expressed as mg of the Cd ion per L, are summarized in Table 4.1. In all tests, 4.0L of each test or control solution was used per treatment. All solutions were renewed daily. In tests with *P. acutus*, *P. clarkii*, and *O. placidus*, checks were conducted every six hours. In tests with *P. alleni*, and *O. virilis*, the animals were checked every 24h. At each check, animals were examined for behavioral response by tapping them lightly on the abdomen five times with a glass rod. The escape or tail flip response was defined as the animal sweeping its tail fully downward at least one time. The predator response pattern or claw raise response was defined as the chelae being raised above parallel to the abdomen (Hayes 1975; Hayes 1977). Lack of either of these behavioral responses was scored as a zero. Complete lack of response to the five taps on

the abdomen (*e.g.* no movement of the legs, tail, scaphognathite, antennae, *etc*.) was counted as mortality.

To measure the effects of Cd on crayfish heart rate and assess this physiological characteristic as a marker of Cd toxicity the electrocardiograms (ECG) of control and Cd exposure groups of crayfish (*P. clarkii*) were monitored continuously over the 96h exposure period. Four organisms were housed individually in 11.3 L polypropylene tanks with 4.0L carbon filtered tap water diluted to 42.9 mg $CaCO_3$/L in an isolated room with 12h light/ 12h dark light cycle. Nine crayfish, including the four that had previously served as controls, were exposed to 26.5 mg Cd/L, 10 times the LC50 value, using similar water and environmental conditions.

Heart rates (ECG) were measured by placing platinum-iridium wires (diameter: 0.127mm; with wire coating: 0.203mm; A-M systems, Inc., Carlsburg, WA) just through the exoskeleton anterior and posterior to the heart along the dorsal midline and securing them in place with fast drying adhesive glue (Eastman, 5-minute drying epoxy) as described by Listerman *et al.* (2000). Crayfish were allowed to recover from this installation for three or more days to allow activity patterns to return to normal. These wires were connected to an impedance detector (UFI, model 2991) measuring dynamic resistance as a monitor of heart rate. Data was continuously recorded on a PowerMac G3 computer via a MacLab/4s interface (ADInstruments, Australia) using MacLab Chart software (version 3.5.6; ADInstruments, Australia) for several hours before the beginning of Cd exposure through the full 96h of the experiment. Data acquisition rates were set to 4kHz. Heart rate data was collected for one hour immediately prior to the start of testing to establish pretreatment conditions. Heart rate counts were determined as averages of 5 one minute segments counted manually one hour prior to the start of Cd exposure, after

6h of exposure, and every 6h thereafter. At each 24h interval after the initiation of the experiment, the effect of Cd on the crayfish's reaction to two stimuli was assessed using methods modified from Kellie *et al.* (2001) and Schapker *et al.* (2002). Heart rate was counted for 30s after a minor stimulus (*i.e.* the dropping of three glass beads (3.9mm diameter) into the exposure chamber from a height of approximately 70cm) or a major stimulus (*i.e.* a tap on the abdomen with a glass rod).

For statisitcal analysis in the acute toxicity tests, the number of crayfish responding with a particular behavior in each treatment was divided by the number of surviving crayfish at the time of that check. These proportions, grouped by treatment, were then used in ANOVA and subsequent pair-wise comparison with the Tukey Honest Significant Difference (HSD) test. The proportions derived for control animals were tested across species with simple linear regression to determine if body mass might have an effect upon the likelihood of performing either behavior. Concentrations lethal to 50% and 10% of individuals (LC50; LC10) were calculated using the probit method with software provided by U.S. EPA (1992). In each case, a chi-square test for heterogeneity was conducted to ensure the data fit the probit model adequately. Student's t-test for dependent samples and Fisher's exact test were used to assess the relationships between Cd exposure and changes in crayfish heart rate.

Water samples were collected from at least two replicates of each exposure concentration daily. The sampling scheme was designed so that some samples were taken immediately before and after a water change to ensure that Cd levels remained constant. These samples were analyzed on either inductively-coupled plasma optical emission spectrophotometry (ICP OES- Varian Vista MPX) or graphite furnace atomic absorption spectrophotometry (GFAAS- Varian SpectrAA-20 with GTA-96 Graphite Tube

Analyzer) to determine actual Cd exposure concentrations. Either a Denver Instruments Model 215 or Orion Research Expandable Ion Analyzer EA-960 was used to determine pH. A YSI Model 51A oxygen meter was used for all dissolved oxygen measurements. Water hardness was checked using the EDTA titrimetric method, then confirmed using ICP-OES (APHA 1995). Temperatures were measured on a daily basis. All quality control measurements were within U.S. EPA acceptability guidelines (1993).

4.4 Results

Cadmium had a significant effect on all five species of crayfish for the tail flip response. One-way ANOVA was performed and each test showed $p<0.05$, although for *P. alleni*, the p value was just barely lower than this criterion (Table 4.2). Each of the tests, except for *P. alleni*, showed multiple cases where by the tail flip response was significantly reduced in frequency relative to controls (Figure 4.1). A general trend, whereby this effect grew larger as Cd concentrations increased, was seen in each test. Between the five tests, the lowest test concentration to have a response significantly different from controls ranged from 0.063 to 3.50 mg Cd/L. None of the tests concentrations was significantly different from controls in the test with *P. alleni* (Table 4.2).

The effect of Cd on the claw raise response was not as clear. Cadmium had a significant effect on all three *Procambarus* species, but not on the *Orconcetes* species when tested with one-way ANOVA (Table 4.3). For *P. alleni* and *P. acutus*, only the highest concentrations of Cd (45.2 - 48.8 mg Cd/L) resulted in a significant increase in the claw raise response (Figure 4.2). *P. clarkii*, on the other hand, showed a significant increase at the middle and second highest concentrations (3.50 and 17.4 mg Cd/L), but

not at the highest. *O. placidus* showed a general decrease in response, although the trend was not significant, while *O. virilis* did not show any clear pattern.

A significant trend existed between species based on body mass. As body mass increased, the frequency of the tail flip response decreased ($r = -0.58$; $p<0.0001$). Conversely, the frequency of the claw raise behavior increased as body mass increased between species ($r= 0.55$; $p<0.0001$). The assay with *P. acutus* had an unusually high frequency of tail flips based on the size of these crayfish. It was significantly higher than assays with all other crayfish except *P. alleni* ($p<0.05$ Tukey-HSD). Given that the data from *P. acutus* were somewhat anomalous because of an unusually high molting rate in the test, these regressions were recalculated omitting data from that assay. The closeness of fit increased for both the tail flip response ($r = -0.87$; $p<0.0001$) and the claw raise response ($r= 0.68$; $p<0.0001$).

Crayfish heart rate was affected by Cd exposure. In organisms that died under Cd exposure, average heart rate was significantly slowed by 56% the last time they were measured before death compared to their pretreatment heart rate (t-test for dependent samples; $p<0.01$). Additionally, in control crayfish, measurements taken between 6:00PM and midnight always had the highest heart rate, while this pattern did not hold for Cd treated crayfish, for whom the time of maximum heart rate was more variable. Cadmium treatment also may have reduced crayfish response to stimuli. In each of four trials, crayfish in control water showed a slowed heart rate relative to the rate taken immediately before a mild stimulus (the dropping of three glass beads into the exposure chamber). At the moment of the stimulus, the heart would pause briefly, skipping 1-2 beats, then resume beating at a slower rate which subsequently returned to the normal rate (Figure 4.3). Also crayfish showed an increase in heart rate in all four trials after a

stronger stimulus, a tap on the tail with a glass rod (Figure 4.3). However, in both cases, only one trial was significantly different from pretrial values ($p<0.01$), while in each case a second one was nearly significantly different ($0.09>p>0.06$). No trends in the response of heart rate after stimulus were evident in crayfish exposed to Cd (Figure 4.4). When any alterations to the rate, shape, or amplitude of the heart rate traces at the time of stimulus were manually counted, indicating some level of physiological response, the frequency of a noticeable change was significant at $p=0.053$ (Fisher's Exact Test) between control (93.8%) and Cd treated animals (63.6%).

4.5 Discussion and conclusions

The effect of Cd exposure on the tail flip behavior was more consistent and more robust than on the claw raise response. Additionally, the tail flip response was typically more sensitive to Cd insult. *O. virilis* was the species with the most sensitive tail-flip response to Cd exposure with a significant effect at 0.063 mg Cd/L. This value is fifty times lower than the concentration lethal to 50% of the population (LC50) and fifteen times lower than the 10% lethality concentration (LC10; Table 4.1). However, the species with the next most sensitive tail flip response was *O. placidus*. This species experienced a significant decrease in tail flip activity at an exposure concentration of 0.128 mg Cd/L. This is less than 4 times lower than the LC50 value and 39% higher than the LC10 value. The minimum significant concentration (3.50 mg Cd/L) for *P. clarkii* was actually 30% above the LC50 value and 7 times higher than the LC10 value. The least sensitive tail flip response relative to controls was measured in *P. acutus*. The lowest significant concentration (1.65 mg Cd/L) was more than 4 times greater than the LC50 value and about 34 times greater than the LC10 value for that species. None of the concentrations in the *P. alleni* test showed a significant response. Members of this

species were, on average, smaller than for any other test, and so the tendency for them to tail flip was likely strongest (Lang *et al.* 1977; Kellie *et al.* 2001; Keller and Moore 2000). A significantly greater percentage of this species' control group tail flipped compared to any other test's control group. Thus, while Cd has clear effects upon crayfish behavior, especially the tail flip response, the variability of that response complicates the use of this behavior as an indicator of Cd exposure. However, it does seem that Cd, in some cases, can have significant effects upon the tail flip behavior at levels below acutely lethal exposure concentrations. Further studies could be done to establish whether these effects can be found at more environmentally relevant concentrations, especially on a chronic timescale.

Explaining the interspecific differences measured in the tail-flip behavior is difficult because there is not a pattern between the tolerance to the lethal effect of Cd and its behavioral effects. Indeed, the most tolerant crayfish to Cd lethality was the must sensitive to depression of the tail-flip response. The next most sensitive to behavioral alteration also had the second lowest LC50 value of the tested species. Since no relationships appear to exist between Cd induced impact on crayfish behavior and overall tolerance to Cd poisoning, crayfish mass, or taxonomy, perhaps the measured differences are related to intrinsic differences in the manifestation of this behavior between species.

The claw raise response, when comparing controls groups across all tests, was a generally less common occurrence than the tail flip response, except in the assay with *P. clarkii*. Individuals in this test were on average larger than those in any other test and actually exhibited a greater proportion of claw-raises than tail-flips. In the test with *P. clarkii*, it is likely that a significant response for the highest exposure group was not seen because of the extreme lethality of this concentration of Cd. All members of this group

died within 48 hours of the start of the assay. Only tests with *Procambarus* species showed treatments that were significantly different from controls. The most sensitive significant response was in the assay with *P. clarkii* at an exposure level of 3.50 mg Cd/L, 32% greater than the LC50 value. The only other significant responses came from the highest exposure concentrations in each of the other two *Procambarus* assays which were many times higher than their respective LC50 values. This indicates that the claw raise behavior is much less susceptible to Cd perturbation than the tail flip response and much less useful as a potential behavioral indicator of Cd stress. Also, the difference in response between *Procambarus* spp. and *Orconectes* spp. indicates that these two genera may have intrinsically different levels of aggressive behavior. *P. clarkii* is known to be an aggressive species that can exclude other species from their native habitats (Holdich 2002b).

When conducting the toxicity tests listed in this paper, it became apparent that the *P. acutus* assay was different from the others. It experienced an unusually high molt frequency (41.7%) compared to the other tests (0.556% for *O. juvenilis* to 13.3% for *P. clarkii*). It has been shown that molting lobsters have altered threat responses compared to non-molting individuals (Cromarty *et al.* 2000). Because of this, *P. acutus* was excluded from the analysis of the interspecific effects of size on tail flip and claw raise behavior. Periods of very high molting rates are known to occur in nature (Taylor and Schuster 2004) and given that they are times of considerable additional stress could represent times of unusually high sensitivity to toxicant insult (Chapter 2).

The crayfish species in this series of tests tended to exhibit the tail-flip response less often as average crayfish mass increased. Conversely, they evinced an increase in the frequency of the claw-raise behavior as average body mass increased. American lobsters

(*Homarus americanus*) increase the frequency of their more aggressive defensive responses as they grow in size (Lang *et al.* 1977; Wahle 1992). Also, *H. americanus,* and *P. clarkii* are less likely to exhibit a tail flip response as body mass increases (Lang *et al.* 1977; Kellie *et al.* 2001). Keller and Moore (2000) found that smaller crayfish *(O. virilis*) tended to tail flip more often and larger crayfish to raise their claws more often when encountering fish. In this series of tests, because the response was strong even between species, it would seem that size was a more important determinant of crayfish response than interspecific variation. The relative level of aggressiveness between crayfish species has been assessed in dominance contests between individuals of different crayfish species. The interspecific level of aggression does seem to be significantly different in some cases, which may be a factor that has aided invasive crayfish species, such as *O. rusticus*, in displacing native species such as *O. immunis* (Tierney *et al.* 2000). However, no pattern was found between body mass and the sensitivity of the behaviors for Cd exposure. Thus while overall trends in behavioral response may be governed by body mass, the individual species seem to have intrinsic differences in how they respond to Cd. Further studies within and between groups would be needed to control for and assess both interspecific variability in behavioral response and the effect of body mass on response.

The assay with *P. acutus*, the second heaviest species, had a significantly higher frequency of tail flips than assays with smaller crayfish, including *O. placidus* and *O. virilis* ($p<0.05$ Tukey-HSD). However, as previously mentioned, this assay experienced an unusually high number of molts which probably explains the altered response (Cromarty *et al.* 2000).

The condition of an individual's chela (*i.e.* both present, one missing, two missing, one or more being a product of regeneration) was not controlled in this study which is a confounding factor (Kellie *et al.* 2001). The complete separation of individuals would also be beneficial, since in the current study, to ensure that Cd was equally available to all individuals, crayfish were housed in perforated beakers in common tanks of exposure medium. This allowed one individual to potentially hear or smell the reaction of another crayfish when tapped, which may have altered its reaction (Schneider and Moore 2000; Gherardi *et al.* 2002).

Crayfish heart rates decreased with Cd exposure. This may have been caused by the direct interference of Cd with muscle function or been more the result of generalized metabolic distress from the high levels of Cd used in this portion of the study. Since crayfish tend to accumulate relatively little Cd in their muscles (Mirenda 1986; Chapter 4) the latter explanation is more plausible. While some alteration to the circadian rhythm of crayfish was indicated by the data in this assay, the low n of the controls precludes strong statistical correlation. Styrishave *et al.* (1995) measured a disruption of normal circadian rhythms in crayfish under Cu and Hg exposure, so it is possible that Cd may have caused a similar effect. Likewise, the low number of the control animals made the significance of the trends observed after mild and strong stimulus questionable. An additional 3-4 control animals would probably have shown this trend to be more strongly significant. Based on this assumption, heart rate does show some promise as an indicator of physiological status under Cd exposure.

Table 4.1. Toxicity values (LC50, LC10) and average mass for five crayfish species used in toxicity and behavioral tests.

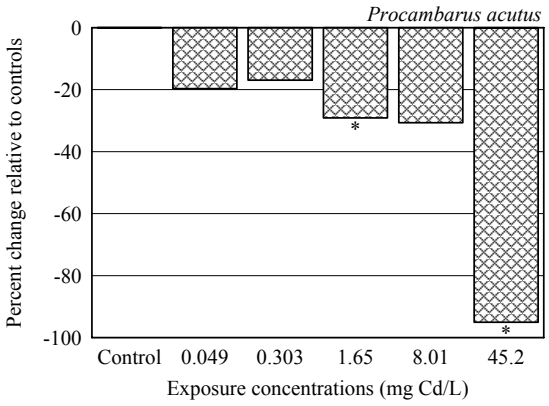
Species	Mass	LC50	LC10
Procambarus acutus	15.5 g	0.368	0.048
Procambarus alleni	5.14 g	3.07	0.386
Procambarus clarkii	18.5 g	2.66	0.486
Orconectes placidus	7.06 g	0.487	0.092
Orconectes virilis	12.8 g	3.30	0.947

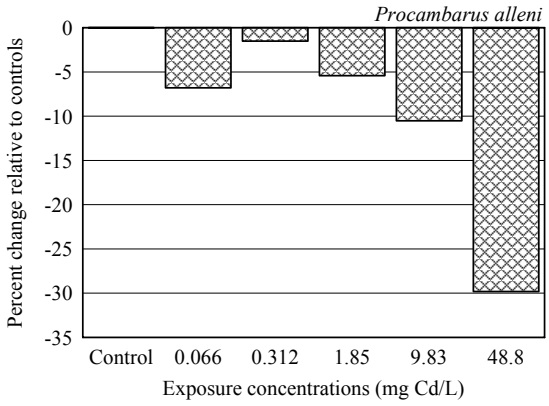
Table 4.2. Tail-flip behavior in crayfish. ANOVA was calculated for change in mean proportion of response between exposure concentrations. Tukey's HSD test (corrected for unequal n) was used to determine significant responses in exposure concentrations relative to controls (mg Cd/L; $p \leq 0.05$).

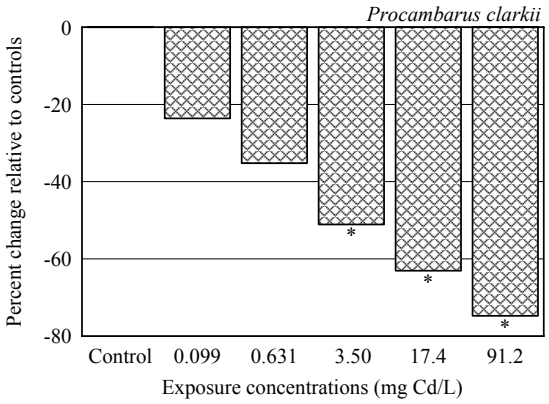
Species	ANOVA	Lowest concentration with significant response
Procambarus acutus	$p < 0.0001$	1.65 mg Cd/L
Procambarus alleni	$p = 0.0454$	none
Procambarus clarkii	$p = 0.0005$	3.50 mg Cd/L
Orconectes placidus	$p < 0.0001$	0.128 mg Cd/L
Orconectes virilis	$p < 0.0001$	0.063 mg Cd/L

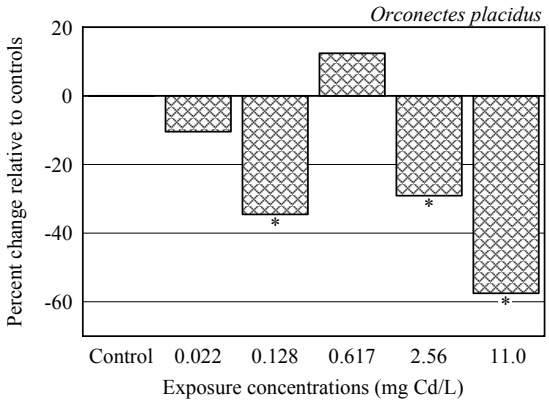
Table 4.3. Claw-raise behavior in crayfish. ANOVA was calculated for change in mean proportion of response between exposure concentrations. Tukey's HSD test (corrected for unequal n) was used to determine significant responses in exposure concentrations relative to controls (mg Cd/L; $p \leq 0.05$).

Species	ANOVA	Lowest concentration with significant response
Procambarus acutus	$p = 0.0002$	45.2 mg Cd/L
Procambarus alleni	$p = 0.0003$	48.8 mg Cd/L
Procambarus clarkii	$p < 0.0001$	3.50 mg Cd/L
Orconectes placidus	$p = 0.3040$	none
Orconectes virilis	$p = 0.6072$	none









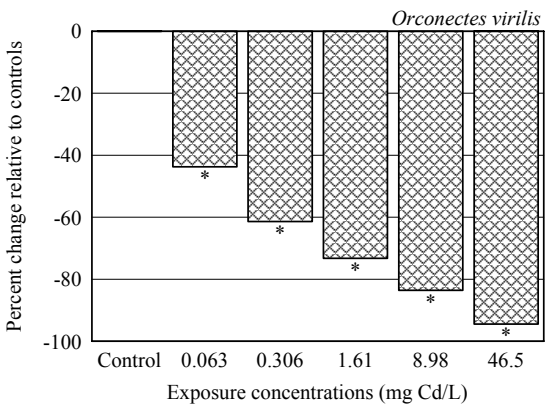


Figure 4.1. Percent change in tail-flip behavior at each exposure concentration for 5 species of crayfish relative to controls. An asterisk (*) indicates a significant difference from control responses ($p \leq 0.05$).

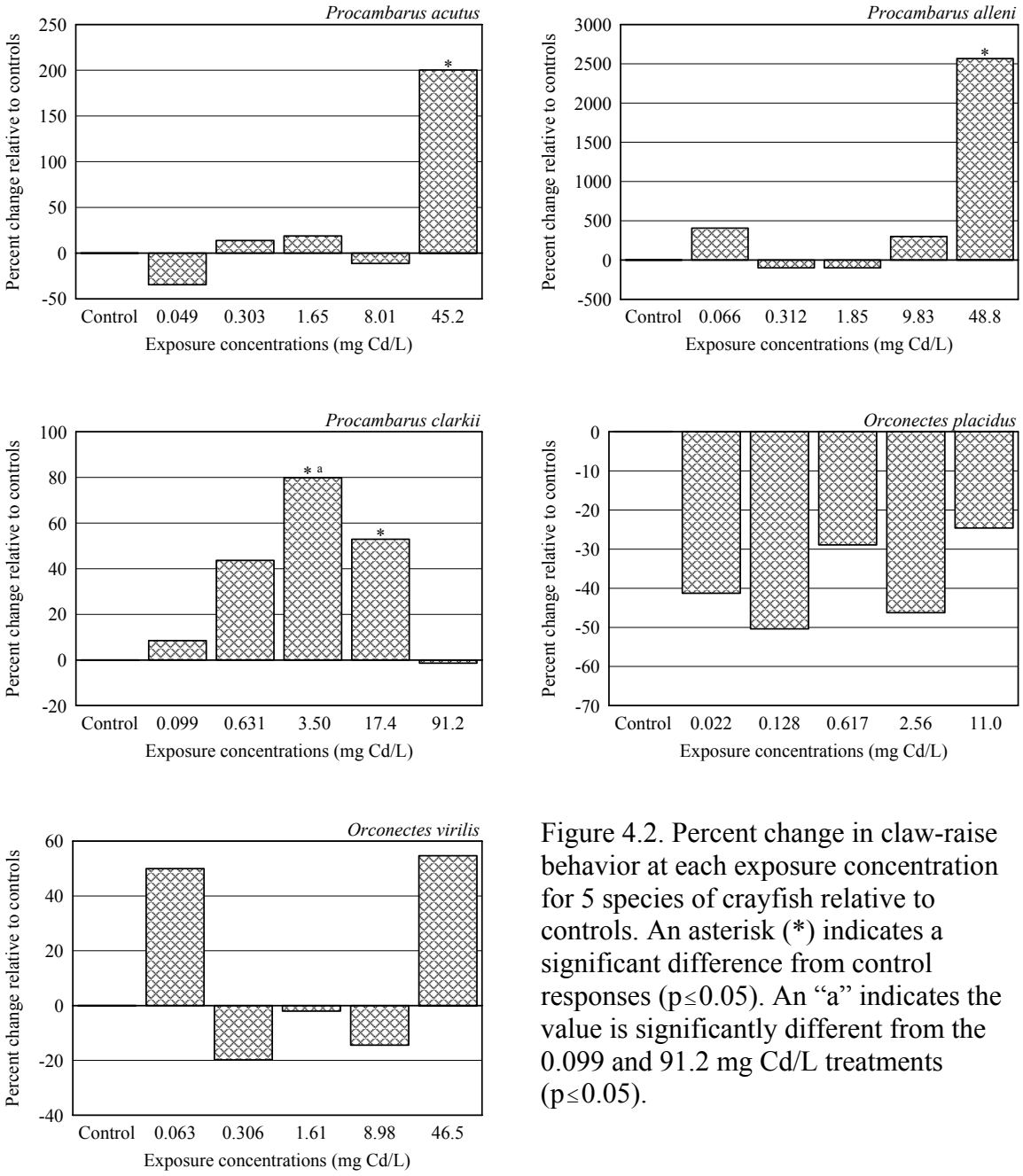


Figure 4.2. Percent change in claw-raise behavior at each exposure concentration for 5 species of crayfish relative to controls. An asterisk (*) indicates a significant difference from control responses ($p \leq 0.05$). An "a" indicates the value is significantly different from the 0.099 and 91.2 mg Cd/L treatments ($p \leq 0.05$).

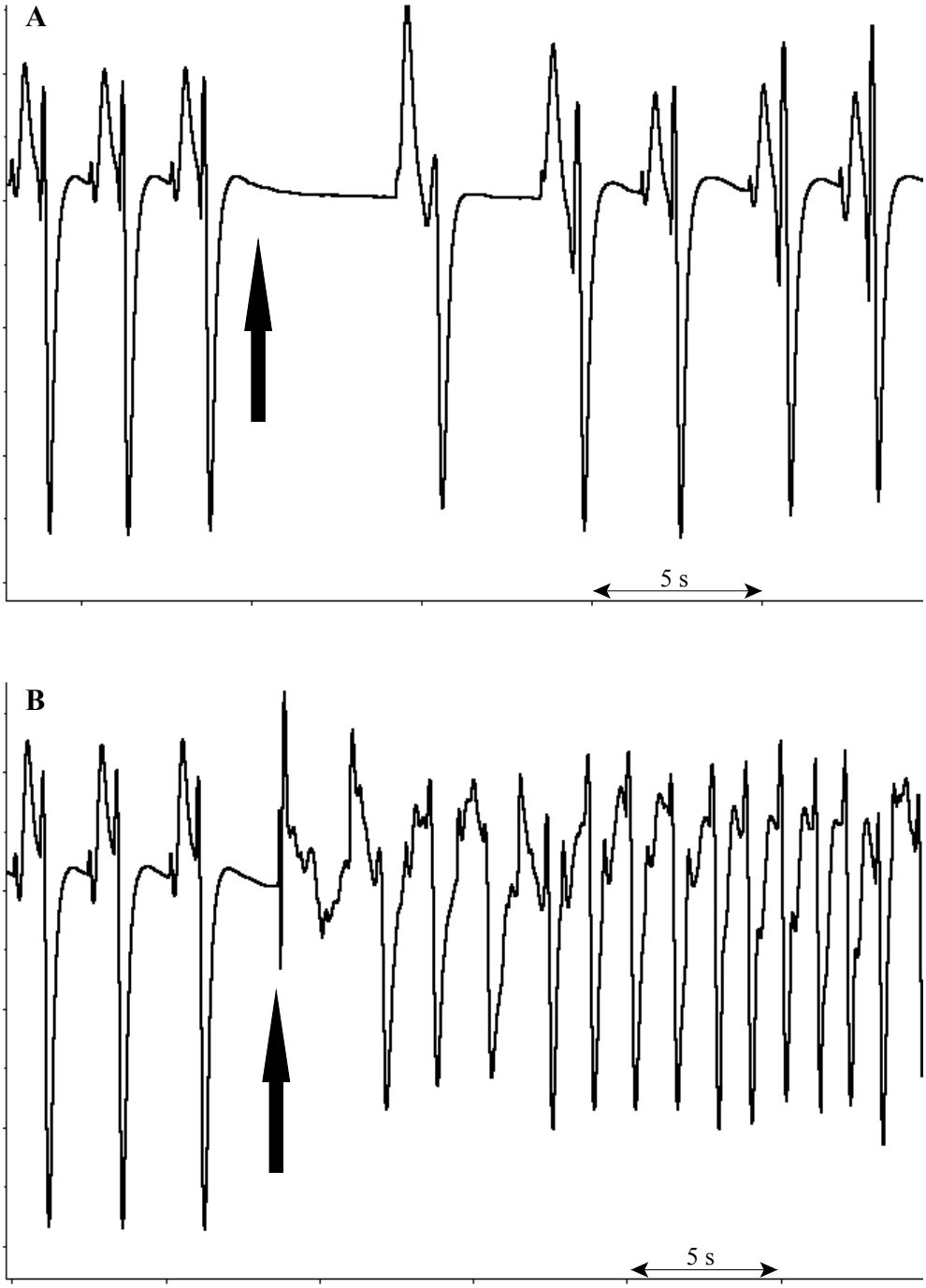


Figure 4.3. Heart rate traces for *P. clarkii* undergoing either a mild stimulus, the dropping of 3 glass beads (A), or a strong stimulus, a tap on the tail with a glass rod (B). The initiation of the stimulus is indicated by the arrow.

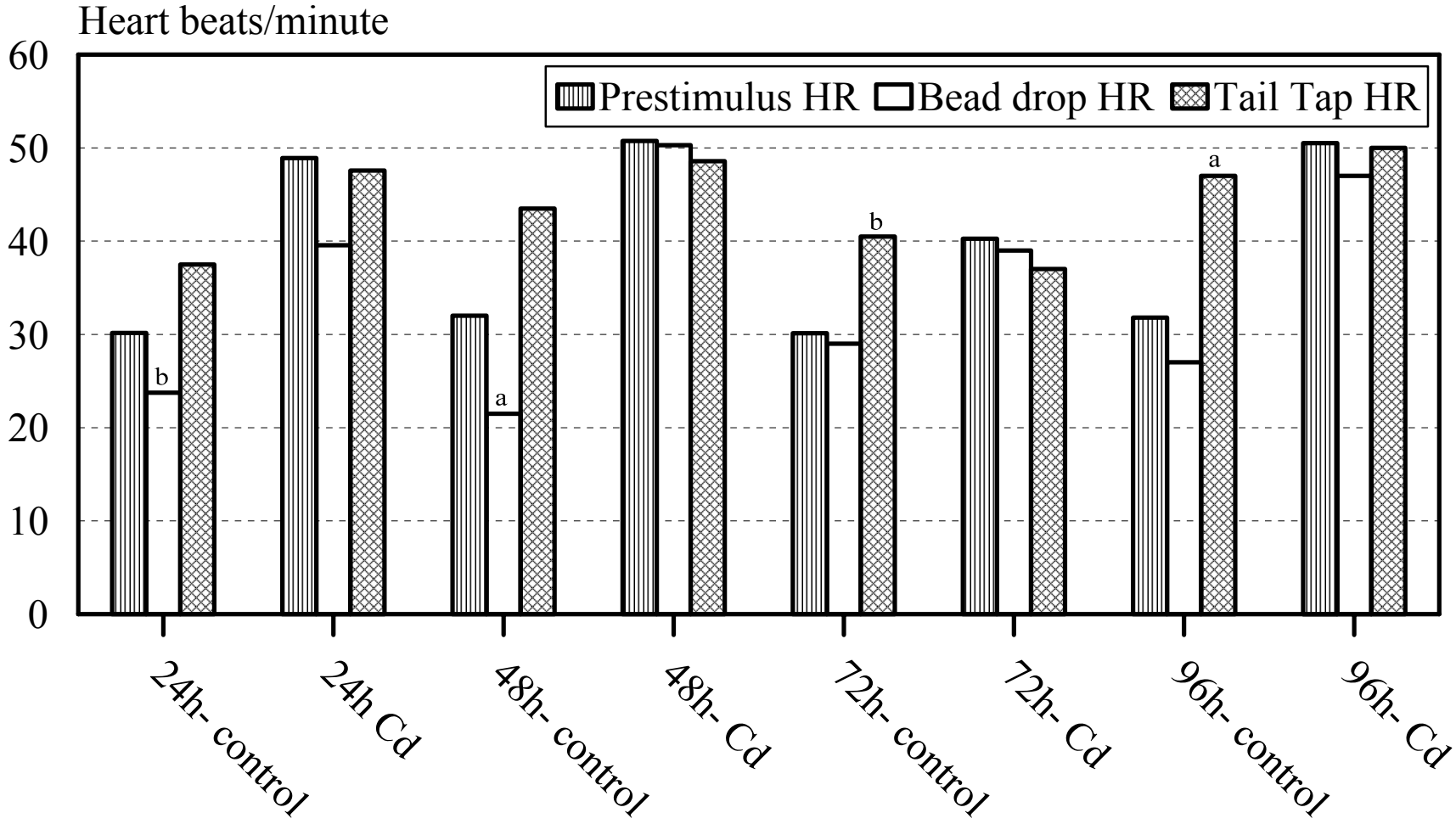


Figure 4.4. Heart rates in *P. clarkii* before stimulus, after a mild stimulus (dropping 3 glass beads into tank), and after a major stimulus (a tap on the tail with a glass rod). An "a" indicates $p<0.05$ and a "b" indicates $0.10>p>0.05$.

Chapter 5: Conclusions

5.1 Introduction

Crayfish are an ecologically important group of freshwater organisms whose greatest global center of diversity is located in the eastern and central United States. Relatively few studies have been conducted on the ecotoxicology of cadmium or other heavy metals to crayfish compared to groups such as the freshwater fish, especially with regards to sensitive early life stages. Additionally, very few species of crayfish in the United States have received any sort of legal protection under the Endangered Species Act, which contributes to their vulnerability. This dissertation addresses several key issues in the ecotoxicology of Cd to the Astacida by 1) expanding the current database of crayfish toxicology for adults and sensitive early life stages to include a wider variety of species, including ones from more limited ranges; 2) assess the impact of Cd on molting survival rate; 3) comparing the differences between how sensitive and tolerant species of crayfish accumulate Cd; 4) exploring how Cd can affect the distribution of other, nutritionally important, metals in the bodies of crayfish, which also gives important clues to the mechanism and scope of the organism's response to this toxicant; 5) investigating how Cd affects two of the key defensive behaviors of crayfish; and 6) examining crayfish heart rate as a useful physiological marker of Cd impact.

5.2 Comparative toxicology of cadmium to crayfish and its effects on molting

Toxicity test values are reported for six species of crayfish. Only one of these species has been reported previously in U.S. EPA's Ambient Water Quality Criteria for Cadmium (2001). Acute toxicity values (96h LC50) for these species ranged from a maximum of 3.30 mg Cd/L to a minimum of 0.368mg Cd/L. The 96h LC10 values for these species ranged from 0.947 to 0.048mg Cd/L. The species fell into two general

groups, the more tolerant group with 96h LC50 values that ranged from 3.30 mg Cd/L to 2.44mg Cd/L and 96h LC10 values of 0.947 to 0.386mg Cd/L. The more sensitive species possessed 96h LC50 values from 0.487 to 0.368mg Cd/L with threshold (96h LC10) values from 0.092 to 0.048 mg Cd/L. The values for the more tolerant group are similar to those for several common tolerant freshwater fish test species, such as goldfish (*Cassarius auratus*) and channel catfish *(Ictalurus punctatus*). The more sensitive group is lower than moderately tolerant freshwater fish test species like the fathead minnow (*Pimephales promelas*). However, sensitive species of fish, like the rainbow trout *(Onchorynchus mykiss*), are more sensitive than any of the adult crayfish studied. However, endangered crayfish species have not been investigated and toxicity values in some sensitive groups like the amphibians can vary by 2-3 orders of magnitude (Birge *et al.* 2000a). Toxicity values for the crayfish species in this dissertation corresponded well to values reported by other authors.

Toxicity values for juveniles of tested crayfish species indicate that they were much more sensitive than adults. The 96h LC50 and LC10 values ranged from maxima of 0.624 and 0.037 mg Cd/L respectively to minima of 0.283 to 0.002 mg Cd/L, respectively. The lower two values are similar to other sensitive invertebrate test species such as *D. magna* and *D. pulex*. The values for *P. clarkii* match well with reported literature values.

Overall, molting increased the susceptibility of crayfish to Cd exposure. When an individual was subjected to Cd during or shortly after molting, it was very likely to die. In experiments with adult animals, at lower exposure concentrations, 14.3% of molting individuals died. At middle exposure concentrations, 75% died if they molted during or before the experiment. This represented 66.7% and 46.5% of all mortalities at those

concentrations respectively. At the highest exposure concentrations, all molting crayfish died. All adult control animals molted successfully during these experiments.

For juvenile crayfish, the same pattern occurred. While 12.5% of molting juveniles died in the control groups, 54.5% of those in the lowest Cd exposure concentrations died and 100% of all other Cd exposed juvenile crayfish perished during or after molting. This indicates that molting is a sensitive life stage in crayfish that enhances their susceptibility to Cd toxicity. Taking molting, the fragility of juveniles, and other factors into account supports the high sensitivity of crayfish to environmental stress.

5.3 Cadmium tissue residues and effects on the distributions of other metals.

While a few researchers have investigated the accumulation of Cd by individual crayfish in the laboratory (*e.g*. Mirenda 1986, Thorp and Gloss 1986), this work is the first to compare tissue accumulation patterns across species. Also it is the first to examine the alterations in the distribution patterns of nutritional metals caused by Cd.

Whole body burden patterns of Cd

Overall (whole body) Cd burdens did not vary much between species. The highest whole body 96h BCF was 136 for *O. placidus* at 0.022mg Cd/L and the lowest was 2.81 for *P. clarkii* at 17.4 mg Cd/L. However differences were apparent in how sensitive and tolerant species achieved similar body burdens of Cd.

Patterns in tissue burden of Cd between species

Cadmium tended to accumulate in a dose response pattern in most tissues under Cd exposure. The pattern of tissue concentrations was similar to literature values for some species. Those that differed from reported values seemed to be in the more sensitive group. Species that had lower LC50 values for Cd tended to have higher

accumulations of the metal in their hepatopancreas than gills while the trend was reversed for more tolerant species. In tolerant species, the exoskeleton and remnant samples tended to show higher Cd accumulation. Curvilinear regression models were used to compare the Cd accumulation patterns from tests with different exposure concentrations. At a hypothetical exposure concentration of 0.303 mg Cd/L, sensitive crayfish had an average hepatopancreas 96h BCF of 348 and average gill 96h BCF of 72.4, while tolerant crayfish only had an average hepatopancreas 96h BCF of 54.5, but an average gill 96h BCF of 60.8. At this same level, exoskeleton and remnant sample 96h BCF values ranged from 8.9-16.8 for sensitive species to 23.3-27.0 for tolerant species. These differences may indicate a fundamental difference between sensitive and tolerant crayfish and may reflect the differing capabilities of their uptake or detoxification machinery. Tolerant crayfish are either able to avoid accumulating as much Cd as sensitive crayfish, or can depurate or detoxify it more quickly, or possibly a combination of these two options. Because crayfish seem to have a strong ability to accumulate metals, they can serve well as monitors of bioavailable metal. Many researchers have used them as such.

Effects of Cd on the distribution of nutritionally important metals

Cadmium seems to have had significant effects on the metabolism of several important dietary metals such as Ca, Cu, Fe, Mg and Zn. It may have been that some of these metals were being displaced from the molecules that normally bind and regulate them, such as metallothionein. Zn levels in the hepatopancreas tended to decrease with increasing exposure concentrations while levels in the exposure media tended to increase during the 24h exposure period. Copper tended to accumulate in the gills and green glands indicating that Cu uptake is being increased or excretion increased. Probably Cu

was being released from the body as was indicated by the build up in the green glands, a known organ of heavy metal depuration. Also, some weak evidence indicated that Cu might have increased in the exposure media over time. Both Zn and Cu seemed to be transported out of the crayfish's body via an excretion pathway. Whether this involved the gills or green glands or something else is not known.

5.4 Cadmium effects on anti-predator behaviors

The data presented in this dissertation are the first that have been collected on the effects of Cd, or any other heavy metal, on anti-preadator behaviors, specifically, the claw-raise behavior and the tail-flip behavior. The tail-flip behavior seemed to be a relatively sensitive marker for Cd stress. One-way ANOVA indicates that Cd has an effect on the tail-flip response in all five species. The Tukey HSD test revealed that multiple Cd treatments showed significant reductions in the frequency of this behavior in four of the five species tested. For two of these four species, the behavioral response was more sensitive than its respective LC50 value and for *O. virilis* the behavioral response was considerably more sensitive than the species' LC10 value.

The claw-raise behavior seems less sensitive to Cd exposure than the tail-flip behavior. One-way ANOVA only showed significant Cd effects on *Procambarus* spp. Only *P. clarkii* showed a response that was similar to the species' LC50 value. *P. acutus* and *P. alleni* only showed a significant response at very high exposure levels. The two *Orconectes* species showed no significant responses, perhaps indicating a fundamental difference in the response pattern of this behavior between genera.

An overall pattern was seen between species with regards to the tail-flip behavior and size. The larger the size of the individual, the less likely it was to attempt to escape via a tail-flip after stimulus. When *P. acutus*, because of its unusually high molting rate,

was excluded from analysis, the trend was strongly negative ($r = -0.87$; $p<0.0001$). An opposite pattern was evident for the claw raise response, with its occurrence increasing as body mass increased. When *P. acutus* was again excluded from analysis, the trend was positive and significant ($r= 0.68$; $p<0.0001$). For these two behaviors, the effect of size seems more important than any intrinsic interspecific differences.

The crayfish heart rate served as a more sensitive marker of crayfish response than simple visual observation. This was especially true of mild stimuli which sometimes did not elicit any visible response, yet caused an alteration in the animal's heart rate. It was found that the heart slowed significantly before death in Cd exposed crayfish. There may have been a shift in the pattern of peak activity from early night to later night under Cd treatment. There also seemed to be an altered response to stimuli in some cases. Often the crayfish failed to slow its heart slightly after a minor disturbance as control animals typically did. Also, after a stronger stimulus, the heart rate would not increase as much as it did in control animals. However, low sample size in the control groups hampered the statistical analysis of this portion of the experiment. Additional control animals would have likely clarify these relationships. These preliminary data on the effects of Cd on heart rate response to behavioral stimuli do indicate that is has some promise as physiological marker of Cd stress.

5.5 General conclusions and future directions

Crayfish are generally excellent model organisms for a variety of types of investigations. They are small and easily handled, yet are among the largest freshwater invertebrates, making them relatively easy to manipulate or dissect. They can be easily maintained in a laboratory and can even breed there. The basic biology has been thoroughly researched and behaviors have been examined for several species.

Additionally, being invertebrates, they do not require Institutional Animal Care and Use Committee (IACUC) oversight resulting in reduced paperwork. Their eggs are large and can easily be counted or manipulated. The juveniles have direct development and thus rearing them in the lab is relatively simple.

Crayfish are a unique group of animals that are extremely important to many ecosystems, not only in the United States, but around the world. Since they are often considered keystone species (*i.e.* of great importance to the normal function of the ecosystems they inhabit), their conservation should be an important part of over all efforts to conserve global species diversity since even rates of biodiversity loss as low as 0.2% per year could be devastating (Ehrlich and Wilson 1991). Ricciardi and Rasmussen (1999) predict that U.S. freshwater extinction rates in the near future could reach 4% per decade in the future. This is five times higher than predictions of extinction rates for terrestrial species and rival extinction rates found in tropical rain forests. Given that between 48% and 51% of species in the United States are threatened in some fashion, more research into and government involvement in their protection and conservation are vital (Taylor *et al*. 1996; Master *et al*. 2000).

From the array of species tested in this dissertation, it is probable that adult crayfish are adequately protected by the current U.S. EPA recommended water criteria for Cd (Criterion Maximum Concentration = 0.001 mg Cd/L). However, endangered or crayfish from extremely limited geographic distributions have yet to be tested. Juvenile crayfish are also protected by this value, at least in the short term. More long term tests, especially with early life stages, should be done to establish the lowest Cd concentrations that have long term effects on juvenile crayfish as they develop. It would also be important to establish in more detail the effects Cd has on the molting process. For

example, might it slow down the hardening of the new exoskeleton or reduce the maximum strength of the exoskeleton, either of which may have serious repercussions on crayfish survival.

Cadmium proved to have effects on crayfish behavior at low exposure levels in some cases. These alterations, especially to the tail-flip behavior, could have negative impacts on crayfish survival. More chronic tests of the effects of Cd on behavior would be useful to determine if behavioral effects can occur at more environmentally realistic concentrations. This is especially relevant for the early life stages of crayfish. Physiological markers such as heart rate could prove useful in measuring responses to subtle behaviors.

The work on the accumulation of Cd and redistribution of other metals offered some interesting possibilities. It showed that the uptake, detoxification, and depuration dynamics in crayfish are very intricate and may have multiple processes affecting several organ systems simultaneously, including physiological alterations to the hepatopancreas, gills, green glands, and possibly the exoskeleton. In the species tested, it seemed that the detoxification mechanisms included the liberation of other metals, especially Zn and Cu which then seem to have been eliminated from the body. The involvement of the gills, and green glands seem likely in toxicant heavy metal depuration, as well as the hepatopancreas.

The ability of crayfish to accumulate metals seems to be higher than most fish species, possibly in part caused by their access to a wide range of exposure pathways (respiratory, oral, sediment, *etc*.), but also because of their considerably different physiology. This indicates that they can serve as effective environmental monitors or sentinel species. They are capable of depurating some metals however, so metal found in

a crayfish might indicate a more recent exposure. Caged crayfish might serve as useful monitors of instantaneously biologically available metal over controlled periods of time, in comparison to wild caught crayfish which may be at steady state conditions. Metallothionein mRNA is a commonly used biomarker for metal exposure in fish species. Given that crayfish have heavy metal inducible MTs, it should be possible to develop crayfish MT mRNA as a useful biomarker for this important group of animals.

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Vita

Andrew J Wigginton
Born: January 7, 1973, Louisville, Kentucky

Educational Background

1987-1991: Saint Francis Xavier High School, Louisville, KY
1991-1995: Bachelor of Science with Honors, Zoology, University of Florida, Gainesville, Fl

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Recent Employment Information

2005- Spring, Teaching Assistant at the University of Kentucky, AS 500, Mammalogy
2004- Fall, Instructor BIO 340- Comparative Vertebrate Anatomy
2003 - 2004: Presidential Fellow at the University of Kentucky
1999- Summer: Instructor, Bluegrass Community and Technical College (formerly Lexington Community College), BSL 110 Human Anatomy and Physiology
1997 - 2003: Instructor at the University of Kentucky, Independent Study Program. BIO 103, Basic Ideas of Biology
1996 - 2003: Teaching Assistant at the University of Kentucky- Classes include: BIO 151, Principals of Biology I, Lab; BIO 153, Principals of Biology II, Lab; BIO 340, Comparative Vertebrate Anatomy, Lab, Lead TA; BIO 570, Invertebrate Zoology, Lab, Lead TA
1995 - 1996: Research Technician at the University of Florida, Center for Aquatic Plants and Nematology and Entomology Department.

Peer Review

2002: ASTM International. Symposium on Multiple Stressor Effects in Relation to Declining Amphibian Populations. Pittsburgh, PA. April 16-17, 2002

Committee Service

2002: Department of Biology, Director Search Committee- Student representative.

Awards

1991 - 1995: University of Florida, National Merit Scholarship ($10,000)
1996 - 1999: University of Kentucky, Daniel R. Reedy Quality Achievement Fellowship (Renewed for three years, $3000 each year)
2001: National Science Foundation - Summer Fellowship for study in Japan (~ $8000)
2002: University of Kentucky Association of Emeriti Faculty - Endowed Doctoral Fellowship ($1000)
2003: University of Kentucky Presidential Fellowship ($15,000)

Publications, Presentations, and Posters

I. Journal Articles

Peer reviewed article, Wigginton, A.J. and W.J. Birge. The toxicity of cadmium to six crayfish species in two genera and the effect of cadmium on molting. Submitted to Archives of Environmental Contamination and Toxicology in April, 2005.

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II. Book Chapters and Technical Reports

Book chapter, Westerman A.G, A.J. Wigginton, G. Linder, and W.J. Birge. 2003. Integrating amphibians into environmental risk assessment strategies. In: Sparling DW, Bishop C, and Linder G, editors. Amphibian Decline: An Integrated Analysis of Multiple Stressor Effects. Society of Environmental Toxicology and Chemistry, Workshop on Amphibian Declines; 18-23 Aug 2001; Racine, WI. Pensacola FL: SETAC. pp 283-314.

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III. Poster and Platform Presentations

Poster, Wigginton, A.J., H.C. Schoepp, D.J. Price, W.J. Birge. Effects of cadmium on metal body burden in four crayfish species in two genera. SETAC 24th Annual Meeting and 4th World Congress. Austin TX. November 14-18, 2004.

Poster, Wigginton, A.J., B.F. Brammell, J.R. Shaw, E.M. Frymann, and W.J. Birge. Variation in the toxicity of cadmium between four crayfish species in two genera. SETAC 24th Annual Meeting. Austin TX. November 9-13, 2003.

Poster, Price, D.J., A.G. Westerman, A.J. Wigginton, M.G. Beiting, G. Linder, and W.J. Birge. Evaluation of a proposed toxicity testing regime for amphibian species, Part- 1 Metals. SETAC 23rd Annual Meeting. Salt Lake City, UT. November 16-20, 2002.

Poster, Wigginton, A.J., D.J. Price, A.G. Westerman, M.G. Beiting, G. Linder, and W.J. Birge. Evaluation of a proposed toxicity testing regime for amphibian species, Part- 2 Organics. SETAC 23rd Annual Meeting. Salt Lake City, UT. November 16-20, 2002.

Poster, Wigginton, A.J., B.F. Brammell, J.R. Shaw, and W.J. Birge. Variation in the Toxicity of Cadmium Between Two Related Crayfish Species. SETAC 23rd Annual Meeting. Salt Lake City, UT. November 16-20, 2002.

Poster, Westerman, A.G., D.J. Price, A.J. Wigginton, M.G. Beiting, G. Linder, and W.J. Birge. Proposed Toxicity Testing Regime for Amphibian Species. Symposium on Multiple Stressor Effects in Relation to Declining Amphibian Populations, ASTM International. Pittsburgh, PA. April 16-17, 2002.

Presentation, Hogstrand, C., J.R. Shaw, A.J. Wigginton, and W.J. Birge. Interactions Within Metal Mixtures Affecting Aquatic Biota. 22nd SETAC Annual Meeting. Baltimore, MD. November 11-15, 2001

Poster, Wigginton, A.J., J. R. Shaw, and W.J. Birge. Variation in the Toxicity of Cadmium to the Crayfish *Orconectes rusticus*. 22nd SETAC Annual Meeting. Baltimore, MD. November 11-15, 2001.

Poster, Price, D.J., A.J. Wigginton, and W.J. Birge. Remediation and Bioassessment of Freshwater Stream Systems. 22nd SETAC Annual Meeting. Baltimore, MD. November 11-15 2001.

Poster, Birge, W.J. and A.J. Wigginton. Relationship of WET and Ambient Toxicity to Ecological Assessments in Impacted Freshwater Systems. 21st SETAC Annual Meeting. Nashville, TN. November 12-16, 2000.

Poster, Wigginton, A.J., W.J. Birge, and J.R. Shaw. Cadmium Effects on the Crayfish *Orconectes rusticus*. 21st SETAC Annual Meeting. Nashville, TN. November 12-16, 2000.

Presentation, Birge, W.J. and A.J. Wigginton. Reliability of Effluent Toxicity Testing (WET) in Estimating Ecological Impact of Surface Waters. 3rd SETAC World Congress and 10th Annual Meeting of SETAC Europe. Brighton, United Kingdom. May 21-25, 2000.

Presentation, Birge, W.J. and A.J. Wigginton. U.S. EPA Effluent Biomonitoring Program (WET)-Reliability in Estimating Ecological Impact in Surface Waters. 20th SETAC Annual Meeting. Philadelphia, PA. November 14-18, 1999.

Presentation, Birge, W.J., A.G. Westerman, and A.J. Wigginton. Use of Toxicity Data in Amphibian Risk Assessment. 20th SETAC Annual Meeting, Philadelphia, PA. November 14-18, 1999.

Presentation, Sofyan A., J.R. Shaw, J. Schmittschmitt, A.J. Wigginton, and W.J. Birge. Accumulation and Trophic Transfer of Metals from Algae to Cladocerans. 20th SETAC Annual Meeting. Philadelphia, PA. November 14-18, 1999.