Acute and Chronic Exposure to Lead: Cellular and Behavioral Effects on the Pond Snail *Helisoma trivolvis*

Virginia T. Bennett

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ACUTE AND CHRONIC EXPOSURE TO LEAD: CELLULAR AND BEHAVIORAL EFFECTS ON THE POND SNAIL HELISOMA TRIVOLVIS

Virginia T. Bennett
Acute and Chronic Exposure to Lead: Cellular and Behavioral Effects on the

Pond Snail *Helisoma trivolvis*

by

Virginia T. Bennett

A Thesis Submitted to the Faculty
of the College of Graduate Studies
At Georgia Southern University
in Partial Fulfillment of the
Requirements for the Degree of
Master of Science
in the Department of Biology

Statesboro, Georgia

July, 1997
Acute and Chronic Exposure to Lead: Cellular and Behavioral Effects on the Pond

Snail *Helisoma trivolvis*

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7-23-97 Date
Acknowledgments

This work could not have been done without the help and support of many people. I thank my family, especially my husband, Bill, for their patience and understanding. I also thank Dr. Alison Morrison-Shetlar for allowing me to do two jobs at once, the one I'm paid for and this research. I extend a special thanks to the many faculty members of the Department of Biology and the Department of Chemistry who instructed me and encouraged me here at Georgia Southern. My gratitude also goes to the graduate student body of the Department of Biology at Georgia Southern, in particular the Teaching Assistants. Their professionalism and camaraderie while I was their Laboratory Coordinator allowed me to continue my research and motivated me to set an example.

I am especially grateful to Dr. Norman Schmidt for working with me in the Department of Chemistry, and to my committee members, Dr. Steven Vives, Dr. Oscar Pung and Dr. Robert Shetlar for their suggestions and guidance. To the chair of my committee, Dr. Jonathan Copeland: A conversation many summers ago sparked what I consider a lifelong interest in animal behavior. I cannot begin to express my gratitude and admiration for your knowledge and guidance throughout the last several years. Finally, I would like to extend my deepest thanks to Ms. Ruth Snider, who persuaded me that you are never too old to set a new course.
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Introduction

The focus of this study is on the effects of lead on Helisoma trivolvis, a pulmonate pond snail. Many species of molluscs are being used as bioindicators of environmental toxins. To provide a background for the reader, a brief history and description of the Phylum Mollusca (at the class level) is presented in Chapter 1. A second chapter (Ch. 2) is concerned with lead and its effects. A synopsis of past and present studies that show the interaction between lead and molluscs is given in Chapter 3, then two experimental chapters follow. The first experimental chapter (Ch. 4) is concerned with the effects of lead on the behavior of H. trivolvis. The second experimental chapter (Ch. 5) is concerned with lead accumulation in the tissue of H. trivolvis.
Phylum Mollusca is the second largest of all the invertebrate groups (Pearse et al., 1987; Brusca and Brusca, 1990). Its name means soft body and many of its members are easily recognized (Pearse et al., 1987; Barnes, 1987; Brusca and Brusca, 1990). Molluscs have been a food item since prehistoric times (Hyman, 1967; Brusca and Brusca, 1990), and their shells used as tools, ornaments and as a means of bartering (Hyman, 1967; Brusca and Brusca, 1990). Aristotle gave the first known description and classification of molluscs, but the present classification was not adopted until the nineteenth century (Hyman, 1967; Brusca and Brusca, 1990).

The most commonly cited classes are Aplacophora, Bivalvia, Cephalopoda, Gastropoda, Monoplacophora, Polyplacophora, and Scaphopoda (Pearse et al., 1987; Barnes, 1987; Brusca and Brusca, 1990)(Fig. 1). A detailed description of the Gastropods, beginning with *Helisoma trivolvis* (the mollusc of interest), is provided. A brief history and description of each of the other classes will follow.

*Helisoma trivolvis*: *Helisoma trivolvis* (Phylum Mollusca, Class Gastropoda, Subclass Pulmonata, Order Basommatophora), the animal of interest in this study, is a planorbid snail with several characteristics desirable in a test animal (Fig. 2).
Figure 1. A general evolutionary tree of the Mollusca. Hypothetical ancestral molluscs are not represented (Reproduced from Brusca and Brusca, 1990).
Figure 2: A. Diagram of *Helisoma trivolvis* with mantle cavity open (approximately 4 times the size of the laboratory snails). B. External view of living snail with foot extended (approximately 3 times the size of the laboratory snails).
Fig. 2

antenna
buccal mass
penis
vas deferens
anus
digestive gland

prostate

oviduct
alburem gland
stomach
intestine

ovotestis A.

pseudobranch

B.
A considerable amount of literature exists concerning its biology: neurobiology by Kater (1974, 1994), Culver et al. (1993), Berdan and Bulloch (1987, 1990), Berdan et al. (1990, 1993), Gelperin (1994) and Goldberg et al. (1993, 1994); physiology by Terwilliger et al. (1976, 1977), Diefenbach (1990, 1991) and Ilan et al. (1986); and behavior by Paraense and Correa (1988), and Covich (1994). Like other species in the Order Basommatophora, it is a simultaneous hermaphrodite that will self-fertilize if left alone and readily cross-fertilize when placed with other individuals (Calow, 1978; Friesen, 1981; Geraerts and Joosse, 1984; Paraense and Correa, 1988) (Fig. 3). It can tolerate sub-optimal conditions such as low oxygen, high carbon dioxide levels and changes in pH, as well as a variety of pollutants (Harman, 1974). Reproductive success under these conditions, however, is limited (Harman, 1974). It has a high molecular weight hemoglobin which may provide for much more efficient oxygen transport than hemoglobin of non-planorbid snails (Terwilliger et al., 1976, 1977; Ilan et al., 1986). Life span of the snail is between one and five years in the field (Morris, 1970; Eversole, 1978; Boerger, 1975), with a slightly lower range seen in laboratory studies (Friesen, 1981). While its reproduction is seasonal in the field, laboratory conditions prolong the reproductive cycle (Geraerts and Joosse, 1984).

Figure 3:  A. Reproductive organs of *Helisoma trivolvis*: 1) ovotestis, 2) hermaphroditic duct, 3) seminal vesicles, 4) albumen gland, 5) fertilization pouch, 6) sperm duct, 7) prostate gland, 8) penial complex, 9) male gonopore, 10) oothecal gland, 11) oviduct, 12) vagina, 13) spermatheca, 14) female gonopore (replicated from Hyman, 1967).

B. Generalized nervous system of a planorbid snail: 1) cerebral ganglion, 2) pleural ganglion, 3) pedal ganglion, 4) pleurovisceral connectives, 5) parietal ganglion, 6) visceral ganglion, 7) buccal ganglion, 8) visceral chain (reproduced from Hyman, 1967).

C. Musculature of the gastropod foot (replicated from Brusca and Brusca, 1990).
Fig. 3

A. [Diagram showing labeled parts 1-13.]

B. [Detailed diagram showing structures labeled from 1 to 13.]

C. [Labeling details: Right retractor, Analus, Mantle cavity, Left retractor, Head, Foot, Operculum.]
Studies examining the neurobiological basis for feeding (Kater, 1974) and intracellular calcium regulation and homeostasis (Rehder et al., 1991; Kater and Shibata, 1994) have provided the foundation for research comparing the interaction of toxicants, such as lead, with normal physiological functioning (Newman and MacIntosh, 1983; Simkiss and Mason, 1983; Bianchi et al., 1993; Truscott et al., 1995). Thus, normal behavior of molluscs, including H. trivolvis, and the normal action of toxicants have been studied. Chapter 2 provides a detailed description of the interaction between lead and calcium homeostasis.

**Molluscan Classes: General Physiology and Habitats**

**Gastropoda:** The class Gastropoda is the largest of all the molluscan classes. It is comprised of about 40,000 living species of marine, terrestrial and freshwater snails and slugs (Russell-Hunter, 1968; Fretter, 1974; Martin, 1983; Barnes, 1987). Along with this large number of living species there are considerable differences in body plans, habitats, and reproductive strategies (Hyman, 1967; Russell-Hunter, 1968; Fretter, 1974; Brusca and Brusca, 1990). To attempt to classify the gastropods in a simple manner, they will be divided into their three subclasses for discussion purposes: Subclass Prosobranchia, Subclass Opisthobranchia and Subclass Pulmonata. Each of these subclasses will further be divided into order. The only species addressed in considerable detail will be *Helisoma trivolvis*.

**Subclass Prosobranchia:** Animals within this subclass generally have a spirally coiled shell or one that is cap-shaped or tubular (Hyman, 1967; Brusca and Brusca, 1990). The majority in this group are marine with only a few land and
freshwater species (Hyman, 1967; Pearse et al., 1987; Brusca and Brusca, 1990). They are torted gastropods with the mantle cavity located anteriorly in front of the visceral mass (Hyman, 1967). The mantle cavity contains the pallial complex, osphradia, ctenidia, hypobranchial glands, anus and nephridiopores (Hyman, 1967; Brusca and Brusca, 1990). The head generally has tentacles with basal eyes (Brusca and Brusca, 1990). Upon retraction of the head and foot, there is a corneous (horn-like texture) or calcareous operculum and the foot consists of a creeping planar sole (Pearse et al., 1987; Brusca and Brusca, 1990). In those prosobranchs with a cleft or perforated shell, the most primitive type of gill structure is seen; this may be a solution to possible sanitation problems caused by torsion (Barnes, 1987). The rectum and anus are removed from the edge of the mantle cavity and open beneath the perforation or cleft, thus keeping excreted waste out of the mantle (Barnes, 1987; Pearse et al., 1987). In general, prosobranchs are dioecious, but sexes are not easily identified (Hyman, 1967). In species where fertilization is external, spawning occurs at various times depending on the habitat of the species (Hyman, 1967). All mesogastropods have internal fertilization and eggs are laid in capsules or oothecae that range from soft to a hard consistency (Brusca and Brusca, 1990). Many prosobranchs brood their eggs; some in the mantle cavity and some have uterine brooding (Hyman, 1967). Development ranges from hatching into a free-swimming planktonic larva to hatching as a small snail complete with shell (Hyman, 1967). Only a few species produce free-swimming trochophore larva (Hyman, 1967).
The most primitive of the prosobranchia belong to the Order Archaeogastropoda (Brusca and Brusca, 1990). These animals are primarily marine and include such members as the abalones (*Haliotis*), keyhole limpets (*Diodora, Puncturella*), true limpets (*Acmaea, Patella*), nerites (*Nerita*), and the trochids (*Calliostoma, Trochus*). Their shell has a pearly or iridescent layer, and the radula has been modified for herbivory and is often found with numerous teeth in transverse rows (Brusca and Brusca, 1990). They have one to two bipectinate ctenidia, a mantle cavity without a siphon, and nervous system is weakly concentrated (Brusca and Brusca, 1990). They also have two hypobranchial glands, two osphradia, two atria and two metanephridia (Brusca and Brusca, 1990). The sexes are usually separate with the male lacking a penis (Brusca and Brusca, 1990). Gametes are discharged via the gonopericardial ducts into the pericardial chamber (Brusca and Brusca, 1990). They are picked up by the kidneys and eventually released into the surrounding sea water (Brusca and Brusca, 1990).

The Order Megogastropoda includes about 10,000 species of marine, freshwater and terrestrial animals (Brusca and Brusca, 1990). Some examples are the periwinkles (*Littorina*), turret shells (*Turritella*), ceriths (*Cerithium, Liocerithium*), conchs (*Strombus*), horse hoof limpets (*Hipponix*), cap limpets (*Capulus*), slipper shells (*Crucibulum*), moon snails (*Natica, Polinices*) and helmet shells (*Cassis*) (Hyman, 1967; Brusca and Brusca, 1990). The shells of these gastropods are mostly porcelain-like and nonnacreous, the operculum rarely calcified, the head with a pair of cephalic tentacles and basal eyes (Hyman, 1967; Brusca and Brusca, 1990). The mantle cavity
is asymmetrical with an incurrent opening on the anterior left which may lead to an
inhalant siphon (Brusca and Brusca, 1990). The right ctenidium is lost, and the left is
usually monopectinate (Brusca and Brusca, 1990). The radula is generally
taenioglossate (number of marginal teeth reduced), and most are dioecious and higher
forms have a concentrated ganglia (Hyman, 1967; Brusca and Brusca, 1990).

Species in the Order Neogastropoda are comprised of about two dozen families
of marine snails, including whelks (*Buccinum, Macron, Metula*), Columbellidae
(*Anachis, Columbella, Nassarina*), tulip shells (*Fasciolaria, Leucozonia*), false
trumpets (*Melongena*), olive shells (*Agaronia, Oliva, Olivella*), cone shells (*Conus*) and
auger shells (*Terebra*) (Hyman, 1967; Brusca and Brusca, 1990). They have shells
without a nacreous layer, a rachiglossate or toxoglossate radula with one to three teeth
in each row, one left monopectinate ctenidium, one osphradium and a chitinous
operculum when present (Hyman, 1967; Brusca and Brusca, 1990). Sexes are separate
with the male possessing a penis. The mantle forms a siphon which is contained within
the siphonal canal or notch of the shell, the heart has the left atrium only and the right
nephridium is lost (Hyman, 1967; Pearse *et al.*, 1987; Brusca and Brusca, 1990).

**Subclass Opisthobranchia:** This group includes the sea slugs which are
primarily benthic marine organisms with a few freshwater species (Brusca and Brusca,
1990). They are detorted with exposed conical shells or a shell concealed in the
mantle. They usually lack an operculum, have one gill or have respiratory substitutes
in the form of dorsal outgrowths (Hyman, 1967). The radula varies from species to
species and is not used as a means of classification (Hyman, 1967; Brusca and Brusca,
1990). Their head has one to two pairs of rhinophores or tentacles, and they possess one hermaphroditic reproductive system (Hyman, 1967). While most are simultaneous hermaphrodites, some are protandric (Hyman, 1967). The size of opisthobranchs range from 1.5 millimeters to 400 millimeters in length (Hyman, 1967).


**Subclass Pulmonata:** There are over 35,000 species of pulmonates living throughout temperate and tropical regions of the world, with 16,000 of them described in some detail (Kaestner, 1967; Barnes, 1987). Most are untorted, found in both freshwater and terrestrial environments, and usually have a spiral shell (Hyman, 1967). Those who undergo torsion only exhibit about 90° of torsion; the mantle cavity never develops an extensive opening (Fretter and Graham, 1976). Pulmonates are considered the most highly evolved gastropods; the mantle cavity has been converted into a lung (Kaestner, 1967; Russell-Hunter, 1968; Barnes, 1987; Brusca and Brusca, 1990). Respiration is facilitated by the arching and flattening of the mantle cavity floor, with the pneumostome generally open at all times in terrestrial species but closed
while submerged in aquatic species (Barnes, 1987; Pearse et al., 1987). The nervous system is highly developed, with the ganglia concentrated in the head, and the shell is usually well developed and whorled rather than spiral (Kaestner, 1967; Brusca and Brusca, 1990). The circulatory system is less open than other gastropods with the pericardial sac enclosing the heart lying in the roof of the pulmonary sac (Hyman, 1967). Most are terrestrial, respiring with atmospheric oxygen, although some aquatic pulmonates take oxygen from the water as well (Kaestner, 1967). Ctenidia are lacking in all species (Russell-Hunter, 1968). There is a wide range of feeding habits: herbivores, carnivores, scavengers, deposit feeders, and suspension feeders (Hyman, 1967; Kaestner, 1967; Russell-Hunter, 1968; Barnes, 1987). Shape and size of teeth in the radula vary depending on feeding preferences (Barnes, 1987; Pearse et al., 1987; Brusca and Brusca, 1990).

Pulmonates are simultaneous hermaphrodites that employ copulation but some species will self-fertilize if necessary (Russell-Hunter, 1968; Barnes, 1987). In many species, eggs develop into the veliger stage within the egg capsule and emerge as juveniles (Buchsbaum et al., 1987). Development in many freshwater species is without the veliger stage (Buchsbaum et al., 1987).

There are three orders within the pulmonates: Archaeopulmonata, Basommatophora and Stylommatophora. The archaeopulmonates are considered the most primitive of the orders with a spirally coiled shell but no operculum. They are mainly littoral genera such as Cassidula, Ellobium and Otina (Brusca and Brusca, 1990). The stylommatophorans may or may not have a shell (Brusca and Brusca,
1990). If present, it is usually spirally coiled, in some partly or completely enveloped by the dorsal mantle (Brusca and Brusca, 1990). Eyes are generally on the tips of the sensory stalks and an osphradium is usually not present (Hyman, 1967; Brusca and Brusca, 1990). They are terrestrial and mostly herbivorous (Kaestner, 1967).

The basommatophorans have one pair of tentacles, the eyes are never stalked or on tentacles and the shell is well developed (Kaestner, 1967). The pneumostome is drawn out into a tube or siphon in some freshwater genera such as Planorbis, Lymnaea and Physa (Hyman, 1967). In some basommatophorans there is an accessory structure called the pseudobranch that is analogous to ctenidium (Hyman, 1967). The nervous system of the basommatophorans is of particular interest to researchers as it is detorted with ganglia concentrated into a ring and is easily manipulated (Hyman, 1967; Kater, 1974).

The reproductive system of the basommatophora is complex (Geraerts and Joosse, 1984). There are separate male and female gonopores; the male gonopore is always anterior to the female pore and close to the right tentacle while the female pore is usually found near the pneumostome (Hyman, 1967). Most basommatophorans have an eversible or protrusible penis (Hyman, 1967; Kaestner, 1967). Male and female gametes are produced in a single ovitestis which is found in the apical area of the visceral hump (Geraerts and Joosse, 1984). Male and female gametes are then passed through separate pathways with the female duct secreting material for the formation of eggs and egg masses, as well as to receive sperm (Geraerts and Joosse, 1984). All species have an albumen gland and a bursa copulatrix (Runham, 1983). In most
basommatophorans, the entire embryonic development takes place within the egg; morula, blastula, gastrula, trochophore and veliger stages (Geraerts and Joosse, 1984). At hatching, the egg membranes are cut open using the radula, and the body is completely released by movements of the foot and body. In planorbid snails, an enzyme is used to liquify the jelly, allowing the juvenile to free itself (Brahmachary, 1983). These snails immediately attach themselves to a substrate and are able to feed (Geraerts and Joosse, 1984).

Aplacophora: Members of the class Aplacophora (the solenogasters) are benthic marine organisms that live in depths from 18 to 8,000 meters and are absent from intertidal zones (Hyman, 1967; Barnes, 1987; Pearse et al., 1987; Brusca and Brusca, 1990). About 250 species have been described thus far, and have been found primarily through dredging (Hyman, 1967; Barnes, 1987; Pearse et al., 1987; Brusca and Brusca, 1990). They are small, worm-like molluscs that lack a shell and a flattened foot and have a poorly developed head (Barnes, 1987; Pearse et al., 1987; Brusca and Brusca, 1990). They range in size from 1 to 50 mm in length, and are covered with calcareous spicules (Hyman, 1967; Barnes, 1987; Brusca and Brusca, 1990). The radula, which classifies them as Mollusca, is imbedded directly in a basal expansion of the foregut, with or without a cuticle (Hyman, 1967; Pearse et al., 1987). With the exception of the Chaetodermatidae, aplacophorans are hermaphrodites, producing trochophore larvae, but they do not fertilize their own eggs (Hyman, 1967; Barnes, 1987; Pearse et al., 1987). Diet seems to consist primarily of cnidarians (Hyman, 1967; Barnes, 1987). Although extensive descriptions of their body plans can
be found, very little is known about their life cycles (Hyman, 1967; Barnes, 1987; Pearse et al., 1987; Brusca and Brusca, 1990).

**Bivalvia:** The class Bivalvia includes clams, oysters, mussels and scallops (Brusca and Brusca, 1990). There are both marine and freshwater species that have a shell that is typically two valves hinged together dorsally by an elastic ligament and shell teeth (Brusca and Brusca, 1990). They have a rudimentary head without eyes, but eyes may occur elsewhere on the body (Brusca and Brusca, 1990). The foot is generally laterally compressed, usually without a sole; they have one pair of large bipectinate ctenidia to aid in ciliary feeding; they possess a large mantel cavity with the posterior edges often fused to form inhalant and exhalant siphons; and they have one pair of nephridia (Brusca and Brusca, 1990). There are over 8,000 living species found at all depths in the marine environment (Barnes, 1987; Brusca and Brusca, 1990). Bivalves lack a radula and are almost exclusively filter feeders (Pearse et al., 1987; Brusca and Brusca, 1990). They range in size from less than a millimeter long to over 1.5 meters long (Pearse et al., 1987). The majority of marine bivalves are dioecious with two gonads encompassing the intestinal loops (Barnes, 1987). Many species have external fertilization with sperm and egg released into the water (Pearse et al., 1987). Those with internal fertilization release their zygotes which then develop into free-swimming trochophores and then veligers (Pearse et al., 1987). Most of the freshwater bivalves are hermaphrodites that self-fertilize; this aids in their wide distribution (Barnes, 1987; Pearse et al., 1987).
Cephalopoda: This class contains the nautili, cuttlefish, squids and octopods. They are marine animals and are, for the most part, adapted for swimming rather than bottom dwelling (Barnes, 1987; Brusca and Brusca, 1990). There are currently around 650 living species and 7500 fossil forms (Barnes, 1987; Brusca and Brusca, 1990). Cephalopods are considered the most highly organized of the molluscs with a head that projects into a circle of large prehensile tentacles that are considered homologous to the anterior of the foot of other molluscs (Barnes, 1987). Only the nautili have a completely developed shell; it is reduced and internal in the cuttlefish and squid and completely lacking in the octopods (Barnes, 1987; Buchsbaum et al., 1987; Pearse et al., 1987). Average sizes range from six to seventy centimeters in length (Barnes, 1987; Pearse et al., 1987; Brusca and Brusca, 1990). However, the cephalopods have the largest known size of any invertebrates because of members such as the giant squid, Architeuthis, which has been reported to have reached 16 meters in length (Barnes, 1987; Buchsbaum et al., 1987). There is a beak-like jaw in addition to the radula located in the buccal cavity that allows the animal to tear off tissue that is then pulled into the buccal cavity by the radula (Barnes, 1987; Buchsbaum et al., 1987; Pearse et al., 1987; Brusca and Brusca, 1990). Cephalopods are predatory carnivores (Brusca and Brusca, 1990). Diet depends on habitat: pelagic squids feed on fish, crustaceans and other squids (Macy, 1982), cuttlefish feed on invertebrates such as shrimp and crab (Barnes, 1987), octopods eat a variety of snails, crustaceans and fish (Barnes, 1987), and nautili are scavengers and chiefly eat decapod crustaceans (Barnes, 1987; Pearse et al., 1987).
Cephalopods are dioecious with a single gonad located at the posterior of the body (Barnes, 1987; Pearse et al., 1987; Brusca and Brusca, 1990). Sperm is packaged into spermatophores found in the Needham's sac, a large storage area which opens into the left side of the mantle cavity (Barnes, 1987; Brusca and Brusca, 1990). Females possess an oviduct that terminates in an oviductal gland; in some species of squid and octopods there are two oviducts present (Barnes, 1987). Fertilization can take place either inside or outside of the mantle cavity and involves copulation (Barnes, 1987; Brusca and Brusca, 1990). Eggs are released into the water or on the substrate, depending on the species (Brusca and Brusca, 1990).

Cephalopods have direct development with eggs absorbing the yolk prior to hatching (Barnes, 1987; Brusca and Brusca, 1990). The hatching cephalopod may be planktonic for a short time and usually lives at a higher level than the adult benthic cephalopods (Barnes, 1987). Many cephalopods die following spawning (Home, 1974), but the nautili may live up to twenty years (Saunders, 1983).

**Monoplacophora:** There are only a few living species of monoplacophorans. Until the early 1950s it was believed that this class was extinct with fossils dating back to the Cambrian (Barnes, 1987; Pearse et al., 1987; Brusca and Brusca, 1990). They are comprised of untorted limpet-like organisms that are found in deep-water oceans (Hyman, 1967; Barnes, 1987; Pearse et al., 1987; Brusca and Brusca, 1990). They are about three cm in length and have a single cap-like shell, a shallow mantle cavity surrounding a foot with five to six pairs of ctenidia, two pairs of gonads, two pairs of heart atria, a radula, and lacking eyes (Hyman, 1967; Barnes, 1987; Brusca and
Brusca, 1990). They are characterized by their repeated organs (Pearse et al., 1987). There are at present eleven known species in three genera (Brusca and Brusca, 1990). The monoplacophorans feed on the bottom mud of the ocean floor which is rich with microscopic organisms (Hyman, 1967). There is little known about their reproductive habits except that they are dioecious with two pairs of lobulated gonads found in a blood sinus below the intestine and midgut gland (Hyman, 1967). It is believed that the second and third pairs of nephridia are used for sexual reproductive purposes (Hyman, 1967). As with the aplacophorans, little is known about their development.

**Polyplacophora:** Known as the chitons, these animals have a series of eight shell valves that resemble armour plating (Hyman, 1967; Pearse et al., 1987). They are sedentary or slow moving marine animals that feed on algal growth or animals that encrust rocks (Barnes, 1987; Brusca and Brusca, 1990). While most live in intertidal zones, a few have been dredged from depths of up to 4,000 meters (Pearse et al., 1987). At present, around 600 species have been described (Brusca and Brusca, 1990).

The generalized body plan of the chitons consists of a flattened, elongated body with a broad ventral foot; eight shell valves as mentioned above; a well developed radula with 17 teeth in the transverse direction (Hyman, 1967); a mantle that forms a thick girdle that borders and may partially cover the shell plates (Brusca and Brusca, 1990); a mantle cavity that contains from six to more than eighty pairs of ctenidia; one pair of nephridia; and lacking eyes, tentacles or a crystalline style (Hyman, 1967; Barnes, 1987; Pearse et al., 1987; Brusca and Brusca, 1990). They range in size from
three millimeters to forty centimeters, with most falling between three to twelve centimeters in length (Barnes, 1987).

Most chitons are dioecious, although one species, *Trachydermon raymondi*, is known to be hermaphroditic (Hyman, 1967). A single gonad is located just anterior to the pericardial cavity under the middle shell plates (Hyman, 1967; Barnes, 1987). Gametes are transported to the outside via two gonducts rather than by nephridia (Barnes, 1987). The gonopore is located in each pallial groove in front of the nephriidiopore (Barnes, 1987; Brusca and Brusca, 1990). Fertilization occurs either in the sea, as sperm is released from the male in the exhalant currents and eggs are released one or two at a time from the female, or within the mantle cavity of the female (Hyman, 1987; Barnes, 1987). There is a free swimming trochophore stage with the exception of a few species who brood their eggs within the mantle cavity (Barnes, 1987). There is no veliger stage (Hyman, 1967; Barnes, 1987; Brusca and Brusca, 1990).

**Scaphopoda**: The class Scaphopoda is comprised of approximately 350 species of burrowing marine molluscs (Brusca and Brusca, 1990). The majority of the scaphopods burrow in sandy ocean bottoms at depths of more than six meters (Barnes, 1987). They are commonly called tusk or tooth shells because of the unique shape (Hyman, 1967; Barnes, 1987; Pearse *et al.*, 1987; Brusca and Brusca, 1990). The scaphopod body is elongated and tube-like along the anterior-posterior axis with both ends of the shell open (Barnes, 1987; Pearse *et al.*, 1987). They live buried in the sand with their head downward, and only the posterior aperture is above the surface of
the sand (Barnes, 1987). The mantle cavity of the scaphopod is large, extending along the entire length of the ventral surface (Pearse et al., 1987; Brusca and Brusca, 1990). Scaphopods lack eyes, a heart and ctenidia but have a well developed radula with large flattened teeth (Barnes, 1987; Brusca and Brusca, 1990). They also possess paired clusters of clubbed contractile tentacles to aid in prey capture, as well as a proboscis and crystalline style (Brusca and Brusca, 1990). They range in size from less than five millimeters to close to fifteen centimeters, the average being between three to six centimeters (Barnes, 1987; Pearse et al., 1987). Scaphopoda feed on microscopic organisms, particularly forams, by capturing them with their many tentacles some numbering up to 100) and then brought to the mouth via cilia (Barnes, 1987; Pearse et al., 1987).

Scaphopods are dioecious with external fertilization (Barnes, 1987). They have one unpaired gonad that fills most of the posterior of the body (Barnes, 1987). Sperm and egg are expelled through the right nephridium with eggs shed singly (Pearse et al., 1987). Eggs are planktonic and develop into a free-swimming trochophore and veliger larva (Barnes, 1987; Pearse et al., 1987; Brusca and Brusca, 1990).

The next two chapters will discuss lead. First, there is a brief history of lead toxicity and the effects of lead on humans. This is followed by a chapter that examines molluscan exposure to lead and will discuss the physiological effects of lead as well as the usefulness of snails as bioindicators of lead and other pollutants.
Work Cited


Chapter 2:

Lead Toxicity

Lead is a widely studied metal toxin (Carrington and Bolger, 1992; Bradbury and Deane, 1993; Preuss, 1993; Simons, 1993). It has been listed as a poison since ancient times (Gilfillan, 1965; Goyer and Rhyne, 1973; Rice, 1990) and, because of its widespread use as a sweetener of wine by Romans, has even been cited as a possible cause of the fall of the Roman Empire (Gilfillan, 1965). The term toxin is generally used to describe toxic substances that are produced naturally and cause harmful changes in behavior and or physiology (Eaton and Klaassen, 1996). There is no known biological need for lead (Goyer, 1996). However, in spite of its known toxicity (Rice, 1990; Tuormaa, 1996), it is still used extensively throughout the world for industrial purposes (Tuormaa, 1996). In 1977, the EPA stated that lead has no known health or biological benefits and must therefore be viewed as a deleterious agent. Until recently, it was thought that lead was toxic only at high concentrations (Rice, 1990; Tuormao, 1994; Goyer, 1996). Results from numerous studies show that much lower levels of lead are toxic (Needleman et al., 1979; Gilbert & Rice, 1987; Needleman & Gatsonis, 1990; Rice, 1990; Needleman, 1994; Goyer, 1996). The question then becomes what are the lowest levels of lead that adversely affect living organisms?
**Sources of Lead**

Exposure to lead is due in part to natural sources, but most of the present lead contamination is through anthropogenic sources (Krigman *et al.*, 1980; Schaule and Patterson, 1981; Boyle *et al.*, 1986; Tuormaa, 1994; Abdel-Moati, 1996; Schuhmacher and Domingo, 1996). Human industrial activity has increased the lead content in Greenland ice almost 200 fold since about 800 B.C. (Ng and Patterson, 1981). Exposure is commonly through food, water, industrial sites, automobile exhaust and mining (Rice, 1990; Linder, 1991; Shank and Carson, 1992; Tuormaa, 1994; Kotsonis *et al.*, 1996). Lead is used in ceramic ware glazes, and this allows it to leach out into food (Shank and Carson, 1992). Although some countries, such as the United States and Great Britain, have banned the use of lead in ceramics (Kotsonis *et al.*, 1996), most of the ceramics purchased in the United States are imported from countries that have no such ban (Kotsonis *et al.*, 1996). Additional lead contamination of food includes wheat flour (Linder, 1991). Here, lead absorbed by the plant survives food processing (Linder, 1991). In 1995, Carvalho found that 79% of radioactive lead ($^{210}\text{Pb}$) ingested by adults in and around Lisbon, Portugal, is through consumption of cereals and vegetables. Vegetables and plants used in the production of cereals in Portugal take up radioactive lead found in naturally occurring uranium series radionuclides in the environment (Carvalho, 1995). In countries that have not yet banned it, lead-soldered seams in food cans contributes to lead exposure (Kotsonis *et al.*, 1996). Small quantities of lead are also found in calcium supplements (Shank and Carson, 1992; Kotsonis *et al.*, 1996). To date, there are no regulations issued by the
United States government to outlaw lead in calcium supplements (Shank and Carson, 1992; Kotsonis et al., 1996).

Lead that is in drinking water is still a major concern in the United States and other countries (Tuormaa, 1994). While the water from pumping stations may have an acceptable lead content, many houses still have lead pipes which release the lead into the tap water (Dojlido and Best, 1993; Tuormaa, 1994). After being left in contact with lead plumbing overnight, Dojlido and Best (1993) reported that water analyzed to have less than 50 μg Pb/L (after running for several minutes) had up to 2600 μg Pb/L when tested at first “run off”. This occurs when lead particles from the lead plumbing bind to stagnant water in the pipes during periods of non-use (Dojlido and Best, 1993; Kendall et al., 1996). This is an even greater problem in areas where water is soft, slightly acidic or the soil has a high peat content, thus promoting the release of lead (Bryce-Smith and Waldron, 1974). Lower pH in these areas increases the solubility of lead, thus adding to its bioavailability (Kendall et al., 1996). Added to the problem are private wells that have little to no regulation concerning lead levels or testing procedures (Montgomery, pers. comm.). At present, the EPA lists the maximum contamination level (milligrams per liter) as under review but sets its ultimate goal at zero (Doull, 1996).

Contaminated drinking water represents only a small percentage of overall water contamination by lead. While much of the focus has been on the effects of lead on humans, there is ongoing research to assess the effect of lead on aquatic organisms living in contaminated waterways (Rainbow and Dallinger, 1993; Timmermans, 1993).
For benthic organisms, exposure to lead is increased due to the fact that they are living in an environment with both dissolved lead and precipitated lead (Rainbow and Dallinger, 1993; Bonaventura, Bonaventura and Bodishbaugh, 1996). While the levels of dissolved lead in surface waters are seldom higher than 20 μg/L (Dojlido and Best, 1993), benthic organisms are also in direct contact with lead precipitate once the saturation point between lead and water is exceeded (Salanki, Balogh and Berta, 1982; Abdel-Moati, 1996; Bonaventura, Bonaventura and Bodishbaugh, 1996). The interaction between lead contaminated waterways and invertebrates will be discussed in detail in Chapter 3.

Perhaps the greatest contributor to the high levels in lead contamination during the post-technology era was the advent of leaded gasoline (Elias et al., 1975; Krigman et al., 1980; Rice, 1990; Tuormaa, 1994; Abdel-Moati, 1996). Prior to the common practice of lead being added as an anti-knock agent to gasoline, the Surgeon General warned in 1926 of the widespread use of lead in gasoline as a potentially health threatening agent (Rosner and Markowitz, 1985). However, it was not until 1983 that lead was officially banned from gasoline in the United States, and since that time airborne lead levels have dropped 90 percent (Costa and Amdur, 1996). There are many countries, however, that still use leaded gasoline (Abdel-Moati, 1996; Costa and Amdur, 1996). The former Soviet Union, many Middle Eastern countries and Central and South America still have not banned leaded gasoline and are experiencing high levels of airborne lead emissions around areas heavily congested with automobiles (Rice, 1990; Abdel-Moati, 1996; Costa and Amdur, 1996). In those areas that are
close to coastal waters, many aquatic organisms have a higher lead concentration in their tissues than those further from the source of lead (Abdel-Moati, 1996; Chevreuil et al., 1996; Schuhmacher and Domingo, 1996). No mention was made concerning the percentage of lead found in tissue samples attributed directly to airborne lead pollution.

Lead has a variety of uses in industry. In addition to being used as an additive in gasoline until 1983, lead was used in the 1950s in hair dyes (Smith and Carson, 1981), and, until the ban that went into effect in 1977, as a pigment enhancer in indoor paint (Krigman et al., 1980; Rice, 1990; Goyer, 1996). At present, 80 percent of lead mined in the United States is used in automobile batteries, of which greater than 95 percent are recycled (Lead Industry Association, pers. comm.). Although no longer used in indoor house paints, lead continues to be used in paint for adherence to metals (Goyer, 1996). It is also used extensively in electronic components such as computer monitors, radar and televisions, and in other commercial and industrial products such as cathode ray tubes; radiation shielding; flat roofs; lead plating; photo developing; ammunition; and in new building foundations as a protection against earthquake damage (Lead Industry Association, pers. comm.; Krigman et al., 1980; Goyer, 1996).

The current production of lead worldwide exceeds 4.5 million tons annually (Tuormaa, 1996), and the American Miner’s Association (1997) estimates that the production of lead will reach 5 million tons by the year 2000. The United States used 1.5 million metric tons of lead in 1995 and close to one million metric tons in 1996; ninety percent of the lead used was in the form of storage batteries (American Bureau of Metal
Statistics, 1997). Current and past world production of lead is higher than any other toxic metal, including zinc, cadmium and nickel (National Academy of Sciences, 1972; Rice, 1990; American Bureau of Metal Statistics, 1997). Despite stringent safety specifications, lead dust consists of such fine particles that it is virtually impossible to keep it all of it from becoming airborne during mining operations (Krigman et al., 1980; Goyer, 1996; Tuormaa).

Pathways and Physiological Effects

Human Symptoms: Symptoms of lead poisoning vary with age, duration of exposure and concentration of lead during the exposure period (Krigman et al., 1980; Rice, 1990; Abou-Donia, 1992; Goyer, 1996). Symptoms of acute toxicity include acute gastroenteritis, with burning of the pharynx, vomiting and diarrhea, possibly resulting in death (Abou-Donia, 1992). Lead encephalopathy occurs sometimes in adults but more frequently in children, and is characterized by lethargy, vomiting, irritability, loss of appetite, and dizziness (Krigman et al., 1980; Goyer, 1996). This condition progresses to decreased alertness and loss of orientation, and in severe cases, a 50 percent mortality rate has been reported (Abou.-Donia, 1992).

Chronic lead toxicity leads to central nervous system symptoms, and, after hours or days, advances to confusion, coma and seizures (National Academy of Sciences, 1972; Whitfield et al., 1972; Krigman et al., 1980). Physical examination sometimes reveals the presence of fluid filled growths on the skin and severe neurological disorders such as blindness (Krigman et al., 1980). Often these patients
die, but in those that recover, there is frequently an onset of epilepsy, mental retardation and optic neuropathy (Perlstein and Attala, 1966).

**Absorption:** Lead enters terrestrial organisms primarily through ingestion and inhalation (Krigman *et al.*, 1980; Goyer, 1996). In humans, lead rarely enters the body via cutaneous absorption unless concentrations are extremely high (Krigman *et al.*, 1980; Abou-Donia, 1992). When cutaneous absorption occurs, hyperpigmentation can develop (Rice and Cohen, 1996). Preuss (1993) estimated that 10-50% of American children (over 3-4 million) had unsafe blood lead levels. Through ingestion, children absorb lead at a much higher rate than adults (Krigman *et al.*, 1980; Needleman *et al.*, 1984; Rice, 1990; Abou-Donia, 1992; Goyer, 1996). In adults, anywhere from 5 to 15 percent of lead is absorbed, and only about 5 percent of that is retained (Abou-Donia, 1992; Goyer, 1996). In infants it is reported that there is a net absorption of ingested lead of 41.5 percent and 31.8 percent of the absorbed lead is retained (Goyer, 1996). While Kelly and Kostial (1973) found that milk increases the absorption of lead in rats, more recent studies contradict those findings (Carrington and Bolger, 1992; Simons, 1993; Bogden *et al.*, 1995; Fullmer, 1997). Children and adults with poor nutrition have a higher absorption rate and retention than those with good nutrition (EPA, 1977; Ziegler *et al.*, 1978). This is thought to be correlated to the amount of calcim in the diet (Ziegler *et al.*, 1978). Bogden *et al.* (1995) supported this theory in experiments using rats. They found that increases in dietary calcium reduced maternal, fetal and neonatal lead accumulation during pregnancy and lactation (Bogden *et al.*, 1995). The interaction of lead and calcium with cellular sites depends
upon the concentration of free ions present (Pb\(^{2+}\) and Ca\(^{2+}\)) (Simons, 1993). An increase in calcium due to diet or dietary supplements acts to decrease lead ion competition for binding sites (Banua et al., 1995). Rice (1990) suggested that the reduction in calcium levels due to demineralization found in many elderly people may contribute to the increased absorption of lead. However, this hypothesis has yet to be supported by experimental data (Spencer, 1990).

Airborne lead in the form of dust particles or lead vapor is absorbed by the lungs at varying rates (Chamberlain et al., 1975; Goyer, 1996). Only a small fraction of particles over 0.5 \(\mu\)m in diameter are absorbed by alveoli; instead they are cleared from the respiratory track and swallowed (Goyer, 1996). However, absorption of particles smaller than 0.5 \(\mu\)m in diameter by alveoli is very efficient (Goyer, 1996).

**Pathways:** Regardless of the site of absorption, lead is transported by the blood where 90 percent of it is located in the red blood cells (Rice, 1990; Goyer, 1996). Lead ions bind to both the membrane of the red blood cell and the hemoglobin, which provide two major compartments for its transportation (Goyer, 1996). From the blood, lead is transported to the liver and the kidneys, finally ending up in the bone (Nogaki, 1958; EPA, 1977; Krigman et al., 1980; Rice, 1990; Abou-Donia, 1992; Goyer, 1996).

Greater than 90 percent of lead in the body is found in the bone (Krigman et al., 1980; Rice, 1990; Abou-Donia, 1992). Lead has a half life in bone of more than 20 years and, through mobilization from bone into the blood stream, may contribute as much as 50 percent of the blood lead (Goyer, 1996). Lead may be readily mobilized in
those afflicted with osteoporosis (Silbergeld et al., 1988) or by maternal bone during pregnancy and lactation (Krigman et al., 1980; Rice, 1990; Abou-Donia, 1992; Goyer, 1996). Lead readily crosses the placenta, allowing it to accumulate in fetal tissue as well as pass through the blood-brain barrier (Goyer, 1990; Rice, 1990; Abou-Donia, 1992; Bradbury and Deane, 1993). The accumulation found in the fetus is proportional to that of the mother (Goyer, 1990).

In the central nervous system, lead is usually concentrated in gray matter and selected nuclei (Goyer, 1996). Lead easily crosses the blood-brain barrier (Collins et al., 1982; Rice, 1990; Abou-Donia, 1992; Goyer, 1996). It accumulates to varying degrees, depending on the portion of the brain in contact with the metal (Collins et al., 1982; Rice, 1990; Abou-Donia, 1992; Goyer, 1996). There is disagreement, however, as to the rate of transport into the brain (Bradbury and Deane, 1993). The rate is dependent on the absence or presence of sulphur or organic ligands for lead, such as nitrogen and oxygen (Lyon et al., 1984; Bradbury and Deane, 1993). Lead binds to protein and sulfides before crossing the blood-brain barrier (Bradbury and Deane, 1993), so the amount of accumulation depends on the protein content and sulfides present in the organism (Lyon et al., 1984). The hippocampus has the highest accumulation (Bondy, 1988; Abou-Donia, 1992; Goyer, 1996), followed by the cerebellum, cerebral cortex and finally the medulla (Goyer, 1996). While cortical white matter appears to contain the least amount of lead, very few studies have been conducted to date which support this finding (Goyer, 1996).
Mechanisms: On a cellular and subcellular level, lead is found to accumulate in a cell’s nucleus and mitochondria (Krigman et al., 1980; Bondy et al., 1988; Abou-Donia, 1992; Goyer, 1996). Lead ions (Pb\(^{2+}\)) have an ionic structure similar to that of calcium ions (Ca\(^{2+}\)) and mimic calcium (Chang and Cockerman, 1994). Calcium ions are required for muscle contractions, microtubule polymerization and regulation of intracellular levels of cyclic nucleotides (Becker and Deamer, 1991; Kandel et al., 1991; Simons, 1993). Ca\(^{2+}\) is complexed with specific proteins such as calmodulin (Becker and Deamer, 1991). Calmodulin is a calcium ion receptor protein that couples to several enzymes such as phosphodiesterase (involved in the hydrolysis of cyclic AMP), and protein kinases (Becker and Deamer, 1991; Simons, 1993). Calmodulin has two binding sites for calcium at each end of the molecule, allowing four calcium ions to bind (Fig. 1) (Becker and Deamer, 1991). The calcium-calmodulin complex is also involved in the contraction of striated voluntary muscle (Becker and Deamer, 1991). Lead ions compete with the calcium ions for binding sites on calmodulin and disrupt these processes (Simons, 1993; Goyer, 1996). In addition, Ca\(^{2+}\) dependent K\(^{+}\) channels in the plasma membrane, which are involved in neurotransmission, are interrupted by the binding of lead and may be associated with human neuropathy and encephalopathy (Simons, 1993). The interaction of Pb\(^{2+}\) with Ca\(^{2+}\) leads to a disruption of calcium homeostasis by decreasing calcium uptake from the cytosol to the mitochondria (Abou-Donia, 1992; Simons, 1993). This, in turn, may result in high lethal levels of calcium in the cell (Abou-Donia, 1992; Goyer, 1996). Lead directly blocks Ca\(^{2+}\) pumps, eventually resulting in inhibition of the cholinergic system.
(involved in nerve transmission) and the activation of the catecholaminergic system (which includes the release of the hormones norepinephrine, epinephrine and dopamine) (Abou-Donia, 1992). Figure 2 depicts the normal functioning of calcium ions involved in synaptic transmission. Studies have found that lead-calcium interactions can lead to the impairment of other neurotransmitters systems such as the noradrenergic system (involved in innervation of the hypothalamus), and the GABA-ergic system (an inhibitory transmitter) (Markovac and Goldstein, 1988; Abou-Donia, 1992). Morphological differences include the impairment of the timed programming of cell-to-cell connections (Goyer, 1996). This results in the modification of neuronal circuitry (Cookman et al., 1988). Because of covalent bonding with protein kinase C and the substitution of Ca\(^{2+}\) as a natural ligand (Gregus and Klaassen, 1996) the interference with neurotransmitter systems, calcium uptake, mitochondrial membrane and the calcium pump are largely irreversible (Simons, 1986; Abou-Donia, 1992; Goyer, 1996). Additionally, workers exposed to high levels of lead may experience Schwann cell degeneration and axonal degeneration (Rice, 1990; Abou-Donia, 1992; Goyer, 1996).

One of the few reversible interferences caused by the replacement of calcium ions by lead ions is in calmodulin-dependent reactions (Goyer, 1996). Calcium binds to calmodulin (a protein) and allows calcium to act as a second messenger (Campbell, 1993). Lead may replace the calcium in this reaction (Simons, 1986), preventing functions such as muscle contractions, microtubule polymerization and disassembly, and regulation of intracellular levels of cyclic nucleotides (Becker and Deamer, 1991).
Figure 1. Structure of calmodulin. Calmodulin has a helix-loop-helix structure (Moran et al., 1994), with two globular ends which provide the binding sites for calcium (reproduced from Becker and Deamer, 1991). to calmodulin (a protein) and allows calcium to act as a second messenger (Campbell, 1993). Lead may replace the calcium in this reaction (Simons, 1986), preventing functions such as muscle contractions, microtubule polymerization and disassembly, and regulation of intracellular levels of cyclic nucleotides (Becker and Deamer, 1991).
Figure 2: Normal functioning of calcium ions at the site of synaptic transmission. a) A nerve impulse arrives at the end of an axon. Calcium ions diffuse inward due to a change in the permeability of the presynaptic membrane to the calcium ions. b) Calcium ions now located inside the axon cause the synaptic vesicles to fuse with the presynaptic membrane and empty acetylcholine into the synaptic cleft. c) Acetylcholine causes the ion channels in the postsynaptic membrane to open, generating an inward rush of sodium. d) The sodium depolarizes the membrane, resulting in an excitatory postsynaptic potential that can now provoke an action potential in the postsynaptic membrane. As this occurs, the acetylcholine is degraded, the calcium is pumped back out of the presynaptic axon. The process can now be repeated (summarized from Becker and Deamer, 1991).
functions such as muscle contractions, microtubule polymerization and disassembly, and regulation of intracellular levels of cyclic nucleotides (Becker and Deamer, 1991). Lead also inhibits membrane-bound Na, K-ATPase (Goyer, 1996), resulting in the disruption of the mitochondrial release of calcium and therefore energy metabolism (Simons, 1986). This interference can be reversed if lead is removed from the active sites (Goyer, 1996).

One of the better known toxic effects of lead is its inhibition of hemoglobin synthesis (Chang and Cockerham, 1994). Lead can interfere with the biosynthesis of hemoglobin by covalently bonding to thiol groups in α-aminolevulinic acid dehydrase (ALAD), the major target enzyme in heme synthesis (Krigman et al., 1980; Goyer, 1996). ALAD and ferrochelatase mitochondrial enzyme (which catalyzes the insertion of Fe^{2+}) are inhibited, resulting in a decrease in the synthesis of protoporphyrin and a reduction in the insertion of Fe^{3+} into heme (Chang and Cockerham, 1994; Goyer, 1996). This reduction of heme stimulates an increase in aminolevulinic acid synthetase (ALAS) activity, leading to anemia (Chang and Cockerham, 1994; Goyer, 1996). Lead causes increased erythrocyte destruction and a decreased hemoglobin production (Krigman, Bouldin and Mishak, 1980). The changes in enzyme activities, particularly ALAD and ALAS found in urine, correlate with actual blood lead levels and can be used as an early biochemical indicator of lead exposure (EPA, 1986).

Renal effects of lead were one of the first recognized health problems (Goyer, 1996). Acute lead nephrotoxicity, a reversible condition, consists of morphological changes in the proximal tubular cells (Goyer and Rhyne, 1973). It is expressed as a
decrease in energy-dependent transport functions, including aminoaciduria, glycosuria and ion transport (Goyer and Rhyne, 1973; Goyer, 1996). Microscopically, inclusion bodies (lead-protein complexes found in the renal tubular cells) are formed (Goyer, et al., 1970). The binding of these lead-protein complexes depends on the strength of the binding properties of the protein (Lyon, Taylor and Simkiss, 1984), and is therefore reversible (Goyer, 1996). However, with the reduction of lead exposure in the workplace, and more sensitive bioindicators of renal toxicity, this condition is rarely seen today (Goyer, 1996).

Lead exposure can lead to hypertension in children, adults and other vertebrates (Weiss, 1990; Bogden et al., 1995; Goyer, 1996). Adult females tend to have a higher threshold for lead than adult males before blood pressure rises (Goyer, 1996). However, some studies indicated the steepest rise in blood pressure occurred at the lowest exposures to lead (Weiss, 1990; Goyer, 1996). Although animal studies conducted by Victory et al. (1982) indicated this inverse relationship, the data was ignored until recently because it conflicted with the previously accepted theory (Weiss, 1990). Following Victory et al. (1982), further research indicated that modest exposure to lead may cause the individual to have higher plasma renin activity than normal, while the more chronic severe exposures may show normal or depressed plasma renin activity (Vander, 1988).

Lead exposure can lead to serious reproductive problems in animals and humans (Stowe and Goyer, 1971; Rice, 1990; Goyer, 1996). Early studies agreed that high levels of lead exposure caused spontaneous abortions, stillbirths and neurological
abnormalities in infants who survived to birth (Rice, 1990; Abou-donia, 1992; Goyer, 1996). However, there is now evidence that at even low levels, reproductive health is compromised (Needleman et al., 1984; McMichael et al., 1986; Rice, 1990; Abou-Donia, 1992; Goyer, 1996). Not only does lead cross the placenta, but lead in maternal bone becomes mobile (Needleman et al., 1984; Rice, 1990; Goyer, 1996). This adds to the blood lead level of both the mother and the fetus (Needleman et al., 1984; Rice, 1990; Goyer, 1996). With increasing maternal blood lead levels, there is an association in infants with abnormal reflexes, poor muscle tone and neurological soft signs such as jitteriness, hypersensitivity and abnormal cry (Emhart et al., 1985, 1986). These infants were thought to have blood lead levels in the normal range (Emhart et al., 1985, 1986). Studies performed as early as the 1950s indicated an alarmingly high rate of miscarriage among women who had some contact with lead (Nogaki, 1958). Japanese wives of lead workers doubled the frequency of miscarriage after their spouses began work at lead handling operations (Nogaki, 1958). Lead dust adhered to their clothing, exposing their spouses to high levels (Nogaki, 1958). Lead causes the uterus to contract, thus contributing to miscarriage and premature births (Needleman et al., 1984; Abou-Donia, 1992; Goyer, 1996). Ongoing studies on premature births associated with high maternal blood lead levels indicate that children in this group have a higher incidence of learning disabilities and behavioral problems when they reach school age (Needleman et al., 1984; Bellinger et al., 1987).

The difficulties in assessing the health problems associated with lead exposure are not in determining if lead is toxic. That has been clear since the era of the ancient
Greeks (Goyer, 1996). The challenge facing science today is to determine the lowest exposure possible that will not induce ill health effects (Rice, 1990; Goyer, 1996).

Although much of the focus of lead toxicity has been on adverse effects on humans, there is an increasing awareness of the effects on all living organisms (Cookman et al., 1988; Enserink et al., 1991; Chang and Cockerham, 1994; Franson et al., 1994; Hayward et al., 1996). Studies such as the Mussel Watch Program (Goldberg, 1986) and an increase in behavioral studies in children are beginning to give us a better understanding of the effects of lead (Goldberg, 1986; Rice, 1990).
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Molluscs are known to accumulate high concentrations of heavy metals from polluted areas (Bryan and Hummerstone, 1978; Ireland, 1979; Moore, 1994; Bou-Olayan et al., 1995; Chevreuil et al., 1996). It is this characteristic of bioaccumulation that prompted the advent of the International Mussel Watch Program in 1978 which uses bivalve filter feeding molluscs as bioindicators (biological organisms that suggest possible sites of pollution) to monitor for chemical contaminants on coastal marine shores (Goldberg, 1986; McConnell and Harrel, 1995).

Studies involving molluscs and heavy metals appear to fall into four categories: 1) known sites of pollution, such as mines and shipping lanes (Bryan and Usal, 1978; Ireland, 1979; Newman and McIntosh, 1983a; Walsh et al., 1995; Mikac et al., 1996); 2) sites suspected of possible contamination (Ismail, 1994; Bou-Olayan et al., 1995; Konar and Stephenson, 1995; Schuhmacher et al., 1995; Shulkin and Kavun, 1995); 3) determination of the usefulness of an organism as a bioindicator (Schulz-Baldes, 1974; Amiard et al., 1995; Pip, 1995; Puente et al., 1996; Schuhmacher and Domingo, 1996); and 4) the effects of contaminants on a specific mollusc (Boyden, 1974; Newman and
In those studies examining known areas of pollution, different organisms were used. Walsh et al. (1995) used the marine gastropod *Austrocochlea constricta* to determine metal distribution in the Newcastle region of New South Wales, Australia. Choice of organism was dictated by its habitat diversity (Walsh et al., 1995). *A. constricta* is found in intertidal regions of rock platforms, estuaries and marine lake systems in Australia (Underwood and Creese, 1976; Walsh et al., 1995). The sites of collection were the harbor at New Castle, a commercial shipping harbor known to have high concentrations of aliphatic hydrocarbons and heavy metals, and Lake Macquarie, a marine lake contaminated with organic resin acids and runoff from residential areas (Walsh et al., 1995). This research was undertaken primarily to determine if the shell of this species should be analyzed for heavy metal contamination along with the soft tissue (Walsh et al., 1995). The authors concluded that both the shell and soft tissue should be analyzed due to variations between metal concentrations of soft tissue and shells at different sites of collection (Walsh et al., 1995).

Two freshwater gastropods, *Physa integra* and *Campeloma deciscum* were used to examine possible physiological differences in an attempt to explain variations in lead levels in soft tissue (Newman and McIntosh, 1983a). These snails were collected from Weston’s Mill Pond in New Jersey, a small reservoir known to be contaminated with lead (Newman and McIntosh, 1983a). Lead elimination rates were examined using laboratory procedures which employed a continuous-flow system of lead free water
(Newman and McIntosh, 1983a). This study illustrated the differences between species in heavy metal analysis. *C. decisum* had a higher lead concentration in smaller individuals than in larger individuals, indicating an inverse relationship between size and metal concentration (Newman and McIntosh, 1983a). Elimination of lead was slow in this animal (Newman and McIntosh, 1983a). By contrast, *P. integra* had no size dependent relationship with lead concentration in soft tissue and showed much more rapid elimination of lead (Newman and McIntosh, 1983a).

In a comparative study using the same species but analyzing lead content from two different sites (a lead polluted site versus an unpolluted site), Ireland (1979) found that lead did not concentrate in any specific tissue in the terrestrial slug *Arion ater*. This was not the case in research using other molluscs such as *Mytilus edulis* (Pentreath, 1973; Schulz-Baldes, 1974; George and Pirie, 1980), *Scrobicularia plana* (Bryan and Hummerstone, 1978), and *Pecten alba* (Wu and Groves, 1995). In each of these aquatic organisms, there was a higher accumulation of lead in the digestive gland than in any other soft tissue (Pentreath, 1973; Schulz-Baldes, 1974; Bryan and Hummerstone, 1978; George and Pirie, 1980; Wu and Groves, 1995).

As with many studies, Mikac et al. (1996) had two objectives using the mussel *Mytilus galloprovincialis*. The first was to determine the concentration of organolead compounds in the mussel, and the second was to establish which lead compounds were bioaccumulated from seawater in the Adriatic to mussels in their natural environment (Mikac et al., 1996). This study was important since research efforts on the effects of lead introduced from gasoline have been declining due to the ban on leaded gasoline in
North America (Mikac et al., 1996). The authors found that alkyllead pollution in urban areas adjacent to the Adriatic Sea was widespread and that there were strong correlations between alkyllead concentrations in seawater and concentrations found in *M. galloprovincialis* (Mikac et al., 1996).

In addition to being used to monitor known sites of lead pollution, molluscs are effective in testing areas suspected of being polluted (Ismail, 1994; Bou-Olayan et al., 1995; Konar and Stephenson, 1995; Schuhmacher et al., 1995; Shulkin and Kavun, 1995). In Malaysia, several freshwater snails such as *Pila scutata, Filopaludina martensi, Indiplanorbis exustus* and *Physa acuta* were used to monitor heavy metals in rice fields (Ismail, 1994). The findings from this study were used to compare lead levels to those considered permissible by the Malaysian government (Ismail, 1994).

To determine the effect of the Gulf War on the Kuwait marine environment, Bou-Olayan et al. (1995) used the oyster *Pinctada radiata* as a bioindicator. Samples were collected prior to and following the Gulf War; results indicated a significant increase in lead, cadmium, nickel and copper between 1990 and 1992 (Bou-Olayan et al., 1995). It was the authors’ belief that the increase could be attributed to oil spills during the Gulf War (Bou-Olayan et al., 1995).

Konar and Stephenson (1995) compared the results of larval toxicity tests using *Crassostrea gigas* with data collected through the International Mussel Watch Program. Their research focused on subsurface waters rather than sediment or accumulation in bivalves and examined abnormal development patterns in these oysters (Konar and Stephenson, 1995). Their results indicated that those sites suspected of toxic
contaminations (Richmond Harbor, Los Angeles Harbor, Newport Harbor and Santa Cruz Harbor) did, indeed, have a wide variety of toxic substances present (Konar and Stephenson, 1995). Additionally, they concluded that their indicator species, *Crassostrea gigas*, was effective in determining toxicity, but that toxicity was probably due to many factors indicating that mussel watch data should be used on a site specific basis (Konar and Stephenson, 1995).

Recently, a considerable amount of research has come out of Russia concerning highly contaminated areas (Tkalin *et al.*, 1993; Shulkin and Kavun, 1995). Shulkin and Kavun (1995) monitored heavy metal contamination from Vladivostok and the Sea of Japan using *Modiolus kurilensis* and *Crenomytilus grayanus*. This study focused on both seasonal and spatial variability of heavy metal concentrations in soft tissue (Shulkin and Kavun, 1995). The indicator organisms were long lived species and are widely distributed in that geographical location (Shulkin and Kavun, 1995). These results indicated a relationship between spawning and lead concentrations, leading them to conclude that increases in body weight during spawning led to an increase in lead concentration (Shulkin and Kavun, 1995).

The third study category involves determination of the usefulness of an organism or a part of the organism as a bioindicator. Schulz-Baldes (1974) conducted an extensive research project using the common mussel *Mytilus edulis*. Rather than just using the whole animal to establish rates of uptake and loss of lead, his work included a comparison of the whole animals with selected organs, the effects on exchange equilibrium and the mechanisms used by the animal for uptake and loss (Schulz-Baldes,
The author concluded that *M. edulis* was an ideal indicator organism for examining the presence of lead based on its ability to bioaccumulate and its sedentary nature. He provided numerous mathematical models to use in determining rates of uptake (Schulz-Baldes, 1974). These results have been utilized extensively by subsequent research using *M. edulis* as well as other species (Bryan and Hummerstone, 1978; George and Pirie, 1980; Newman and McIntosh, 1983a,b; Amiard *et al.*, 1995; Prakash and Rao, 1995).

Research conducted by Pip (1995) examined nine freshwater mussels found in the Assiniboine River, Canada, for usefulness as bioindicators of metal pollution. This work was done to support the findings of Hinch and Stephenson (1987) and Green *et al.* (1989) who used many of these organisms to determine their usefulness. The author concluded that these animals were valuable as bioindicators because they were exposed to both the surrounding water and sediments (Pip, 1995). However, the results from this study also indicated that there was a great deal of variability in metal concentrations between species and that environmental factors such as pH, temperature, water turbulence, reproductive activity and others impact on these concentrations (Pip, 1995).

Amiard *et al.* (1995) examined the usefulness of the oyster *Crassostrea gigas* using both *in vitro* techniques and an experimental model of a food chain. They hoped to determine if enzymes were responsible for the elimination of lead from the gut, as well as whether direct or indirect exposure to lead would lead to higher concentrations (Amiard *et al.*, 1995). The results of their *in vitro* study suggested that the effect of
pH changes during digestion rather than enzyme interactions aided in the release of lead from the gut of the oyster (Amiard et al., 1995). In experiments using a food chain model, direct contamination rather than contamination from a trophic source led to greater metal retention (Amiard et al., 1995).

Finally, Puente et al. (1996) attempted to determine if the nacreous shell of *Mytilus galloprovincialis* should be used, in addition to soft tissue, in lead analysis. Despite other studies to the contrary (Newman and McIntosh, 1983b; Walsh et al., 1995; Laskowski and Hopkin, 1996) the authors concluded that not only should the nacreous shell should be used in lead analysis, it should be used instead of soft tissue (Puente et al., 1996). The results indicated that analysis of lead concentrations in the shell were more precise than those using soft tissue (Puente et al., 1996).

Whereas research to determine a species usefulness as a bioindicator has been primarily focused on bivalves and other filter feeders (Schulz-Baldes, 1974; Bryan and Hummerstone, 1978; George and Pirie, 1980; Newman and McIntosh, 1983a,b; Pip, 1995), there is a vast amount of research being conducted to determine lead toxicity in a variety of species (Boyden, 1974; Newman and McIntosh, 1983b; Udoidiong and Akpan, 1991; Bianchi et al., 1993; Babukutty and Chacko, 1995; McConnell and Harrel, 1995; Nelson et al., 1995; Prakash and Rao, 1995; Black et al., 1996).

Boyden (1974) recognized that the possible correlation between metal concentration and body weight should be examined. He developed a mathematical model to assess whether results of heavy metal measurements were accurate or merely a reflection of body size. His study used six different estuarine species (limpets, cockles, mussels and
clams) and six heavy metals (Cd, Cu, Fe, Ni, Pb and Zn). The regression coefficient indicated that the relationship between metal concentration and body size might be useful (Boyden, 1974).

Other estuarine species, such as the clam *Rangia cuneata* (McConnell and Harrel, 1995) and *Villorita cyprinoides* (Babukutty and Chacko, 1995), have been used in lead studies. McConnell and Harrel (1995) focused on both field and laboratory studies. The authors found that lead was rapidly accumulated (5 days) by *R. cuneata* and that lead levels in this species accurately reflected changing concentrations of lead in the environment (McConnel and Harrel, 1995). Research conducted by Babukutty and Chacko (1995) concentrated on the correlation between lead in water and sediment of an estuarine system and lead in the soft tissue of *V. cyprionoides*. From their analysis of these components, they were able to generate a bioconcentration ratio (BCR) and a metal partitioning ratio (MPR) to be used in future studies (McConnel and Harrel, 1995).

Newman and McIntosh (1983b) and Bianchi *et al.* (1993) used freshwater gastropods to study whole animal and neuronal effects of lead respectively. Newman and McIntosh (1983b) conducted a two year study in which their focus was the accumulation of lead from a contaminated food source in *Physa integra* and *Campeloma decisum*. Their results showed that *P. integra* rapidly accumulated lead and had an equally rapid elimination of lead. *C. decisum*, however, had a slow accumulation of lead and elimination was slow as well (Newman and McIntosh, 1983b). Bianchi *et al.* (1993) looked at the effects of lead on *Planorbarius corneus* at a
cellular level. They cultured neurons and found that neurite outgrowth was greatly affected by lead, and that few neurites sprouted in the presence of lead (Bianchi et al., 1993).

Finally, Black et al. (1996) used the freshwater mussel *Anodonta grandis* to study DNA strand breakage following exposure to lead. Four concentrations were used (0, 50, 500 and 5000 μg/L Pb), with analysis of accumulation in tissue as well as DNA analysis of strand breakage (Black et al., 1996). Their results showed that while no detectable lead accumulation occurred in the tissue at the lowest concentrations (0 and 50 μg/L Pb), there was significant DNA strand breakage (Black et al., 1996). However, at higher concentrations (500 and 5000 μg/L Pb), there was significant accumulation but no DNA strand breakage (Black et al., 1996). The authors speculated that the enzymatic process for DNA repair may be subject to a threshold effect and that this threshold is not reached with exposure to low lead concentrations (Black et al., 1996).

While the research may be varied, the purpose in using molluscs and lead seem to be aimed at the same thing: monitoring the concentration of lead in the environment and effects of environmental lead on organisms that live in that environment.
Work Cited


Chapter 4:

Behavioral Changes in the Pond Snail *Helisoma trivolvis* Following Chronic and Acute Exposure to Low Concentrations of Lead

Abstract

The pond snail *Helisoma trivolvis* was used to study behavioral changes that occurred following exposure to 0.40 ppm (acute) and 0.05 ppm (chronic) lead. Acute exposure (fifteen minutes) resulted in a contortion of the foot. Foot contortions lasted from twenty seconds to fifteen minutes. Foot contortions were not seen in untreated snails. Head and radula movements were not affected by acute exposure. Chronic exposure (twelve days) significantly reduced the number of head movements but did not significantly affect the number of radula movements.
Introduction

Molluscs are being used as bioindicators to determine the presence of toxicants in aquatic environments (Goldberg, 1986; Viarengo, 1993). Many marine molluscs are known to accumulate large concentrations of heavy metals (Phillips, 1977; Bryan and Hummerstone, 1978; Schuhmacher et al., 1995; Schuhmacher and Domingo, 1996). Freshwater and terrestrial molluscs have been used less extensively but are also considered efficient accumulators of heavy metals (Newman and McIntosh, 1983; Pip, 1995; Truscott, 1995; Chevreuil et al., 1996). The majority of the research in both marine and freshwater environments has concentrated on tissue analysis (Schulz-Baldes, 1974; Pentreath, 1973; Bryan and Hummerstone, 1978; Schumacher and Domingo, 1996). Few studies have looked at behavioral effects as an indicator of heavy metal contamination because of both the difficulty in assessment and the lack of standardized methods (Little et al., 1985; Little, 1990; Truscott, 1995). Instead, the focus has been on the traditional LC$_{50}$ (Tchounwou et al., 1991 a,b; Dr. Foster Mayer, EPA, pers. comm.; Dr. James Keating, EPA/OST, pers. comm.). In addition, the concentrations used in studying heavy metals have usually been much higher than EPA guidelines for safe drinking water (currently 10 ppb) (Goyer, 1996). Lead concentrations in some studies have ranged from 0.40 ppm (Laskowski and Hopkin, 1996 a,b), 32 ppm (Newman and McIntosh, 1983a), and 647 ppm Newman and MacIntosh, 1983b) to 12,700 ppm (Laskowski and Hopkin, 1996 a,b).
With this in mind, *Helisoma trivolvis*, a freshwater pulmonate, was used to study behavior following exposure to lead. This snail was chosen because of its extensively studied physiology (Berdan *et al.*, 1987; Berdan *et al.*, 1990; Culver, 1993; Kater and Shibata, 1994). It is experimentally hearty and is able to tolerate unfavorable environmental conditions, such as low dissolved oxygen, high dissolved carbon dioxide and a wide pH range (Harman, 1974; Pennak, 1989), and it has a distinct red coloration, making it fairly easy to identify (Boycott, 1936; Lodge, 1987). In addition, it has a worldwide distribution, making it potentially useful as an indicator organism.

The objectives of this study were to 1) observe and describe behaviors common to this snail under laboratory conditions, 2) determine if these behaviors would be affected after exposure to lower concentrations of lead than had been used in previous studies (Newman and MacIntosh, 1983 a,b; Laskowski and Hopkin, 1996 a,b), and 3) see if the behaviors observed under acute conditions (15 minutes at 0.40 ppm lead) differed from those under chronic conditions (12 days at 0.05 ppm lead). This paper describes these results as well as a new assay that is simple and inexpensive.
Methods and Materials

Laboratory reared *Helisoma trivolvis* were obtained from the laboratory of Dr. Stanley Kater, then on the faculty of Colorado State University.

**Snail Culturing:** Snail culturing conditions were provided by Friesen (1981) and Kater (1995). Snails were placed in 19 and 38 L glass aquariums equipped with an undergravel filter, an airstone and SeaFlor seashells as a substrate. Water was dechlorinated, and 1 gram per gallon of sea salt was added to the water to approximate natural pond water. Whisper 500 air pumps were used, and each air pump provided air for four tanks. Tanks were cleaned on an as needed basis, with frequency depending on the size of the snail population. Food was provided five days per week in the form of catfish pellets or trout chow, depending on availability.

In addition, two other conditions were described but not used in this experiment: calcium carbonate added to the water (only in soft water conditions), and recommended densities of ten snails per 19 L tank to encourage egg laying. Due to monitary and space constraints, tanks averaged up to 75 individuals per 38 L tank. The pH of the water was between 7 and 9, and the temperature of the water ranged between 16 and 24° C. Twenty eight degrees was considered optimum for egg laying under laboratory conditions according to Friesen (1981).

**Determination of Lead Concentrations:** Granular lead nitrate (Pb(NO₃)₂) was obtained from Fisher Scientific. All lead concentrations were made from a stock
solution of 0.286M Pb(NO₃)₂ diluted with water from an aquarium containing no snails (see Appendix E).

To determine the lowest concentration of lead needed for a behavioral change to be seen, four concentrations of lead were tested for acute exposure (from $1.93 \times 10^{-3}$ M to $1.93 \times 10^{-6}$M), and seven concentrations were tested for chronic exposure ($0.286M - 2.86 \times 10^{-7}$M) (see Appendix D for results of preliminary tests). Initial criteria were for the snail to survive in the lead solution for at least fifteen minutes for acute tests and five days for chronic tests. After survival was determined, behaviors were observed to see if they were normal (those seen under control conditions). The exact concentrations were $1.93 \times 10^{-6}$M Pb (0.40 ppm) for acute tests and $2.42 \times 10^{-7}$M Pb (0.05 ppm) for chronic tests.

Concentrations of lead solutions were tested prior to and after experimental runs using a BAS 100 automated Anodic Stripping Voltammetry unit (Bioanalytical Systems, Inc.) set on DPSV mode. Differential pulse stripping voltammetry (DPSV) operates in the following manner: a single drop of mercury (Hg) is used to concentrate the analyte from a diluted solution by electroreduction. The analyte is then dissolved in the drop by applying a constant potential more negative than the half-wave potential for the analyte (Harris, 1991). After concentrating the analyte in the drop for a specified time, the potential is made more positive at a constant rate which reoxidizes the analyte from the drop (Harris, 1991). The maximum value of the current measured during reoxidation is proportional to the quantity of analyte that is deposited (Harris, 1991).
A 3 ml sample of each lead solution was diluted to 8 ml with 25% acetic acid. The following settings were used: Initial E = -800 mV, Final E = -200 mV, Deposit Time = 60 sec., Sensitivity = $1.0 \times 10^{-6}$ ng/ml, Rate of stirring = 0, Mode = DPSV.

Each sample was analyzed three times before a standard addition of 1.0 M Pb was added to the sample to determine the unknown concentration. Sensitivity was reduced to $1.0 \times 10^{-5}$ ng/ml and analysis was run three times after standard addition (see Appendix A, Table 6).

For all tests, a snail was taken from stock and placed in a 250 ml beaker (containing either 150 ml of tank water or 150 ml of lead solution) and the beaker suspended over a magnifying mirror.

**Preliminary Behavior Testing:** Based on the ethogram (Fig. 1), the three most frequent behaviors were head movement, radula movement and antenna movement (see Results for description of behaviors). These behaviors were recorded and a frequency distribution generated (see Appendix B). Head and radula movements were chosen as the behaviors of interest because of frequency of occurrence and ease of observation. Visual observations rather than video recordings were used because of problems with resolution.

**Acute Toxicity Testing:**

**Control:** Each snail was placed in 150 ml of tank water. The occurrence of head movement, radula movement and other behaviors of individual snails was recorded (see Results). Observations were made for fifteen minutes per snail, the
Figure 1: Ethogram of *Helisoma trivolvis* under laboratory conditions. There were nineteen behaviors noted over twelve and one half hours of visual observation (n=63). Average number of occurrences for each behavior is seen on the graph. A detailed description of each behavior is found in Appendix C.
Fig. 1

Ethogram of *Helisoma trivolvis* under laboratory conditions

Average # of Occurrences

Behavior

- head mvt.
- radula mvt.
- antenna retraction
- antenna wave
- shell twist
- crawling over
- crawling on
- full retraction
- partial retraction
- no movement
- copulation
- grouping
- scooting
- floating
- rising
- sinking
- falling
- defecating
- laying down
temporal standard for acute testing of aquatic invertebrates (Burton, 1991; Toussaint, 1995).

Beakers were acid washed after each test to prevent a build up of lead in the glassware. Snails were used once (n=40) for a total of 600 minutes of observations.

**Lead Treatment:** Each snail was placed in 150 ml of 1.93 M (0.40 ppm) Pb solution. The occurrence of head movement, radula movement and other behaviors was recorded. Observations were made for 15 minutes per snail. A clean beaker was used for each snail. Snails were used only once (n=40) for a total of 600 minutes of observations. **Chronic Toxicity Testing:** Each snail was left in the beaker for five days with water added as needed if evaporation occurred. Food was withheld throughout the experiment to clear the digestive track. Control snails were placed individually in beakers containing 150 ml of tank water from an aquarium containing no snails. On the sixth day, the beaker containing the snail was placed over the mirror. Head movement and radula movement (see Results) were counted for five minutes. Each snail was observed once per day for seven consecutive days, and the two behaviors were scored. Water temperature was also recorded. Snails were used only once (n=40) for a total of 200 minutes of observations. Lead treated snails were placed individually in beakers containing 150 ml of $2.42 \times 10^{-7}$ M (0.050 ppm) lead solution. Observations were made starting on day six and continued for seven days as with control snails. Snails were used only once (n=40) for a total of 200 minutes of observations.
Results

Behaviors counted during experiments were head movement, radula movement and foot contortions (seen only in acute testing). Head movement and radula movement were seen in both control and lead treated snails. A head movement was scored when there was any change in direction of the head and was tallied regardless of duration (Fig. 2). Radula movement was counted only when the radula was seen, not just when the mouth opened. Foot contortions were only seen during acute testing and only in lead treated snails. They were not seen in any of the control snails. The behavior consisted of the foot being folded in on itself to varying degrees (Fig. 3).

To demonstrate the acute effect of Pb on snails, forty snails were exposed to 1.93M (0.40 ppm) Pb. Thirty nine snails showed foot contortion behavior. Only one lead treated snail did not show a foot contortion as described above. This snail remained curled up in its shell for the entire fifteen minute testing period. Foot contortions were not seen in any of the control snails.

When foot contortions occurred, the behavior lasted from 20 sec-15.0 min (median duration 4 min, 58 sec) (Fig. 4). A Spearman’s Rank Correlation test was used to examine the relationship between delay (the time between placing the snail into the solution and the time the behavior was first seen) and duration (how long the behavior lasted). These results were not significant (r = -0.2558, p = 0.0672), so no correlation can be made. However, several of the snails had foot contortions as soon as they were placed in the lead solution (showing a delay time of 0). By leaving out the snails in this group, a Spearman’s Rank Correlation test showed a negative correlation
Figure 2: Quantification of head movements. Each change in direction of the head counts as one head movement. a) represents one head movement (head moved in a clockwise direction) b) represents one head movement (also clockwise) and c) represents two head movements (first clockwise, then counterclockwise).
Figure 3: Foot contortion behavior during acute toxicity testing. Foot contortions were only seen in snails exposed to 0.40 ppm lead or higher. The different classifications of contortion are represented by normal, mildly contorted, moderately contorted and severely contorted.
Fig. 3

Normal foot  Mildly contorted

Moderately contorted  Severely contorted
Figure 4. The correlation between delay (time behavior was first observed after testing began) and duration (how long the behavior lasted) in foot contortions following acute exposure to lead (0.40 ppm for 15 minutes). There was no significant correlation between delay and duration using Spearman's Rank Correlation ($r = -0.2553$, $p = 0.0672$). When data showing a delay = 0 were discarded, there was a significant correlation between delay and duration ($r = -0.3162$, $p = 0.0468$).
Foot Contortions: Delay vs Duration after Acute Lead Exposure
between delay and duration (r = -0.3162, p = 0.0468). This indicates that the shorter the delay, the longer the duration, and the longer the delay, the shorter the duration.

During chronic testing, when the average number of head movements under control conditions and after Pb exposure were compared, a significant change in behavior was seen (p < 0.0001, Mann-Whitney)(Fig. 5). The percent decrease in the average number of head movements after exposure to lead ranged from 53.8 on day six to 48.5 on day twelve with the biggest decrease seen on day eight (56.6%) (Table 1). There was no difference in the number of head movements among snails under control conditions by day (p = 0.7482, Mann-Whitney). However, there was a significant difference in the number of head movements among snails exposed to lead (0.05 ppm) by day (p = 0.0029, Mann-Whitney).

Because snails are poikilothermic and their behavior affected by temperature, the possible effects of temperature variation in the laboratory were also analyzed. Temperatures of solutions ranged from 16 to 24°C (Table 2). Comparisons were made among snails under control conditions using ANOVA. There were no significant differences in the number of head movements under control conditions throughout the temperature range (p = 0.7708, ANOVA). The Q_{10} for control conditions (a factor measuring the reaction velocity or kinetic activity increase in an organism required for a rise in 10°C) (Prosser, 1973) was 1.18. For snails exposed to lead, there was no significant difference in the number of head movements throughout the temperature range (p = 0.0907, ANOVA). The Q_{10} for lead exposed snails was 1.00.
Figure 5: Comparison of the average number of head movements in snails exposed to 0.05 ppm Pb (chronic test) and snails not exposed (control) observed for 7 consecutive days following a 5 day acclimation period. There were no significant differences in the number of head movements in control snails by day ($p = 0.7482$, Mann-Whitney), but a significant difference was seen in lead treated snails by day ($p = 0.0029$, Mann-Whitney). In addition, there was a significant decrease in the number of head movements of control snails when compared to that of lead treated snails ($p < 0.0001$, Mann-Whitney).
Chronic Effect of Lead on Head Movement

Fig. 5

Average Number of Movements per 5 min.

Day

control

2.42 x 10^{-7} M Pb
Table 1. Effect of Chronic Exposure to Lead on Head Movement

<table>
<thead>
<tr>
<th>Day**</th>
<th>Lead S.E.</th>
<th>Tank Water S.E.</th>
<th>% Decrease</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>10.050±0.310</td>
<td>18.675±0.249</td>
<td>53.8</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>7</td>
<td>9.550±0.334</td>
<td>18.359±0.178</td>
<td>52.0</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>8</td>
<td>10.375±0.242</td>
<td>18.325±0.212</td>
<td>56.6</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>9</td>
<td>9.450±0.206</td>
<td>18.539±0.238</td>
<td>51.0</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>10</td>
<td>9.800±0.243</td>
<td>18.487±0.194</td>
<td>53.0</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>11</td>
<td>9.500±0.215</td>
<td>18.625±0.155</td>
<td>51.0</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>12</td>
<td>8.925±0.361</td>
<td>18.400±0.175</td>
<td>48.5</td>
<td>&lt;0.0001*</td>
</tr>
</tbody>
</table>

* Significant at =0.05 using Mann-Whitney Wilcoxon, n=40 for lead and 40 for tank water. **Observations began on day 6 of exposure.
Table 2. Effects of Temperature on Mean Number of Head Movements

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Lead S.E.</th>
<th>S.E.</th>
<th>Tank Water S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>10.87 ±0.295</td>
<td>18.53 ±0.273</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>10.06 ±0.266</td>
<td>18.50 ±0.200</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>10.54 ±0.340</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>9.86 ±0.595</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>8.78 ±0.466</td>
<td>18.24 ±0.183</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>9.54 ±0.167</td>
<td>18.64 ±0.213</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>9.49 ±0.169</td>
<td>18.54 ±0.171</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>10.88 ±0.386</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>***</td>
<td>18.19 ±0.332</td>
<td></td>
</tr>
</tbody>
</table>

*** There were no animals observed at this temperature. Temperature was tested as a covariate using ANOVA. n=80, p=0.2775
When radula movement and head movement in all snails was compared, there was no significant difference between the number of radula movements seen when the chronic testing was compared to acute testing (p=0.8808, n=80 ANOVA) (Fig. 6). The variability contributing to each mean number of movements was large (Fig. 6). This occurred, in part, because there seemed to be two distinct groups within the data: snails that had many radula movements and snails with few radula movements.

Therefore, the groups were separated, and a t-test was used to test for any significant difference in the number of radula movements between the two groups. There was a highly significant difference in the acute tests between the group with few radula movements (n=14) and the group with many radula movements (n=15) for both control and lead treated snails (p < 0.0001, t-test). Differences in the average number of radula movements between the group with few (n=25) and the group with many radula movements (n=25) in chronic tests was also significant (p < 0.0001, t-test).

Since there was a difference between these groups, a statistical comparison was made between the group with few radula movements in acute tests vs the group with few radula movements in chronic tests. There was a significant difference in the group with few radula movements between acute (n=14) and chronic (n=25) tested snails (p < 0.0001, t-test). Similar results were seen in the comparison between the groups with many radula movements: there was a significant difference in the number of radula movements between acute (n=15) and chronic (n=25) testing (p <0.0001, t-test) (Fig. 7).
Figure 6: A comparison of the number of radula movements seen in chronic tests versus acute tests. The concentration of lead was higher in acute tests (0.40 ppm) than in chronic tests (0.05 ppm), but the chronic tests lasted 12 days and acute tests for 15 minutes. There were no significant differences in the number of radula movements seen between chronic and acute tests (p=0.8808, n=80, ANOVA) among all snails.
Fig. 6

Number of Radula Movements: Chronic vs Acute Testing

Average Number of Movements per 5 min.

Control  Lead

- Acute
- Chronic

- Number of Radula Movements: Chronic vs Acute Testing

- Average Number of Movements per 5 min.

Control  Lead

- Acute
- Chronic
Figure 7: Comparison of radula movements for acute and chronic tests. Snails were divided into classes, those that showed few radula movements and those that showed many radula movements. There was a significant difference in the group with few radula movements in the chronic tests when compared to the same group in the acute tests ($p < 0.0001$, t-test). There was also a significant difference in the group with many radula movements in the chronic tests when compared to the same group in the acute tests ($p < 0.0001$, t-test).
**Fig. 7**

**Radula Movement After Lead Exposure: Chronic vs Acute**

- **Axes:**
  - **Y-axis:** Average Number of Movements per 5 min.
  - **X-axis:** Few Mvts. vs Many Mvts.

**Legend:**
- **Acute**
- **Chronic**

**Legend Symbols:**
- **Bars:**
  - Acute: Shaded
  - Chronic: Unshaded

**Significance:**

* Significant at $\alpha = 0.05$
There were no significant differences in the number of head movements between control snails in acute tests and control snails in chronic tests ($p = 0.1187$, Mann-Whitney) (Fig. 8). There was a highly significant difference between the number of head movements seen in lead treated snails in the acute test when compared to the snails in chronic test ($p < 0.0001$, Man-Whitney) (Fig. 8).

A summary of the effects of lead on behavior following both acute and chronic lead exposure is seen in Table 3. The plus (+) signs indicate a significant difference in that behavior, while the minus (-) signs indicate no significant difference.
Figure 8: A comparison of the number of head movements between acute tests and chronic tests. There were no significant differences in the number of head movements seen in control snails ($p = 0.1187$, Mann-Whitney). However, there were highly significant differences in the number of head movements seen in lead treated snails between acute and chronic tests ($p < 0.0001$, Mann-Whitney).
Comparison of Head Movements: Chronic vs Acute

Fig. 8

Average Number of Movements per 5 min.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Lead</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute</td>
<td>▣</td>
<td></td>
</tr>
<tr>
<td>Chronic</td>
<td>▣</td>
<td>▣</td>
</tr>
</tbody>
</table>

* Significant at $\alpha = 0.05$

Table 3. Summary of Snail Behavior Following Acute and Chronic Exposure to Lead

<table>
<thead>
<tr>
<th>Dosage</th>
<th>Head Movement</th>
<th>Radula Movement</th>
<th>Foot Contortions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute (0.40 ppm)</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Chronic (0.050 ppm)</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Discussion

There is an ongoing debate over acceptable lead levels in the environment (Needleman and Gatsonis, 1990; Doull, 1996; Rice, 1996). Research involving molluscan exposure to lead has been performed using high concentrations (concentrations up to parts per thousand) (Schulz-Baldes, 1974; Amiard et al., 1995; Puente et al., 1996), but recent studies by Black et al. (1996) showed that there was damage to DNA at low levels of lead exposure. Additionally, very few studies on behavioral toxicology have been done at any level (Truscott et al., 1995).

This research showed that behavior is also affected by chronic and acute exposure to low levels (0.05 and 0.40 ppm, respectively) of lead (Fig. 5). Acute responses (those seen in the first fifteen minutes of exposure) were limited to foot contortions not seen under control conditions (Fig. 3). In 10 out of the 40 snails tested, the response was immediate, indicating exceptional sensitivity. When this hypersensitive group was removed from the data set altogether, there was a negative correlation between delay and duration ($r = -0.3162$, $p = 0.0468$). The fact that all but one of the lead treated snails exhibited this behavior implies that lead is interrupting neuromuscular function because of the influence of calcium on movement. The foot is made up almost entirely of striated muscle (Hyman, 1967) and is controlled by a set of pedal retractor muscles which in turn are controlled by the pedal ganglion (see Chpt. 1, Fig. 3) (Brusca and Brusca, 1990). The striated muscles of many molluscs are regulated by calmodulin (Becker and Deamer, 1991), including Helisoma trivolvis (Geraerts and Joose, 1984). Any disruption of the calcium-calmodulin complex would
lead to the loss of voluntary muscle contraction necessary for this animal to maintain its position on the substrate (in this case, the glass of the beaker). Foot contortions may also be a result of the inhibition of synaptic transmission caused by lead entering the cerebral ganglion of the snail (the disruption of calcium influx into the synaptic cleft is discussed in Chapt. 2).

In addition to having a known neurophysiology, *Helisoma trivolvis* also has a unique hemoglobin with ten heme subunits (Terwilliger *et al.*, 1976; Ilan *et al.*, 1986). Since lead is transported on both the membrane of the red blood cell and hemoglobin (Goyer, 1996), the large number of subunits may aid in the distribution of lead throughout the animal's body.

There were more behavioral changes seen in response to chronic exposure than those seen in acute exposure. The number of head movements was significantly reduced after chronic lead exposure, but not following acute exposure (Fig. 5). Head movement is controlled by the right retractor muscle which is connected to the mantle and is connected to the head and throughout the visceral tissue (Brusca and Brusca, 1990). Movement of this muscle is governed by the circumoesophageal nerve ring located in the buccal mass (Brusca and Brusca, 1990). Since it is striated muscle, it is also regulated by calmodulin, and, as in the foot contortions, disruption of this complex would likely lead to an inhibition of voluntary movement. Once the lead enters the animal's open circulatory system via both ingestion and diffusion through epithelial tissue (Geraerts and Joose, 1984), it easily penetrates the buccal mass and into the cerebral ganglion (Simkiss and Mason, 1983). This penetration of the cerebral
ganglion may be analogous to the passing of lead through the blood-brain barrier in humans. The reduction in head movement seen in this research may have been a response to the introduction of lead into the nervous system. This depression in behavior is contrary to the findings of Truscott et al. (1995) using the freshwater pond snail *Lymnaea stagnalis*. At a higher concentration (200 ppm), they found an increase in locomotive activity which they termed as hyperactivity (Truscott *et al*., 1995). The differences in rates of behavior, *H. trivolvis* exhibiting a depression of behavior and *L. stagnalis* an increase, may be a result of the length of exposure to lead. Truscott *et al.* (1995) had an exposure time of over 50 days compared to the twelve days of this experiment. The difference may also be explained, in part, to the findings of Black *et al.* (1996) whose results indicated DNA strand breakage in freshwater clams is seen at low concentrations but not in higher concentrations. Their hypothesis was that in order for repair of DNA strand to occur, a threshold of some sort must first be reached in order to activate the necessary enzyme. Perhaps the low concentration (0.40 ppm and 0.05 ppm) of lead is this experiment had the same effect: a threshold was not reached in order to trigger hyperactive behaviors.

Temperature effects were not as expected. The eight degree temperature range was close to that of the $Q_{10}$ as described by Prosser (1973). He reported the freshwater mussel *Cardium* $Q_{10}$ at rest to be 1.20 and during activity 1.84. The $Q_{10}$ under control conditions in this study was 1.18 and after lead exposure 1.00. The $Q_{10}$ is a measure of kinetics, so differences in values reflect a difference in metabolic rate between the animals. Over a temperature range of 6°C, Truscott *et al.* (1995) found that there was
increased activity at the higher temperature in the range and carried out their experiments at 21°C rather than at their original 15°C. It is possible that by increasing the temperature to above 24°C (the highest temperature in this study) that the number of head movements and or radula movements would increase rather than decrease in the presence of lead.

In addition to a reduction in head movements, there was a significant increase in the number of radula movements seen during chronic testing when compared to acute testing. This increase may be due to a hyperactive effect caused by lead as is seen in humans (Needleman et al., 1979; Rice, 1990; Rice, 1996) or may simply be a response to food being withheld for the duration of the experiment. Differences in radula movements in the two groups may also be due to variance in sensitivity to changes in the environment such as being taken from an aquarium and placed in a beaker containing either tank water or lead treated water.

It is clear that chronic and acute exposure to lead affect the behavior of *Helisoma trivolvis*. This is shown by the foot contortions seen during acute tests (Fig. 3) and by the reduction in head movements (Fig. 5). Since these changes in behaviors were seen at lead concentrations 500 to 4000 times that used in other behavioral studies (Truscott et al., 1995), *Helisoma trivolvis* can be considered particularly sensitive to lead.
Conclusions

The results of this study indicate that *Helisoma trivolvis* has common behaviors that have been easily quantified, and these behaviors are affected by exposure to low concentrations of lead. Acute exposure to 0.40 ppm lead causes foot contortions that are not seen in control snails but does not affect head movements. Chronic exposure to 0.05 ppm lead results in a depression in the number of head movements. This depression is due to either the interference of lead in synaptic transmission, the interference of lead in voluntary muscle contraction or both. Foot contortions were not seen during the chronic exposure observation period. There were fewer radula movements seen in acute tests when compared to chronic tests, and, in both acute and chronic tests, there were two groups: those with few radula movements and those with many.

*Helisoma trivolvis* proved to be useful as an indicator organism for lead. It is easily maintained in the laboratory, it reproduces readily and is widely distributed throughout the world. Further study needs to be conducted to determine if the behaviors seen in the laboratory are also seen in the field. This assay could then be adapted for field use in determining lead contamination in freshwater.
Work Cited


Sunderland, Massachusetts.


Chapter 5:

Lead Concentrations in Selected Tissue and Organs of the Pond Snail Helisoma trivolvis (Experimentally Exposed to Pb)

Abstract

To determine if lead was being sequestered (bioaccumulated) in the pond snail Helisoma trivolvis, selected tissue and organs were analyzed for concentrations of lead following exposure to 0.050 ppm Pb for twelve days. There was a significant increase in lead concentration between control and lead treated organs and tissue. The highest concentration was found in the digestive gland followed by the reproductive organs, the salivary gland, cerebral ganglia, and the remainder of the soft tissue. Tissue and organs of the whole snail contained the least amount of lead. The shell was not tested for lead content.

Concentrations of lead in tissue were significantly higher than the concentrations of lead in the treatment solution. The high concentration of lead in untreated snail tissue suggests that further testing must be done to ensure that methods and analysis are correct.
Introduction

Studies have been done on molluscs to determine if they bioaccumulate heavy metals, and if metal concentration in their soft tissue is related to body size (Bryan, 1973; Boyden, 1974; Laskowski, 1996a,b). Interest has also focused on which tissue metals are sequestered and which molluscs can be considered useful as bioindicators of polluted sites (Bryan, 1973; Boyden, 1974; Laskowski, 1996a,b). While there is general agreement that many molluscs, such as mussels, oysters, and scallops can be used as monitors of polluted sites (Schulz-Baldes, 1974; Hopkin, 1989, 1993; Schuhmacher et al., 1995; Chevreuil et al., 1996), there is disagreement as to particular species, methods of analysis and physiological interactions among species in the presence of metals (Lobel et al., 1991; Lukyanova et al., 1993; Puente et al., 1996). Much of the work has been done using mussels, both freshwater, such as (Dreissena polymorpha pallas, Quadrula quadrula, Ligumia recta, Lampsilis radiata siliquoidea and L. ventricosa) (Chevreuil et al., 1996; Pip, 1995), and marine (such as Perna viridis, Mytilus galloprovincialis, M. edulis, and Modiolus demissus) (Pentreath, 1973; Boyden, 1974; Nelson et al., 1995; Yan, Teo & Sin, 1996)). Other species under investigation are clams (Scrobicularia plana) (Bryan & Uysal, 1978); oysters (Crassostrea angulata, Pinctada radiata, and Crassostrea virginica) (Bou-Olayan et al., 1995; Schuhmacher & Domingo, 1996; Shuster & Pringle, 1969); scallops (Pecten alba) (Wu & Groves, 1995); slugs (Arion ater) (Ireland, 1979); terrestrial snails (Helix aspersa, H. pomatia, Bradybaena fruticum, and Aegopis verticillus) (Laskowski &
Hopkin, 1996a,b; Rabitsch, 1996); and aquatic snails (*Pila scutata, Filopaludina martensi, Melanoides tuberculata, Lymnaea rubiginosa, L. stagnalis, Indoplanorbis exustus, Physa acura, P. integra and Campeloma decisum*) (Ismail, 1994; Newman & McIntosh, 1983 a,b; Truscott et al., 1995).

The internal characteristics of a species as well as the binding properties of a metal will dictate which metals bind to tissue and cells and at what concentration metal is sequestered within a given animal (Cheng & Sullivan, 1974; Lyon, Taylor & Simkiss, 1984; Simkiss & Mason, 1983). There is an ongoing debate over the source of variability of metal concentrations within the tissue of a species in the same environment (Lobel et al., 1991; Puente et al., 1996).

This research examines the lead content in the tissue of an aquatic pond snail following chronic exposure (twelve days) to 0.45 ppm Pb. It addresses the issues of possible body size relationships, bioaccumulation and difficulties in obtaining consistent results.
Methods and Materials

Lead Solution: a 0.05 ppm lead solution was prepared by mixing 8.46 L of 0.286M Pb(NO₃)₂ with 1500 ml of tank water from a tank containing no snail. Tank water was prepared by adding 1g of Instant Ocean sea salt to 1 L of dechlorinated tap water. Analysis of solutions is described in Chapter 2 Methods (see Appendix D Table 7 for analysis).

Physiological saline consisted of 5.74 g NaCl, 2.64 g CaCl₂2H₂O, 0.89 g KCl, 5.62 g MgCl₂6H₂O, 2.05 g NaHCO₃. All chemicals were mixed and diluted to 1 L with distilled water (Cavanaugh, 1956).

Procedure: lead solution from the above stock was placed in 250 ml beakers; one snail was added to each beaker. Beakers with snails were left in the laboratory for twelve days. Snails were not fed or aerated during this time.

Five petri dishes were prepared by partially filling with Sylgard brand silicone. Snails were removed from the beakers and rinsed with distilled water. Their shells were removed using dissecting scissors and snail tissue was pinned to the silicone dish. Tissue was covered with molluscan physiological saline (see above). Dissections were completed under a dissecting scope. The following tissues were dissected and placed individually in 1.5 ml microcentrifuge tubes: digestive glands, cerebral ganglia, reproductive organs, salivary glands and remainder of the soft tissue (kidney, stomach, intestine, mantle and all muscle). Note: salivary glands were pooled together from
five snails. Whole snails were also placed in microcentrifuge tubes. All specimens were marked with date, Pb concentration, tissue type and snail number. Tubes were then placed in the freezer until ready for analysis.

Sample preparation: Tissue samples were brought to room temperature. Pre-scored 5 ml glass ampules were marked with snail number and tissue type and weighed. Tissue was then placed inside the ampule using a Pasteur pipet to remove it from the centrifuge tube. If necessary, distilled water was added to remove tissue from the walls of the ampule. Tissue was dried by placing ampules on a modified metal test tube rack and placed in a 100°C oven overnight. Ampules were allowed to cool prior to re-weighing for dry weight determination.

After samples were re-weighed, 1 ml of trace metal nitric acid was added to digest tissue. Ampules were sealed using a hot flame and placed back in oven on test tube rack. Samples were allowed to digest overnight.

Samples were removed from oven and tops snapped off under a hood. Ampules were then placed on a hot plate and nitric acid was heated until just dry, releasing it in vapor form and leaving any metals in ampule. Distilled water was added to the sample and then pipetted out into a 10 ml volumetric flask, thus washing the sample into the flask. If necessary, the sample was diluted to the mark with additional distilled water. Flasks were sealed with ground glass stoppers.
Blanks were used to test for background lead. Ampules were prepared as in tissue samples but were empty during drying process. Nitric acid was added as described in tissue sample preparation and all other procedures remained the same.

Samples were prepared by diluting 3 ml of sample with 5 ml of 25% glacial acetic acid. Samples were analyzed with a BAS 100 automated Anodic Stripping Voltammetry unit (Bioanalytical Systems, Inc.), set on the differential pulse mode. All samples were analyzed at least twice before adding a standard addition of 1.0 mM Pb. Settings for limits of detection (sensitivity) varied depending on tissue type and were limited by impurities in the background (Table 1). A listing of the general settings is as follows:

- Initial $E = -800 \text{ mV}$
- Final $E = -400 \text{ mV}$
- Deposit Time 60 sec.
- Sensitivity $= 1.0 \times 10^6$ before addition, $1.0 \times 10^3$ after addition
- Rate of stirring $= 0$
- Mode: DPSV

A flow meter was used to control the amount of bubbling of sample.
<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Treatment</th>
<th>Sensitivity (ng ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral Ganglia</td>
<td>0.050 ppm Lead</td>
<td>$1.0 \times 10^{-6}$</td>
</tr>
<tr>
<td>Cerebral Ganglia</td>
<td>None</td>
<td>$1.0 \times 10^{-6}$</td>
</tr>
<tr>
<td>Digestive Gland</td>
<td>0.050 ppm Lead</td>
<td>$1.0 \times 10^{-6}$</td>
</tr>
<tr>
<td>Digestive Gland</td>
<td>None</td>
<td>$1.0 \times 10^{-6}$</td>
</tr>
<tr>
<td>Reproductive Organs</td>
<td>0.050 ppm Lead</td>
<td>$1.0 \times 10^{-5}$</td>
</tr>
<tr>
<td>Reproductive Organs</td>
<td>None</td>
<td>$1.0 \times 10^{-5}$</td>
</tr>
<tr>
<td>Remainder of Tissue</td>
<td>0.050 ppm Lead</td>
<td>$1.0 \times 10^{-5}$</td>
</tr>
<tr>
<td>Remainder of Tissue</td>
<td>None</td>
<td>$1.0 \times 10^{-5}$</td>
</tr>
<tr>
<td>Salivary Gland</td>
<td>0.050 ppm Lead</td>
<td>$1.0 \times 10^{-6}$</td>
</tr>
<tr>
<td>Salivary Gland</td>
<td>None</td>
<td>$1.0 \times 10^{-6}$</td>
</tr>
<tr>
<td>Whole Snail</td>
<td>0.050 ppm Lead</td>
<td>$1.0 \times 10^{-5}$</td>
</tr>
<tr>
<td>Whole Snail</td>
<td>None</td>
<td>$1.0 \times 10^{-5}$</td>
</tr>
</tbody>
</table>
Results

Digestive glands, cerebral ganglia, reproductive organs, salivary glands and the remainder of tissue and organs (kidney, stomach, intestine, mantle and all muscle) were dissected and analyzed for lead content. Whole snails without their shells were also analyzed for lead content. In all tissue (with the exception of the salivary gland) there were significant differences between tissue from control snails and tissue from lead treated snails (Table 2). Differences between lead concentration of control tissue and lead tissue ranged from eight times as much lead in treated cerebral ganglia compared to control) to nine million times as much lead in the remainder of the soft tissue following lead exposure.

In addition to the differences in the amount of lead found in the tissue, there was variation within tissue types (Fig 1). In all tissue except the treated cerebral ganglia and the treated remainder of the soft tissue, the standard deviation was greater than twice that of the mean ppm Pb, indicated a high degree of variability. This was seen in both treated and control tissue.

Total ppm Pb in the analyzed treated tissue exceeded the 0.05 ppm of the solution each snail was placed in for testing. There were highly significant differences in final concentration of lead in the treated tissue when compared to initial lead concentration (Table 3). When control tissue was analyzed, the range of four out of five samples was unexpectedly high (Table 4). There were highly significant differences in final lead concentrations of control snail tissue, with the exception of the digestive gland, when initial ppm lead was set at zero. In all calculations for both
control and lead treatment, background Pb of 0.0001 ppm obtained from analysis of a blank was subtracted out.
Figure 1. Final lead concentration of soft tissue of treated and untreated snails.

C.G. = cerebral ganglia; D.G. = digestive gland; Rep. = reproductive organs; Rem. = remainder of soft tissue; Sal. G. = salivary gland; W.S. = whole snail without shell). Untreated snails n = 5; treated snail n = 10; salivary glands were pooled.
Fig. 1

ppm Pb in Soft Tissue: Lead vs Control

Note: values for remaining soft tissue and whole snail were < 50 ppm Pb
Table 2. Mean ppm Pb in Soft Tissue

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Mean Dry Wt. (g)</th>
<th>ppm Pb Control</th>
<th>ppm Pb Lead</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>cerebra ganglia</td>
<td>0.00037</td>
<td>289.00</td>
<td>2419.53</td>
<td>0.00048</td>
</tr>
<tr>
<td>digestive gland</td>
<td>0.00031</td>
<td>159.85</td>
<td>6796.90</td>
<td>0.0011</td>
</tr>
<tr>
<td>reproductive organs</td>
<td>0.00412</td>
<td>122.06</td>
<td>5586.67</td>
<td>0.0272</td>
</tr>
<tr>
<td>remainder soft tissue</td>
<td>0.00038</td>
<td>0.0001</td>
<td>942.665</td>
<td>0.0047</td>
</tr>
<tr>
<td>salivary gland</td>
<td>0.00023</td>
<td>343.33</td>
<td>3018.40</td>
<td>0.2207*</td>
</tr>
<tr>
<td>whole snail</td>
<td>0.03217</td>
<td>7.3093</td>
<td>856.800</td>
<td>0.0022</td>
</tr>
</tbody>
</table>

includes kidney, stomach, intestine and all muscle (does not include shell)

*significant at $=0.05$ using Mann Whitney
Table 3. Initial ppm Pb vs Final ppm Pb in Treated Tissue

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Initial [Pb]</th>
<th>Final [Pb]</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>cerebral ganglia</td>
<td>0.05</td>
<td>2419.53</td>
<td>&lt; 0.0001*</td>
</tr>
<tr>
<td>digestive gland</td>
<td>0.05</td>
<td>6796.90</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>reproductive organs</td>
<td>0.05</td>
<td>5586.67</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>remainder soft tissue</td>
<td>0.05</td>
<td>942.67</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>salivary gland</td>
<td>0.05</td>
<td>3018.4</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>whole snail</td>
<td>0.05</td>
<td>856.80</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

includes kidney, stomach, intestine and all muscle (does not include shell)
*significant at = 0.05 using Kruskal-Wallis
Table 4. Initial ppm Pb vs Final ppm Pb in Control Tissue

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Initial [Pb]</th>
<th>Final [Pb]</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>cerebral ganglia</td>
<td>0.00</td>
<td>289.00</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>digestive gland</td>
<td>0.00</td>
<td>159.85</td>
<td>0.1329</td>
</tr>
<tr>
<td>reproductive organs</td>
<td>0.00</td>
<td>122.06</td>
<td>0.0053*</td>
</tr>
<tr>
<td>remainder soft tissue</td>
<td>0.00</td>
<td>0.0001</td>
<td>0.4800</td>
</tr>
<tr>
<td>salivary gland</td>
<td>0.00</td>
<td>343.33</td>
<td>0.0032*</td>
</tr>
<tr>
<td>whole snail</td>
<td>0.00</td>
<td>7.3093</td>
<td>0.5778</td>
</tr>
</tbody>
</table>

includes kidney, stomach, intestine and all muscle (does not include shell)

*significant at =0.05 using Kruskal-Wallis
Discussion

With the exception of the salivary gland, there were significant differences in the final lead concentrations between control snails and lead treated snails (Table 2). Because of the ability of molluscs to accumulate large concentrations of heavy metals within their tissues (bioaccumulation) via ingestion or through diffusion through epithelial tissue (Schulz-Baldes, 1974; Simkiss and Mason, 1983; Hopkin, 1989, 1993; Schuhmacher et al., 1995; Chevreuil et al., 1996), these differences were expected. However, the magnitude of the differences was unexpected. There was over nine million times as much lead in the soft tissue which included the kidney, stomach, intestine and foot) of the lead treated snails than that seen in the control (untreated) snails. Chevreuil et al. (1996) found that on four out of six days of sampling, zebra mussels (Dreissena polymorpha) had slightly lower lead concentrations in their tissue than that of the water they lived in continuously in the River Seine. Lead levels of the raw water ranged from $<0.5$ to 54 ppm (Cheveruil et al., 1996). Since the concentration of lead used in this study was low (0.05 ppm), and the length of exposure did not exceed twelve days, it seems unlikely that bioaccumulation in this molluscs could account for the large quantity of lead found in this analysis.

The magnitude of the lead bioaccumulation might be due to contamination or faulty analytical methods. The high concentration of lead in the control snail tissue suggests that this is the cause. Anodic stripping voltammetry is the most sensitive of the polarographic techniques (Harris, 1991). However, because of the low limits of
detection achieved, it is necessary to use extreme precautions to avoid contamination (Harris, 1991). Possible areas of contamination in this study may have originated from impurities in the chemical reagents used such as the glacial acetic acid or the mercury. Neither of these chemicals were trace metal grade. In addition, all glassware should have been acid washed after each sample was run, and the equipment should have been placed in an ultra clean environment, wiped free of residual mercury and kept covered when not in use (Harris, 1991). The most likely source of contamination, however, was the mercury used in the instrument.

Besides the large increase in the amount of lead seen in treated snail tissue when compared to untreated snail tissue, there was also variation in the quantity found within tissue types (Fig. 1). These results are consistent with other studies, such as those done by Boyden and Phillips (1981), Boyle et al. (1986) and Lobel et al. (1991), where high degrees of variability in heavy metal content were seen in soft tissue, whole snail and selected organs, in species from the same environment. Lobel et al. (1991) found that elements which are stored primarily in an insoluble form, such as lead and other heavy metals, have more variability in concentration among species from the same environment than those stored in soluble form. In the mussel *Mytilus edulis*, variability in metal concentration in tissue types was found to be in the following order: kidneys > digestive gland, foot > gills (Lobel et al., 1991). However, in *Helisoma trivolvis*, the order was digestive gland > reproductive organs > cerebral ganglia > whole snail > remainder of the soft tissue (which includes kidney, stomach, intestine,
mantle and all muscle). This difference may be due to the differences in physiology between the two species, the method of analysis, or manner of feeding behavior.

The digestive gland contained the highest concentration of lead of all tissues examined. This is consistent with many studies using both freshwater and marine molluscs (Schulz-Baldes, 1974; Bryan and Uysal, 1978; Bryan and Hummerstone, 1978; Newman and McIntosh, 1983). The digestive gland is the molluscan equivalent of the liver where metabolic processing of absorbed nutrients occurs as well as detoxification of poisons (Fretter and Graham, 1976; Simkiss and Mason, 1983). The digestive gland acquires food and particles from two sources, the blood and the stomach (Fretter and Graham, 1976). Materials gained from the blood are either passed through to the stomach, bind to the walls of the digestive gland or are excreted (Fretter and Graham, 1976). When lead enters the snail through epithelial tissue, it is extracted from the blood into the digestive gland where some of it binds to the walls of the gland and some is excreted along with other waste material (Simkiss and Mason, 1983). When lead is ingested, it passes through the digestive system from the stomach to the digestive gland (Fretter and Graham, 1976). Following the digestive gland, the reproductive organs were found to contain the next highest amounts of lead. Only one study found examined the gonads or reproductive organs (Pip, 1995). The results of their study indicated that this tissue had the lowest concentration of lead (Pip, 1995). Methods of analysis were similar to those used in this study with the following exceptions made: samples were freeze dried prior to analysis, tubes were agitated during the digestion process to control foaming and a digital anodic stripping
voltammeter was used (Pip, 1995). However, the test animals used by Pip (1995) were monoecious. Therefore, there was less tissue used in the analysis, which might account for these differences.

Finally, the lead concentrations obtained through analysis in both control and lead treated snails far exceeded expectations. Control snails were never treated with lead, thus it would be thought that any lead found in their tissue would originate from their environment. However, when tank water was tested prior to behavior studies see Chpt. 4), the results indicated lead levels within the acceptable EPA range of 10 ppb see Appendix D). Tank water was again tested after completing the tissue studies, and while it was much higher than the original results 75 ppb), it is believed that contamination originated from the testing equipment rather than the water itself. The equipment used in this study was also being used for undergraduate chemistry classes while this research was underway. The mercury electrode is made in such a way that if too high a concentration of lead is analyzed, the mercury becomes contaminated with lead. This significantly increases the lead concentration reported in the analysis.
Conclusions

The results of this study indicated that there is bioaccumulation of lead occurring in *Helisoma trivolvis*. The digestive gland and reproductive organs appear to be the major sites of bioaccumulation. It is unclear, however, how much lead is accumulating due to the believed contamination of both equipment and reagents used. Further analysis needs to be performed to determine the amount and possibly the rate of bioaccumulation of lead. If anodic stripping voltammetry is used, much greater care needs to be taken to ensure that the equipment runs at peak performance. In addition, all reagents should be trace metal or ultra pure to prevent any unwanted addition of lead to the samples. Finally, there should be a parallel study conducted using atomic absorption equipped with a lead lamp to confirm results obtained with anodic stripping.
Work Cited


Cavanaugh, G. 1956. Formulæ and Methods V. of the Marine Biological Laboratory Chemical Room. Woods Hole, MA.


### Table 1: Temperature Ranges and Head Movements-Chronic Tests

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Appendix B: Graphs of Raw Data

Figures 1 - 40: The chronic effect of lead on head movement on forty individual snails over seven consecutive days. Control is depicted by the solid line and treatment by the dotted line.

Figures 41 - 49: The chronic effect of lead on radula movement on nine individual snails over seven consecutive days. Control is depicted by the solid line and treatment by the dotted line.

Figure 50: The acute effect of lead on head movement on forty snails. Control is depicted by the solid line and treatment by the dotted line.

Figure 51: The acute effect of lead on radula movement on forty snails. Control is depicted by the solid line and treatment by the dotted line.
Snail 1: Effect of Lead on Head Movement

Snail 2: Effects of Lead on Head Movement

Snail 3: Effects of Lead on Head Movement

Snail 4: Effect of Lead on Head Movement

Snail 5: Effect of Lead on Head Movement

Snail 6: Effect of Lead on Head Movement
Snail 25: Effect of Lead on Head Movement

Snail 26: Effect of Lead on Head Movement

Snail 27: Effect of Lead on Head Movement

Snail 28: Effect of Lead on Head Movement

Snail 29: Effect of Lead on Head Movement

Snail 30: Effect of Lead on Head Movement
Snail 31: Effect of Lead on Head Movement

Snail 32: Effect of Lead on Head Movement

Snail 33: Effect of Lead on Head Movement

Snail 34: Effect of Lead on Head Movement

Snail 35: Effect of Lead on Head Movement

Snail 36: Effect of Lead on Head Movement
Snail 37: Effect of Lead on Head Movement

Snail 38: Effect of Lead on Head Movement

Snail 39: Effect of Lead on Head Movement

Snail 40: Effect of Lead on Head Movement
Appendix C: Ethogram

Definition of Behaviors:

- head movement - any change in direction of the head
- radula movement - mouth opens and radula is seen scraping or attempting to scrape
- antennal retracts - movement is toward the head or inside the shell
- antennal wave - antenna moves from side to side or flows
- shell twist - shell twists to keep up with movement in a circular direction
- crawling over another - walks over another snail but does not stay
- crawling on another - crawls on another snail and stays for longer than 30 seconds
- full retraction - entire snail retracts into shell
- partial retraction - partially retracted into shell but foot can still be seen
- no movement - snail is completely still with no movement of foot, head or antenna
- copulation - two snails actively copulating with penile penetration
- grouping - three or more snails attached to one another by their feet
- scooting - forward movement in any direction
- floating - on surface of water, completely unattached
- rising - rising rapidly from the substrate to the surface of the water; unattached
- sinking - dropping unattached from the surface of the water to the substrate
• falling - falling from an attached surface to the substrate
• defecating
• laying down - shelled laid sideways on substrate with foot and head drawn into shell

Snails were observed in community aquariums at different times of the day.

Small five gallon tanks were used for the observations with usually ten to twelve snails being viewed during any given time period. A total of twelve and one half hours of observations were made.
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Appendix D: Preliminary Behaviors in Chronic Tests

≈0.286 M Pb(NO$_3$)$_2$ (> 5 E5 ppm Pb): Snail disintegrated immediately at this concentration.

≈0.0286 M (> 5000 ppm): Snail remained unmoving for thirty minutes of observation. At the end of two hours the snail was dead.

≈0.00286 M (> 500 ppm): Snail remained unmoving for one hour and died between three and eleven hours after being placed in solution.

≈2.86 E-4 M (> 50 ppm): Snail not moving much, but not curled up either.

- Antenna (left side) was bent and partially retracted at all times.
- No radula movements seen.
- After five hours, a white precipitate seen along the edge of the mantle.
- After seven hours there is no movement.
- Antenna (left side) is fully retracted and tissue no longer appears red; instead is slightly greyish.
- Snail survived the night but continued to show little movement.
- Food introduced, but the snail was unable to grasp with foot; it was contorted too much.
- Snail died after four days; color was almost entirely gone.

≈2.86 E-5 M (> 5 ppm): Snail had foot contortions, but not continuously.

- Color after two days light pink instead of bright red.

- Not as much movement as control snails, but head and radula moved much more than at other concentrations.

- White precipitate around edge of mantle by day four.

- Head and radula movements increased but still not at control levels by day six.

- Able to eat food and grasp with foot.

≈2.86 E-6 (> 0.05 ppm): Snail activity increased over other concentrations.

- No white precipitate after ten days.

- No foot contortions observed.

- Snail able to move freely and eat well.

- Head and radula movements continued to be depressed.
Appendix E: Solution Calculations

Stock solution was prepared in the following manner: 94.76 g of granulated Pb(NO₃)₂ was measured on an analytical balance and placed in a 1 L volumetric flask. Distilled water was added to the 1 L mark.

\[
94.76 \text{ g Pb(NO}_3\text{)}_2 \times \frac{1 \text{ mol Pb(NO}_3\text{)}_2}{331.336 \text{ g Pb(NO}_3\text{)}_2} = 0.286 \text{ M Pb(NO}_3\text{)}_2
\]

Solutions used in chronic and acute tests followed the formula \( M_1 V_1 = M_2 V_2 \)

\[
(0.286 \text{ M}) (x) = (1.93 \times 10^{-6} \text{ M})(1200 \text{ mL}) \quad x = 8.1 \mu\text{L for acute}
\]

ppm were calculated in the following manner:

\[
(1.93 \times 10^{-6} \text{ M})(207.2 \text{ g/mol})(1000 \text{ mg/g}) = 0.40 \text{ ppm for acute}
\]