FEASIBILITY FOR UTILIZATION OF A FRESHWATER PULMONATE SNAIL, *PHYSA ACUTA*, AS A MODEL ORGANISM FOR ENVIRONMENTAL TOXICITY TESTING, WITH SPECIAL REFERENCE TO CADMIUM ION TOXICITY

by

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Presented to the Faculty of the Graduate School of

The University of Texas at Arlington in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

THE UNIVERSITY OF TEXAS AT ARLINGTON

December 2005

ACKNOWLEDGEMENTS

I sincerely wish to thank all of those who participated in this study through their ideas, support and time. I would like to thank my committee members: Dr. Robert F. McMahon, Dr. James P. Grover, Dr. Daniel Formanowicz, Dr. Jonathan Campbell and Dr. Gerald Carney, with special thanks to Dr. Doyle L. Hawkins. I would like to thank members of Dr. McMahon's laboratory: with special thanks to David Britton. I would like to thank laboratory assistants Oleg Lebanov, Rachel Alford and Natalie Hubbard. And finally, I would like to thank my husband, Larry for his assistance in the laboratory and his support throughout my graduate career. This research was partially funded by a grant from the University of Texas at Arlington's chapter of Phi Sigma.

July 25, 2005

ABSTRACT

FEASIBILITY FOR UTILIZATION OF A FRESHWATER PULMONATE SNAIL, *PHYSA ACUTA*, AS A MODEL ORGANISM FOR ENVIRONMENTAL TOXICITY TESTING, WITH SPECIAL REFERENCE TO CADMIUM ION TOXICITY

Publication No. _____

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The University of Texas at Arlington, 2005

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The benefits of developing the pond snail, *Physa acuta*, as a model species for testing acute and chronic toxicity of aquatic pollutants include its wide North American distribution, ease of field collection and laboratory maintenance, and efficacy in testing toxicity responses including mortality, crawling behavior, reproduction and metabolism under both field and laboratory conditions.

Mortality, as 50 % sample lethal concentration (LC_{50}) and 50 % sample lethal time (LT_{50}) values with increased cadmium chloride ($CdCl_2$) concentration in both acute 192 h and chronic 672 h tests. A new endpoint of 50 % sample crawl-out

response (CO_{50}) proved to be a highly efficacious measure of $CdCl_2$ toxicity. It was an order of magnitude more sensitive than mortality testing with endpoint response being rapidly determined at 1-12 h exposure periods.

Both maximum likelihood probit and logistic regression analysis revealed a significant effect of $CdCl_2$ concentration on egg mass oviposition and egg hatching. Similar effects were revealed by general linearized regression analyses on the number of egg masses and eggs oviposited and number of days to egg hatching; by logistic regression on number of embryos developing from eggs; and mixed model least squares regression on the new endpoint of egg diameter. All reproductive responses were significantly different from the control at 5 μg $CdCl_2 \cdot L^{-1}$.

Oxygen consumption rate differed from controls in snails exposed to 1200 μg $CdCl_2 \cdot L^{-1}$, while there was no significant effect on oxygen regulation during progressive hypoxia response. No significant effect on progressive hypoxia resulted from exposure to cadmium chloride.

Shell length was significantly correlated with mortality, crawl-out response, number of egg masses and eggs oviposited, egg hatching, egg diameter and acute oxygen consumption.

The results strongly indicated that *Physa acuta* could be an excellent new model organism for aquatic toxicity testing.

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CHAPTER 1

INTRODUCTION

An investigation was conducted to determine the feasibility of using the common, North American, freshwater, pulmonate snail *Physa* (*Physella*) *acuta* as a model organism for testing acute and chronic toxicological effects of environmental pollutants.

Development of alternative screening methods for determination of lethal concentrations of toxic chemicals have been proposed (Purchase *et al.,* 1998). One alternative is to utilize invertebrates rather than mammals. A similar proposal has recently been sugested to limit toxicological testing of lower vertebrates including amphibians and fish. Toxicological testing with lower vertebrate species requires submission of animal use/care protocols and maintenance of records on care/disposition of test animals similar in extent to that required for higher vertebrates, making use of invertebrate species increasingly more attractive. Advantages for using invertebrates for toxicological testing include decrease in higher vertebrate usage, reduction in space needed for testing and reduced cost of animal acquisition and care.

Several authors have suggested that freshwater gastropods be utilized for toxicological testing. Freshwater snails are common and widespread and easy to

maintain in the laboratory making them readily obtainable and of low cost for toxicity and pollution testing (Cheung and Lam 1998, Melo *et al.* 2000).

A toxicological test species must display measurable responses to the toxic compounds tested (Smith and Hall, 1994). This study investigated the efficacy of *Physa acuta* as a model species for environmental toxicology testing. Specifically, acute and chronic tests of its survival and its metabolic, reproductive and behavioral responses on exposure to a wide concentration range of the common freshwater metallic pollutant, Cadmium (Cd^{2+}), as dissolved cadmium chloride ($CdCl_2$) were investigated.

Toxicology

Studies of environmental toxicology were based on the premise that data collected from laboratory animal studies could be used to predict effects of toxins on endemic species populations or ecosystem faunas (U.S. Environmental Protection Agency [USEPA], 2001). This assertion is based on the assumption that the lethal and sub-lethal responses of animals exposed for short periods of time to elevated concentrations of a toxin in the laboratory could be used to predict the effects of exposure to smaller concentrations of the toxin for longer periods in natural populations (U.S. Environmental Protection Agency [USEPA], 2001). The toxicity of a substance is determined by the ability of that substance to produce undesirable effects through concentration, duration of exposure, chemical form of the substance and route of exposure (i.e., dermal contact, inhalation or ingestion). Substance toxicity is measured through concentration-response, which is based on the concentration and/or duration of exposure and magnitude of the toxic response.

Toxin exposure routes include inhalation, ingestion or dermal contact. Responses range from symptoms of minor irritation to severe effects ultimately leading to death. Many toxic effects are reversible on cessation of toxin exposure, but other impacts are permanent. Toxins affect the organism by altering capacity for behavior, fecundity, growth, metabolism and survivorship. The endpoint or observable effect of a toxin on an organism must be determined in order to conduct a toxicology test. The researcher may choose the endpoint, but there are several accepted standard observed effects, such as the concentration for 50 % sample mortality (LC_{50}), the no observed effect concentration (NOEC), and the lowest observed effect concentration (LOEC) (USEPA, 2001).

The LC_{50} is the concentration of toxin statistically estimated to be lethal to 50 % of a sample of test organisms over a specified period of time (typically 24, 48 or 96 h) (George and George, 1994). The lower the LC_{50} of a substance, the greater is its toxicity to the test organism and presumed toxicity to other species (Shane, 1994). The acute toxicity of a chemical is based on its ability to damage the organism in a one-time exposure to high concentrations of the chemical (George and George, 1994). Acute toxicity testing, defined by the Toxic Substances Control Act of 1976 as amended in 1994 (TSCA), is a method used to determine the concentration of a substance that produced a toxic effect on a reported percentage of test animals over an abbreviated exposure period. In acute testing, exposure periods generally ranged from 24 to 96 h and the measure of toxicity is death. The most common test datum is the LC_{50} value, the concentration, calculated from experimentally derived mortality data, estimated to

be lethal to 50 percent of a test population during continuous exposure over the specified exposure period.

In contrast to short-term, acute, lethal toxicity testing, chronic toxicity determinations measure long-term, often sub-lethal, as well as lethal responses of test organisms to prolonged exposure to low levels of toxin. Because of the complexity, long durations and elevated expense of such chronic tests, they are conducted less frequently than static tests (USEPA, 2001). There is no standard measure for chronic effects. In addition to the LC_{50} endpoint of sample mortality after a specified period of toxin exposure utilized in acute toxicity testing, endpoints for chronic testing include the time period required to achieve a specific level of mortality (usually greater than 50 % mortality). Other endpoints utilized in chronic toxicity testing include relative changes in reproductive, developmental, growth, metabolic, and behavioral responses over extended exposure periods. It is suggested that the most accurate chronic tests are conducted for at least a species' complete life cycle (USEPA, 2001). *Physa acuta* could be raised from hatching to maturity and reproduction within periods of as little as five-six months in the laboratory (personal observation) making this species highly suited for life cycle chronic testing.

The Test Organism

Several factors should be considered when choosing a model organism for toxicity testing. The test organism needed to be sensitive and responsive to the toxic agents being tested. Other requirements included being easy and inexpensive to collect and maintain in the laboratory. Acute toxicity to aquatic animals varies even between

closely related species, so several different species should be tested. Data from several animal species provide more information and is considered more useful than single-species studies (U.S. Environmental Protection Agency [USEPA], 2001). A method for comparative toxicity testing in response to the USEPA water quality guidelines (1975) required that the responses of species from at least eight different freshwater families of animals be tested in order to determine the toxicity of a substance, with guidelines being based on the responses of the most sensitive species (USEPA, 2001). Such comparative testing provided toxicity data for target and non-target species allowing determination of species sensitivity levels.

The advantage of utilizing locally collected organisms is that they best represent native populations impacted by pollutants (Melo, 2000). Thus, it has been suggested that several invertebrate species, including *Daphnia* and snails, be used in freshwater acute toxicity testing. *Physa integra* and *Physa heterostropha* (now combined into *Physa acuta* by Dillon et al., 2002) were examples of snails used for toxicity testing with death as the endpoint (Cheung and Lam, 1998). While use of locally available species offer a number of advantages for toxicity testing, it is also important to choose local species with widespread continental distributions so that they can be used in other geographical areas. Widely distributed test species allowed direct comparison of the results of toxicity data of specific toxins and whole effluents across broad geographic ranges.

Freshwater gastropod molluscs (snails) occurr in a wide variety of habitats**,** including terrestrial habitats, temporary and permanent ponds and rivers. *P. acuta* is

found throughout the North America east of the Rocky Mountains (Burch 1989). In Texas and surrounding states, it was previously identified as *Physella virgata* (Burch, 1989), but, it has been recently documented that all species in the genus, *Physella*, should be collapsed into *Physa acuta* (Dillon *et al*., 2002) which is utilized throughout the remainder of this study. This species is readily cultured in the laboratory and like some other mollusc species described below is particularly amenable to tests of aquatic toxins (Cheung and Lam, 1998, Gomot, 1998, Wilson and McMahon, 1981). The presence of physid snails is as an indicator of poor water quality and nutrient rich conditions (U.S. Environmental Protection Agency [USEPA], 2001).

For this study the utility of the common eastern North American, freshwater, pulmonate pond snail, *Physa acuta*, as an aquatic toxicological model test organism was investigated (Appendix A.1.). Individuals of *Physa acuta* have an ocellus at the base of each of a single pair of tentacles and a thin-walled shell, characteristic of the suborder Basommatophora in the order, Pulmonata, of the molluscan class, Gastropoda (Burch 1989). *P. acuta* have a medium-spired uniquely sinestrally coiled shell characteristic of the family *Physidae*. Like other members of the family, Physidae, *P. acuta* is characterized by a psuedobranch gill formed from elongate extensions of the mantle edge projected over the outer shell whorl, setting it off from the family, Lymnaeidae, which has a dextrally coiled shell and no pseudobranch (Burch, 1989). Physid snails such as *P. acuta* lay gelatinous egg masses just under the water's surface on hard substrata. This species has direct development with fully-formed juveniles hatching

from the egg resembling miniature adults that utilize the same ecological niche as adults (Pechenik, 1996) (Appendix A.2.).

Common Endpoints

Survival

Survival is the most common endpoint of toxicity testing (Phipps and Holcombe, 1985). Acute survival in the freshwater pulmonate *Aplexa hypnorum* to cadmium chloride was investigated and a 96 h LC_{50} of 152 µg $CdCl_2 \cdot L^{-1}$ (88-261 µg $CdCl_2 \cdot L^{-1}$) was determined (Holcombe *et al.,* 1984). Acute toxicity testing has been carried out for *Physa acuta* (Cheung and Lam, 1998). Snails were collected from flooded furrows in Hong Kong and placed in aerated artificial water at 25 °C. Four-day old juveniles were separated from the adults and tested at 25 °C. Ten juveniles were placed in a plastic petri dish with 12 ml of test solution and covered with wire mesh. The acute 24 h LC_{50} for juveniles was 1320 µg $CdCl_2 \cdot L^{-1}$ (1130-1540 µg $CdCl_2 \cdot L^{-1}$) and the 48 h LC_{50} was 1050 µg $CdCl_2 \cdot L^{-1}$ (810-1360 µg $CdCl_2 \cdot L^{-1}$).

There are genotypic impacts on the tolerance of test organisms to toxins. The effects of chronic cadmium exposure on juvenile growth, adult survival and reproduction varied among four naturally occurring genetic clones of the parthenogenetic prosobranch snail, *Potamopyrgus antipodarum* (Jensen and Forbes, 2001). Such results suggested that genetic background and degree of genetic variation among test organisms including snails could impact outcomes of toxicity testing. Thus, use of genetically different cultures of the same species could generate significant

differences in toxicity test results between laboratories even though similar testing protocols were utilized.

Behavior

While crawl-out behavior was investigated in regard to chemical cues associated with predators in freshwater snails, to date, no studies of crawl-out behavior to avoid aquatic pollutants have been carried out for any marine, estuarine or freshwater gastropod species.

Previous studies demonstrated partial or weak avoidance behaviors in response to adverse conditions in freshwater snails (Alexander and Covich, 1991). Gastropod avoidance behaviors included: crawling to the waterline, crawling out of the water and altering crawling speed (McCarthy and Fisher, 2000). Physid snails responded to chemical cues through the use of avoidance behaviors such as crawling to the waterline or out of the water (Dewitt *et al.,* 1998; McCarthy and Fisher, 2000).

McCarthy and Fisher (2000) tested the responses of *Physella heterostropha pomila* to four levels of predation risk. Adult snails were collected from two sites in Pensacola, Florida and placed into 38 L aquaria. The treatments were based on risk level varied from the control with no predation cues (low), cues from non-foraging crayfish (intermediate) to cues from crushed snails (high) and from foraging crayfish (high). Water from tanks housing crayfish was used to provide the chemical cues for crayfish fed snails and those fed algae wafers. “Crawl-out behavior” was defined as the snail being completely above the waterline, “surfacing” was defined as the snail floating or in contact with surface water and “exposed” was defined as snails attached below the

water line to the sides or bottom of the aquaria. Aquaria were observed at intervals of 20, 25 and 30 min. The values for each behavior were averaged over these time periods to provide the proportion for each behavior type.

Crawl-out behavior was analyzed as the proportion of snails completely out of the water of those that moved to the surface or crawled out. Prior to performing an ANOVA, the data was arcsine transformed. Population was defined as the snails collected from the two sites. The crayfish and crushed-snail factors were indicated by presence or absence of cues. The results indicated that weakest crawl-out response occurred in controls, an intermediate response in snails exposed to media from non-foraging crayfish, and the strongest response from media exposed to crushed snails or foraging crayfish. The ANOVA revealed a significant interaction for population x crayfish x injured snails ($F= 4.520$, $p= 0.043$) for crawl-out behavior.

Based on the literature, end points such as size (shell length - SL) (McMahon, 1985), fecundity (Cheung and Lam, 1998), metabolic rate (oxygen consumption rate) (McMahon, 1985) and behavior (avoidance) (Alexander and Covich 1991, McCarthy and Fisher, 2000) are readily quantified in *Physa.* The ability to measure such end points make *Physa* species good candidates for toxicology testing.

Reproduction and Embryonic Development

Reproductive and developmental responses to cadmium exposure in snails have been investigated in several studies (Cheung and Lam, 1998; Gomot, 1998; Holcombe *et al.,* 1984). The effect of exposure to cadmium as $CdCl_2$ on the development of embryos of *P. acuta* was investigated at test concentrations of 0, 1000, 2000, 3000, and

4000 μg $CdCl_2$ L^{-1} over an exposure period of 48 h (Cheung and Lam, 1998). One egg mass laid by an adult snail in artificial water was placed in an aerated 100 ml glass beaker with 80 ml of test solution. The acute 24 h LC_{50} for the embryonic development was 1270 μgL^{-1} $CdCl_2$ (1130 - 1420) and the 48 h LC_{50} was 850 μg $CdCl_2$ L^{-1} (710 - 1010 μg $CdCl_2$ L^{-1}) (Cheung and Lam, 1998). A chronic toxicity test was also conducted at exposure concentrations of 100, 200, 300, 400 and 500 μg $CdCl_2$ L^{-1} in order to compare the number of embryos hatching after exposures of 8 and 28 days. Eggs not hatching after 28 days were considered unhatchable. ANOVA indicated a significant decrease in percent hatch with increasing $CdCl_2$ concentration. Hatching was completely inhibited at concentrations greater than 210 μg L^{-1} $CdCl_2$ L^{-1}.

The toxic effects of cadmium on sub-lethal responses of egg-laying and embryo development were studied in the freshwater snail *Lymnaea stagnalis* (Gomot, 1998). Snails were fed lettuce supplemented with fish food. Egg masses were collected twice a week during water change and placed in Petri dishes with the same cadmium concentration as the adult. The number of egg masses and number of eggs per mass were counted and embryo development recorded weekly. Length of incubation and number of hatched juveniles were recorded for each treatment. At the end of seven weeks, the mean number of eggs per mass for each concentration was determined and the percentage of eggs hatched in each treatment was compared to the control. The results demonstrated no significant difference between controls and concetrations of 0, 25, 50 and 100 μg Cd^{+2} (as $CdCl_2$) L^{-1} for number of egg masses and number of eggs per mass. At a concentration of 200 μg Cd^{+2} (as $CdCl_2$) L^{-1} there was a significant difference

for number of eggs per mass relative to controls, but not in number of masses oviposited. At a concentration 400 of μg Cd^{+2} (as $CdCl_2$) L^{-1} there was a significant difference in both number of masses oviposited and number of eggs per mass relative to controls. This study demonstrated more frequent and more deleterious effects as Cd concentration increased. The control group exhibited hatching response in 12-14 days over a two-day period. Treatment groups of 25 and 50 μg Cd^{+2} (as $CdCl_2 \cdot L^{-1}$) started hatching 4-5 days later over a 10 d period. A hatching rate of 8 % was recorded at 100 μg Cd^{+2} (as $CdCl_2$) L^{-1} which was significantly reduced relative to the 15-21 % hatching rate recorded at 0, 25 and 50 μg Cd^{+2} (as $CdCl_2$) L^{-1}. Time to hatching, number of eggs per mass and hatching rate (0.4%) were all significantly reduced at 200 μg Cd^{+2} (as $CdCl_2$) L^{-1}. There was also a decrease in the size of embryos compared to controls. The study also revealed an inhibition concentration gradient within egg masses such that hatching success was reduced among eggs at the edge of the egg mass and increased among eggs towards the center of the mass. This hatching gradient suggested that the gelatinous material surrounding the eggs provided protection from the inward diffusion of external toxins from the surrounding medium. The egg envelope may have also inhibited inward diffusion of toxins, allowing the embryo to survive to an advanced stage of development. Prior to oviposition, maternal tissues may have provided an initial level of protection from toxins, so that eggs were not fully exposed to cadmium until after being oviposited in contaminated water (Gomot, 1998).

Exposure to heavy metals also has negative sub-lethal impacts on reproduction in other freshwater snails. In a 26-day reproductive study using individuals of the

freshwater pulmonate, *Aplexa hypnorum,* no oviposition occurred at cadmium exposure concentrations that significantly impacted survival determined from a 96 h LC_{50} of 152 μg $CdCl_2 \cdot L^{-1}$ (88-262 μg $CdCl_2 \cdot L^{-1}$) (Holcombe *et al.,* 1984). In a 26 d embryo development study, eggs were added to cadmium concentrations ranging from 0 - 21.6 μg $CdCl_2 \cdot L^{-1}$. Eighty four percent of all eggs developed normally after four days of chronic exposure to these cadmium concentrations. Hatching occurred after 6-9 days only at concentrations of 7.82 μg $CdCl_2 \cdot L^{-1}$ and below. No hatching occurred at higher concentrations (>11.71 μg $CdCl_2 \cdot L^{-1}$) after 10 days of exposure, but 11 embryos survived to the end of the study in the 12.46 μg $CdCl_2 \cdot L^{-1}$ treatment and 3 embryos in the 19.12 μg $CdCl_2 \cdot L^{-1}$ treatment. At 26 days, no embryos hatched in the 21.07 μg $CdCl_2 \cdot L^{-1}$ treatment, although one embryo hatched in the 21.56 μg $CdCl_2 \cdot L^{-1}$ treatment. There was a significant difference between the control and treatment levels of 7.82, 11.71, 12.46 and 21.56 μg $CdCl_2 \cdot L^{-1}$ in the number of embryos surviving to the end of the toxicity test period (Holcombe *et al.,* 1984).

Respiration

Exposure to heavy metals has been demonstrated to impact metabolic rates of molluscs measured as weight specific oxygen uptake rates (Mo_2). The dry tissue weighted oxygen consumption rates of specimens of the inter-tidal blue mussel, *Mytilus edulis L.*, and inter-tidal snail, *Littorina rudis*, were determined for specimens collected from 13 seashore sites of varying copper concentration along a 60 km copper pollution gradient extending from Avoca to Dublin along the eastern coast of Ireland (Wilson and McMahon, 1981). There was a significant negative correlation between environmental

Cu exposure and dry tissue weight in *M. edulis*, but no correlation with Mo_2. In contrast, there was a strong positive correlation between environmental Cu concentration and Mo_2 among samples of *L. rudis* suggesting that environmental copper pollution impacted metabolic rate in this snail species.

Allometric Impacts on Toxicity Testing

Allometry, the study of size and its consequences, attempted to describe and explain the relationship between body size and individual responses or morphology. During the 1990s, the concepts and techniques developed in physiological and morphological allometric studies were adopted in studies of heavy metal accumulation and toxicity (Newman and McIntosh, 1991). Several factors were shown to influence the relationship between body size and tissue metal concentration. Metabolic rate, respiratory rate and relative surface area have been demonstrated to have allometric relationships with body burdens of toxic metals. Temporal factors including age, growth and exposure duration have been demonstrated to influence metal body burden. Age related changes in physiology, cytology and biochemistry associated with maturation and reproduction impact size-dependent body metal concentrations (Newman and McIntosh, 1991).

Comparison of Toxicity Responses to Other Species

The published toxic responses of *Daphnia magna* to heavy metals relative to other species have been reviewed (Mark and Solbe, 1998). This review demonstrates that there were several obstacles to comparing the results of published toxicity values among different species. Several endpoints or exposure periods could not be compared

because no results for other species were available. Exposure periods and toxicity test protocols differed across studied species making it difficult to directly compare results. In order to allow better inter-specific comparisons it was recommended that the same exposure period or periods be used for all species in acute toxicity testing.

Several investigators have reported species differences in cadmium toxicity. Phipps and Holcombe (1985) reported 96 h LC_{50}s for $CdCl_2$ for various organisms including the fathead minnow (1500 $\mu g \cdot L^{-1}$), goldfish (748 $\mu g \cdot L^{-1}$), Rainbow trout (30 $\mu g \cdot L^{-1}$), Bluegill (6470 $\mu g \cdot L^{-1}$) and a snail (930 $\mu g \cdot L^{-1}$). Cadmium lethal tolerance (LT) values were determined for several different exposure periods in the pulmonate pond snail, *Physa gyrina* Say. The LT_{50} value for a 24 h exposure was 7600 $\mu g \cdot L^{-1}$, for 48 h exposures, 4250 $\mu g \cdot L^{-1}$, for 96 h exposures, 1370 $\mu g \cdot L^{-1}$, and for 228 h exposures, 830 $\mu g \cdot L^{-1}$ (Wier and Walter, 1976).

The effects of any one specific toxin can vary among different species and species groups. For example, the responses to a chronic 28 d exposures to various concentrations of $CdCl_2$ (control <0.05 $\mu g \cdot L^{-1}$, 3.0 ± 0.3 $\mu g \cdot L^{-1}$, 8.3 ± 0.3 $\mu g \cdot L^{-1}$, 27.5 ± 2.7 $\mu g \cdot L^{-1}$, 85.5 ± 7.6 $\mu g \cdot L^{-1}$ and 238 ± 16.4 $\mu g \cdot L^{-1}$) were determined for the freshwater pulmonate the snail, *Physa integra*, and stonefly larvae, *Pteronarcys dorsata*, caddisfly, *Brachycentrus sp*. and mayfly, *Ephemerella sp*, using an intermittent flow design (Spehar *et al.,* 1978). Water from Lake Superior was maintained at 15 °C. An LC_{50} of 10.4 μg $CdCl_2 \cdot L^{-1}$ was recorded for *Physa integra* while the LC_{50} of <3.0 μg $CdCl_2 \cdot L^{-1}$ for *Ephemeralla* sp. was based on greater than 50 % mortality in the

lowest concentration of 3.0± 0.3 μg $CdCl_2 \cdot L^{-1}$. Although the concentrations used in this study were not high enough to cause a significant decrease in mortality of stonefly or caddisfly larvae, a change in behavior from the controls involving convulsive flexing of the body was observed at concentrations of 85.5 μg $CdCl_2 \cdot L^{-1}$ and 238 μg $CdCl_2 \cdot L^{-1}$ respectively.

Experimental Design in Toxicity Testing

Toxicity testing designs include static, renewal or flow-through exposures to the toxin. Static or flow-through methods can be used for toxicity testing of aquatic organisms (U.S. Environmental Protection Agency [USEPA], 2001). In static tests, the toxin and organisms are added to the test solution without further change of the test medium. In renewal testing, the medium is periodically replaced during the course of exposure. In flow-through tests, fresh testing medium continuously flows through the chamber holding the test organism.

Each of these toxicity test methodologies has associated advantages and limitations. Static testing has the disadvantage of allowing waste products and metabolic degradation products to accumulate in the test medium potentially negatively impacting survivorship. Its advantage is low maintenance requirements. Renewal testing partially overcomes waste/metabolic degradation product accumulation by periodic replacement of the medium, but increases maintenance requirements. Flow-through testing involves continuous renewal of the medium, maintaining a constant concentration and preventing accumulation of wastes and metabolic degradation products. However, the constant conditions in flow-through systems may not be

representative of more variable natural environments and these systems generally require extensive maintenance. Variation in test conditions associated with static testing designs can be reduced by renewal designs incorporating regular, frequent changes of the test media in order to avoid accumulation of deleterious waste products. Static or renewal test designs are simpler and less expensive than flow-through tests, reducing the overall costs and manpower requirements of toxicity testing (U.S. Environmental Protection Agency [USEPA], 2001).

Detection and Measurement of Cadmium Concentrations

Cadmium is included in a list of known or suspected toxins at super-fund sites on the National Priority List (NPL) that pose a significant threat to human health (U.S. Environmental Protection Agency [USEPA], 2001). Cadmium is used in fertilizers, in industry as an anticorrosive agent and is a waste product from zinc and lead mining (Chang and Cockerham, 1994). Cadmium may enter natural water bodies and soil from spills or leaks at hazardous waste sites. Cadmium is usually present in nature as complex oxides, sulfides or carbonates in zinc, lead, or copper ores. It is also found in smaller amounts as chloride or sulfate salts that are relatively water-soluble (U.S. Environmental Protection Agency [USEPA], 2001).

There are several accepted analytical methods for detection and measurement of cadmium or cadmium compounds (Agency for Toxic Substances and Disease Registry [ATSDR], 1999). Two common methods for analyzing cadmium in environmental and biological samples are atomic absorption spectroscopy (AAS) and atomic emission spectroscopy (AES). AAS uses a flame or furnace to atomize cadmium compounds.

The resulting ground-state cadmium vapor absorbs monochromatic radiation. The intensity of radiation from a constant source is detected by a photometer with reduction in monochromatic light transmission being directly related to cadmium vapor concentration. AES uses a flame or inductively coupled plasma to produce cadmium atomization. The AES method provides a lower detection limit due to increased light emission from the higher temperatures (Manahan 1994).

Toxicity Tests Selected for the Present Investigation

In this section, determinations for acute and chronic survival were estimated utilizing two end points, lethal concentration and lethal time. Both LC_{50} and LT_{50} values were estimated with and without size measured as shell length (SL) as a covariate on the same survival data for samples of *P. acuta* utilizing SAS® (Cary, NC) computer program codes developed in collaboration with Dr. D. L. Hawkins of The University of Texas at Arlington. Values of LT_{50} were estimated from a modified Discrete Logistic Failure Time Model developed by (Hicks *et al.,* 2000). Analysis of the same survival data sets with and without size measured as SL as a covariate allowed comparison of the impacts of sample individual size distributions of *P. acuta* on LC_{50} and LT_{50} determinations and analysis of the degree to which the size of individuals tested could impact decisions regarding environmentally acceptable levels of aquatic toxicants.

Based on the studies of Alexander and Covich (1991), Dewitt *et al.* (1998) and McCarthy and Fisher (2000) which indicated that crawl-out is a general response in freshwater pulmonate snails to irritating, water-borne chemical cues, this study

investigated the possibility of using crawl-out response as a new toxicity testing methodology in *P. acuta*. Toxicity testing based on the crawl-out response would be more rapid (< 12 h), require fewer test individuals, provide an accurate, readily observable endpoint and be more cost-effective and readily adapted to the field.

In addition, development of a 50 % crawl-out value (CO_{50}) allows direct integration of crawl-out response data with LC_{50} values determined from standard mortality testing as required for a new toxicity testing methodology (Purchase *et al.,* 1998). Probability of crawl-out was determined over a wide range of $CdCl_2$ concentrations in order to provide the basis for comparison with the LC_{50} values determined for exposure of specimens of *P. acuta* to $CdCl_2$ in this study. Values of CO_{50} were estimated as were survival LC_{50} using both Maximum Likelihood Probit and Logistic Regression analyses, allowing direct comparison between the CO_{50} endpoints estimated by these two statistical tests.

In addition to the standard toxicity tests of survival and the introduced study of crawl-out response, this study investigated several aspects of reproduction as fecundity and development. The research described in this chapter also examines several statistical methodologies for analyzing the reproduction and development data resulting from these studies. The impacts of $CdCl_2$ concentration on long-term reproductive responses were assessed in *P. acuta*, using effect concentration for 50 % sample egg mass oviposition (EC_{50}) as a point estimator of toxicity. The EC_{50} value, the concentration estimated to induce 50 % reduction in individuals ovipositing one or more egg masses, was calculated by both the more standard maximum likelihood probit

regression analysis and the more recently developed and less commonly used maximum likelihood logistic regression analysis. Use of logistic regression analyses to estimate EC_{50} values has been recommended by statisticians (Finney, 1971), but has not been generally adopted in toxicity studies. One of the main objectives of this study was to analyze the same data sets for reproduction of samples of *P. acuta* on exposure to the toxic effects $CdCl_2$ and compare their outcomes in order to determine if there would be major differences in the EC_{50} predictions generated by probit versus logistic regression analysis that would justify use of one statistical technique over the other.

Based on the studies of Cheung and Lam (1998), Gomot (1998) and Holcombe *et al.* (1984), which indicated that the oviposition decreased as a general response in freshwater pulmonate snails to increasing $CdCl_2$ concentrations, this study investigated the possibility of using fecundity measured as number of egg masses and eggs oviposited·individual^{-1} as a response variable for estimating the sub-lethal toxic effects of $CdCl_2$ exposure for *P. acuta*. A general linearized model regression analysis provided a determination of the effect of $CdCl_2$ on the number of egg masses and eggs oviposited over a 672 h (28 d) exposure period. Mean number of egg masses·individual^{-1} and number of eggs·individual^{-1} were estimated at each tested $CdCl_2$ concentration along with Bonferroni multiple pair-wise comparisons between concentrations.

In addition to the parameters discussed above, the impact of $CdCl_2$ exposure was also recorded for several developmental parameters in *P. acuta.* Among these was the fraction of eggs developing into embryos determined at weekly intervals over an observation period of 672 h (28 d). Regression analysis provided determinations of the

effect of cadmium chloride on the fraction of eggs developing into embryos based on $CdCl_2$ concentration, shell length and weeks after oviposition. Toxicity testing based on fraction of eggs developing into embryos would also require fewer test individuals than standard survival testing, provide an accurate, readily observable endpoint and be cost-effective.

Based on the studies of Cheung and Lam (1998), Gomot (1998), and Holcombe *et al.* (1984), which indicated that the percent of eggs hatching decreased as a general response in freshwater pulmonate snails to $CdCl_2$ exposure, this study investigated egg hatching as a toxicity testing methodology in *P. acuta* using two different developmental endpoints. These two endpoints included, 1) the concentration of $CdCl_2$ resulting in 50 % of individuals ovipositing egg masses in which at least one egg hatched into a juvenile (EC_{50}) and 2) the number of days to hatching over a 672 h (28 d) exposure period. Toxicity testing based on the egg hatching response would require fewer test individuals than standard survivorship testing, provide an accurate, readily observable endpoint and be more cost-effective and readily adapted to the field.

Previous studies (Cheung and Lam, 1998, Gomot, 1998, and Holcombe *et al.,* 1984) have revealed a temporal delay in embryonic development to hatching with increasing $CdCl_2$ concentration in several species of freshwater pulmonate snails. This study investigated the possibility of using the time to hatching of eggs oviposited by specimens of *P. acuta* as a potential endpoint variable in determining sub-lethal $CdCl_2$ toxicity in this species. A general linearized regression model provided a determination

of the effect of $CdCl_2$ exposure on the number of days to egg hatching over a 672 h (28 d) exposure period.

This chapter also describes the efficacy of a new toxicity testing methodology involving the variation in the size of eggs oviposited by specimens of *P. acuta* measured as egg diameter when exposed to a range of sub-lethal $CdCl_2$ concentrations. To date, a thorough literature search conducted as part of this research has revealed no published toxicity studies of aquatic pollutants that have used egg diameter as an endpoint parameter for any freshwater animal test species, although Gomot (1998) reported an observation that embryos in a concentration of 200 µg $Cd^{+2} \cdot L^{-1}$ appeared smaller than those in the control. Toxicity testing based on the egg diameter response would be more rapid (< 96 h), require fewer test individuals than standard survival testing, provide an accurate, readily observable endpoint and be cost-effective.

In their review of the lethal and sub-lethal impacts of heavy metal toxicity, Newman and McIntosh (1991) noted that animal body size impacted tissue metal concentration and level of toxicity. In spite of the widely accepted fact that there is an allometry between body size/age and toxicity tolerance, size of test specimens is rarely considered in aquatic toxicity testing (USEPA 2001). In this chapter, all reproductive endpoints were estimated with and without size measured as shell length (SL) as a covariate on the same reproductive data for samples of *P. acuta* utilizing SAS® (Cary, NC) computer program codes developed in collaboration with Dr. D. L. Hawkins of The University of Texas at Arlington. Analysis of the same reproductive data sets with and without size measured as SL as a covariate allowed comparison of the impacts of

individual size distributions in samples of *P. acuta* on EC_{50} analysis and on the degree to which the size of individuals tested could impact decisions regarding environmentally acceptable levels of aquatic toxicants.

Following the reproductive studies, a section will describe a study of the possibility of utilizing changes in the oxygen consumption rate of adults of the common, pulmonate pond snail, *Physa acuta*, as a new testing methodology to examine the toxicity of $CdCl_2$. Oxygen consumption rate was chosen for investigation as a possible sub-lethal indicator of toxicity because of the impact demonstrated by exposure to low levels of two molluscicides in the freshwater bivalves zebra mussels (*Dreissena polymorpha*) and Asian Clams (*Corbicula fluminea*) (Moeller, 1993). Similarly, exposure to low Cd^{2+} concentrations (5-10 μg $Cd^{2+} \cdot L^{-1}$) depressed oxygen consumption rates in the freshwater cladoceran, *Daphnia magna* (Barber *et. al.,* 1994). In contrast, short-term feeding of cadmium-impregnated food did not significantly affect oxygen consumption rates in juvenile rainbow trout (*Oncorhynchus mykiss*) (Hollis *et al.* 1999, 2000), nor did it impact the oxygen consumption rates of rainbow trout hepatocyte cell cultures even though it is impacted by exposure to copper ions (Manzel *et al.,* 2003). In this section, oxygen consumption values were estimated with size measured as dry tissue weight (DTW) as a covariate on acute oxygen consumption rate at full air O_2 saturation and oxygen consumption rate in response to progressive hypoxia after a 48 h exposure to lethal concentrations of $CdCl_2$. The impacts of $CdCl_2$ concentration on acute oxygen uptake rates at near air saturation with oxygen and oxygen consumption

rates in response to progressive hypoxia was assessed in *P. acuta*, using Analysis of CoVariance (ANCOVA).

CHAPTER 2

EFFECT OF CADMIUM CHLORIDE ON SURVIVAL AND BEHAVIOR CRAWL-OUT RESPONSES

Introduction

The requirements for development of new toxicity test methodologies to replace or supplement existing standard methodologies have been reviewed (Purchase *et al*, 1998). They state that a basic requirement for development of new environmental toxicity test methodologies is that they must be capable of supplying useful information not produced by existing tests and/or produce the information more rapidly, accurately and/or less expensively in terms of direct costs and/or labor time than do existing standard tests (Purchase *et al.,* 1998). In addition, any newly proposed test methodology should be able to be integrated with existing toxicity tests since no single test can provide all required information. Information resulting from a new methodology should also be capable of being converted into predictive models that allow decision makers to resolve issues more quickly or accurately or to use less information than required from traditional test results when making decisions. Thus, data generated from any new test format must be capable of being linked to that produced by existing standard tests. Any newly developed testing methodology must also be readily and inexpensively transferable among laboratories and produce

accurately repeatable results among different laboratories (Purchase *et al.,* 1998). In addition, Purchase *et al.* (1998) highly recommend that any new methodologies allow replacement of vertebrate test species with invertebrate species whenever possible to avoid both the paperwork and public concerns associated with vertebrate toxicity and mortality testing. Finally, statistical analyses utilized to analyze data produced by any new toxicity testing methodology should allow reduction of error leading to more accurate predictions of environmental toxicity and, thus, better decision-making on the part of regulators and industry (Purchase *et al*., 1998).

This section describes a study of the utility of the common, pulmonate pond snail, *Physa acuta*, as a new model species for acute and chronic survival testing in aquatic toxicity studies utilizing cadmium chloride ($CdCl_2$) as the toxicant. It also examines new statistical methodologies for analyzing survival. The impacts of $CdCl_2$ concentration on both short-term and long-term survival responses were assessed in *P. acuta*, using lethal concentration for 50 % sample mortality (LC_{50}) and lethal time for 50 % sample mortality (LT_{50}) as parameters of toxicity. The LC_{50} value, the concentration estimated to induce 50 % sample mortality, was calculated by both the more standard maximum likelihood probit regression analysis and the more recently developed, and less commonly used, maximum likelihood logistic regression analysis. Use of logistic regression analyses to estimate LC_{50} values has been recommended by survival statisticians (Finney, 1971) but has not been generally adopted in toxicity studies. One of the main objectives of this study was to analyze the same data sets for survival of samples of *P. acuta* on exposure to the toxic effects of $CdCl_2$ and compare

their outcomes in order to determine if there would be major differences in the LC_{50} predictions generated by probit versus logistic regression analysis, that justify use of one statistical technique over the other.

In their review of the lethal and sub-lethal impacts of heavy metal toxicity, Newman and McIntosh (1991) noted that animal body size impacted tissue metal concentration and level of toxicity. In spite of the widely accepted fact that there is an allometry between body size/age and toxicity tolerance, size/age of test specimens is rarely considered in aquatic toxicity testing (USEPA, 2001). In this section, both LC_{50} and LT_{50} values were estimated with and without size (measured as shell length (SL)) as a covariate, on the same survival data for samples of *P. acuta* utilizing SAS® (Cary, NC) computer program codes developed in collaboration with Dr. D. L. Hawkins of The University of Texas at Arlington. Values of LT_{50} were estimated from a modified Discrete Logistic Failure Time Model (Hicks *et al.,* 2000). Analysis of the same survival data sets, with and without SL as a covariate, allowed assessment of the impact of size distributions of *P. acuta* on LC_{50} and LT_{50} determinations and analysis of the degree to which the size of individuals tested could impact decisions regarding environmental acceptable levels of aquatic toxicants.

This section also describes the application and utility of a new toxicity testing methodology. The tendency of specimens of *P. acuta* to crawl above the water's surface (i.e., crawl-out response) when exposed to irritating conditions such as those induced by exposure to toxicants such as $CdCl_2$ was investigated based on responses to chemical cues associated with predators in freshwater snails (McCarthy and Fisher, 2000). To

date, no studies of crawl-out behavior to avoid aquatic pollutants have been carried out for any marine, estuarine or freshwater gastropod species.

Previous studies have demonstrated partial or weak avoidance behaviors in response to adverse environmental conditions in freshwater snails (Alexander and Covich, 1991). Gastropod avoidance behaviors included: crawling to the waterline, crawling out of the water and altering crawling speed (McCarthy and Fisher, 2000) including physid snails which respond to noxious chemical cues by crawling to the waterline or out of the water (Dewitt *et al.,* 1998; McCarthy and Fisher, 2000). McCarthy and Fisher (2000) tested the responses of *Physella heterostropha pomila* to four levels of predation risk including no predation cues (low); cues from non-foraging crayfish (intermediate); cues from crushed snails (high); and from foraging crayfish (high). Their results indicated that weakest crawl-out response occurred in controls, an intermediate response occurred in snails exposed to media from non-foraging crayfish, and the strongest crawl-out responses from media exposed to crushed snails or foraging crayfish.

Based on the studies of Alexander and Covich (1991), Dewitt *et al.* (1998) and McCarthy and Fisher (2000), which indicated that crawl-out was a general response in freshwater pulmonate snails to irritating, water-borne chemical cues, this study investigated the possibility of using crawl-out response as a new toxicity testing methodology in *P. acuta*. Toxicity testing based on the crawl-out response would be more rapid (< 12 h), require fewer test individuals, provide an accurate, readily observable endpoint and be more cost-effective and readily adapted to the field than the

standard 96 h LC_{50}. In addition, development of a 50 % crawl-out value (CO_{50}) allowed direct integration of crawl-out response data with LC_{50} values determined from standard mortality testing as required for a new toxicity testing methodology (Purchase *et al.,* 1998). Probability of crawl-out was determined over a wide range of $CdCl_2$ concentrations in order to provide the basis for comparison with the LC_{50} values determined for exposure of specimens of *P. acuta* to $CdCl_2$ in this study. Values of CO_{50} were estimated as were survival LC_{50} using both maximum likelihood probit and logistic regression analyses, allowing direct comparison between the CO_{50} endpoints estimated by these two statistical procedures.

Methods

Acute and Chronic Survivorship

Specimen Collection and Holding Conditions

Species of *Physa acuta* for toxicity testing were collected from a section of Trader Horse Creek in Arlington, Texas, which flowed along the south side of the University of Texas at Arlington campus, Arlington, Texas. Individuals were collected by removing them from the upper surface of rocks on the creek substratum or from rocks lifted from the substratum. After collection snails were returned immediately to the laboratory and held in 21 x 21 x 12.5 cm plastic aquaria filled with 5 L of artificial pond water (composition described below) at a constant temperature of 25 °C. Tank media was replaced every three days until snails were used in experiments. Snails were fed *ad libitum* with flake aquarium fish food (Total Tropical Gourmet Flake Blend® fish food, Wardley, Secaucus, NJ) after each replacement of holding media. Snails were

held in the laboratory for no less than 10 days and no longer than 60 days prior to use in experiments.

Testing Media

Holding and testing medium for all toxicity testing was artificial pond water. The artificial pond water was composed of 29 $mg \cdot L^{-1}$ sodium chloride (NaCl), 17 $mg \cdot L^{-1}$ sodium bicarbonate ($NaHCO_3$), 12 $mg \cdot L^{-1}$ potassium chloride (KCl), 15 $mg \cdot L^{-1}$ magnesium sulfate ($MgSO_4$), and 50 $mg \cdot L^{-1}$ Ca^{+2} as calcium chloride ($CaCl_2$) dissolved in distilled water (Thomas Dietz personal communication). Based on the amount of calcium in this formula, this medium is considered soft water (US EPA, 2001).

Survival Testing Conditions

Acute and chronic survival toxicity testing of $CdCl_2$ to specimens of *Physa acuta* involved utilization of control and experimental samples, composed of 150 individuals each. Control and treatment samples were held at constant temperature at 25 °C (±1 °C). Media was replaced with fresh media previously brought to the test temperature every three days during the chronic test period. During acute testing of 24 h and 48 h exposures to $CdCl_2$, the medium was not changed. In acute 96 h exposures, media was replaced after 48 h of exposure. Media was again replaced at 120 h and the exposure terminated after 192 h (i.e., eight days).

During chronic and acute survival testing, snails were observed at each monitoring period. Those not locomoting or with an extended, unattached foot were tested for mortality by stimulating of the foot with the bristles of a fine brush. Snails not responding to such stimulation by pedal withdrawal were considered dead. Individuals

appearing dead were removed from the test container and allowed to recover for 1 h in untreated media in a Nalgene© container (4 oz, 6'' diameter). If snails did not display a return to normal activity after the 1 h recovery period, they were considered dead and their shell lengths measured to the nearest 0.1 mm with a dial micrometer. Any snails that recovered after not displaying a pedal withdrawal reflex were excluded from data analysis (28 of 4,950 individuals or 0.566 %). At each determination of sample mortality in acute and chronic testing, the behaviors of snails were recorded in control and treated samples (i.e., whether attached, unattached or locomoting) as well as their positions in the sample chamber (i.e., on floor, side, or top of the chamber or on the air stone).

Acute Toxicity Testing

In order to determine acute toxicity tolerance to $CdCl_2$, 150 snails were placed individually in lidded plastic boxes (2.19 x 1.30 x 0.41 cm) submerged in a plastic tank (21 x 21 x 12.5 cm) holding 5 L of test solution at a specific test $CdCl_2$ test concentration (Fig 2.1). The lid of each box had a 2 cm diameter opening covered with a 1 mm mesh screen to allow media exchange but prevent snail escape. In order to prevent snail escape, the lid remained closed unless dead snails were being removed. Control and treated samples of 150 randomly selected individuals ranging from approximately 3.3 to 15.081 mm SL were used for each concentration tested. This size range included juvenile (<4.0 mm) and adult snails (>4.0 mm) (McMahon, 1975). The concentrations tested were determined from prior range-finding testing of acute mortality response of *P. acuta* to $CdCl_2$, based on a previously published observation

that the acute 48-h LC_{50} for *Physa gyrina* is 1370 μg $CdCl_2 \cdot L^{-1}$ (U.S. Environmental Protection Agency (USEPA, 2001). This preliminary range testing indicated that nearly 100 % mortality occurred in a sample of 30 adult individuals of *P. acuta* subjected to 2000 μg $CdCl_2 \cdot L^{-1}$ for 48 h, while no mortality occurred in a similar-sized sample exposed to 10 μg $CdCl_2 \cdot L^{-1}$. Based on this preliminary test, the acute toxicity responses of *P. acuta* were tested at dosages of 0, 100, 200, 300, 400, 600, 800, 1000, 1200, 1400, 1600, 2000, 2600, 3000, 4000 and 5000 μg $CdCl_2 \cdot L^{-1}$.

For acute toxicity determinations, snails were exposed to test $CdCl_2$ concentrations for a maximum of eight days without feeding, based on prior studies demonstrating no feeding resulted in no mortality over an observation period of 8 d (personal observation). During acute toxicity testing, snail mortality was monitored every hour for the first 12 h of exposure and subsequently every 4 h until either 100 % mortality was achieved or for eight days, if 100 % mortality was not achieved within that time period. Dead individuals were removed at each monitoring period and their shell lengths (greatest linear distance from the tip of the spire to the anterior edge of the aperture) measured to the nearest 0.1 mm with a dial micrometer. During acute testing of 24 and 48 h exposures to $CdCl_2$, medium was not changed. In acute 96 h exposures, media was replaced after 48 h of exposure. Media was again replaced at 120 h and the exposure terminated after 192 h (i.e., eight d). The SL of all individuals surviving the eight-day exposure period was also measured.

Chronic Survival Testing

Chronic survival testing on exposure to $CdCl_2$ employed a snail holding methodology similar to that used in acute testing (see above) except that exposure durations were increased to 28 days. At each concentration tested, 150 snails were placed individually in lidded plastic boxes (2.19 x 1.30 x 0.41 cm) submerged in a plastic tank (21 x 21 x 12.5 cm) holding 5 L of test solution. The range of dosages of $CdCl_2$ utilized in survival determinations were chosen based on the results of the acute toxicity study and a preliminary 28-day exposure of snails to a variety of $CdCl_2$ concentrations. Snails were fed Total Tropical Gourmet Flake Blend® fish food (Wardley, Secaucus, NJ) *ad libitum* during chronic testing with food renewed every three days immediately after media renewal. Behavioral observations of snails in control and test media were as described for acute testing conditions above. Control and treated samples of 150 randomly selected individuals ranging from approximately 3.3 to 15.1 mm SL were used for each concentration tested. This size range included juvenile (<4.0 mm) and adult snails (>4.0 mm) (McMahon, 1975). The shell length of each snail was measured to the nearest 0.1 mm as described above. A control and experimental samples of snails each were tested at concentrations of 0, 10, 50, 100, 150, 200, 250, 300, 350, 400, 450, 600, 800, 1000, 1200, 1600, 2000 μg $CdCl_2 \cdot L^{-1}$. Dosages above 2000 μg $CdCl_2 \cdot L^{-1}$ were not chronically tested because acute testing indicated that 100 % mortality was achieved within an 8 d testing period.

Sample mortality was monitored every 24 h over the course of the 28 d exposure period as described in the section above on "Acute Toxicity Testing". The SL of each

dead individual was measured to the nearest 0.1 mm after it was removed from the test chamber. The SL of all individuals surviving the 28 d exposure period was also measured.

Survival Analysis

Sample mortality was analyzed utilizing maximum likelihood probit and logistic regression methods (Shane, 1984) to reveal the influence of $CdCl_2$ concentration and time on survival as LC_{50} (i.e., the lethal concentration of a toxin resulting in 50 % sample mortality within a fixed time period) or LT_{50} (i.e., the lethal time period resulting in 50 % sample mortality at a fixed toxin concentration).

Probit analysis is the conventional methodology for determination of acute or chronic survivorship LC_{50} values (Finney, 1971). The probit transformation has been utilized to straighten cumulative curves, such as survival curves. Binomial response data is transformed into normal equivalent deviations (NED) to produce a linear function. The NED for 50 % mortality is 0, +1 for 84.1 % mortality and –1 for 15.9 % mortality based on the standard deviations from the normal frequency distribution. NED values were converted to probit units by adding +5, thus avoiding negative numbers. These values can be graphed and the LC_{50} values are then determined by estimating the equivalent of 50 % survivorship (Sokol and Rohlf, 1995). In this research maximum likelihood analyses was used to estimate LC_{50} values, using the probit function. The likelihood is defined as being proportional to the joint probability of all observations in the study. Estimators from this analysis are efficient using large samples. Given a large number of subjects per concentration, maximum likelihood

provided estimates with greater precision, smaller variance, than other methods (Finney, 1971).

Logistic regression also uses maximum likelihood to estimate LC_{50}. The logistic regression function is recommended by Floyd (2001) to transform constrained data, rather than using ordinary least squares (OLS) regression for determination of LC_{50} values. Logistic regression transformation removes the violation of regression assumption of a continuous variable. Logistic regression is used to predict values with constrained response values, usually coded as 0 and 1. For this analysis snails surviving a particular test period were coded as 0 and snails dying during the test period were coded as 1. The proportion of 0 or 1 is determined using a logit transformation. The logit converted values with limits (0, 1) to a limitless scale. Logistic regression allowed the use of covariates such as individual size in determination of LC_{50} and LT_{50} values (Dytham, 2003; Floyd, 2001).

Lethal Concentration$_{50}$ (LC_{50}), the statistically estimated concentration causing the death of 50 % of the sample population over a fixed time was calculated from maximum likelihood probit and logistic regression methods for several time periods (i.e., at 24 h intervals) in both acute and chronic tests. To determine the the probability of an individual dying in a given interval without SL as a covariate the following generalized linear regression model with either the probit or logistic distribution is utilized: $\Gamma^{1}(p) = b_0 + b_1(Lconcentration+1)$. To determine the the probability of an individual dying in a given interval with SL as a covariate the following generalized

linear regression model with either the probit or logistic distribution was utilized: $\Gamma^{1}(p) = b_0 + b_1(Lconcentration+1)+b_2\ (SL-SL^0)+b_3[(Lconcentration+1)*(SL-SL^0)]$.

A discrete logistic failure time model (DLFTM) was used to investigate the Lethal $Time_{50}$ (LT_{50}), the estimated median survival time, calculated using concentration as the only factor and with SL as a covariate (Hicks et al., 2000). LT_{50} determinations with specified toxin exposure durations may result in censored data, in which not all subjects exhibit mortality within the experimental time frame. A "censored survival time" methodology was utilized in analysis of survival time in this study that included data on individuals that did not die by the end of the observation period. An *a priori* fixed time period of 8 days for acute survival and 28 days for chronic survival in the toxicity testing resulted in all surviving individuals having the same time under observation. A logistic failure time model (DLFTM) (Hicks *et al*., 2000) rather than Kaplan-Meier survival analysis, another common survival technique, which requires the exact survival times of individuals, was used to investgate the LT_{50}. The DLFTM model allowed the response (survival) to be measured at intervals, which prevented knowledge of the individual's exact time of death. Serious bias could have resulted from applying continuous response methodology to the interval-level response data utilized in this study (Hosmer and Lemeshow, 1989). This model provided a maximum likelihood analysis for response variables that are categorical due to observation intervals. The maximum likelihood method, known to produce statistically optimal estimates for large samples, was used to estimate survival probabilities.

To provide a method for comparison between concentrations, a one-number summary of survival, the covariate-adjusted median survival time (as detailed in Hicks *et al.*, 2000) was generated by SAS's interactive matrix language (IML, SAS, Cary, NC). Differences in medians of death were compared for pairs of treatment coefficients using theWald statistic [$W = (CM)^{\gamma} [Cvar(M)C^{\gamma}]^{-1} (CM)$]. The DLFTM test allowed analysis of such data and was used to determine acute and chronic LT_{50} values for *P. acuta* (Hicks *et al.*, 2000).

Shell length (SL) was both included and excluded as a covariate in both LC_{50} and LT_{50} analyses to reveal size effects and allow comparison with other published data. SL effect was demonstrated utilizing a published methodology developed by D.L. Hawkins of the University of Texas at Arlington for computerized LC_{50} and LT_{50} estimations (Hicks et al., 2001). The impacts of adding SL and the differences between SL on acute and chronic survival LC_{50} values and LT_{50} values were analyzed by comparing LC_{50} and LT_{50} values for individuals representing snails without SL as a covariate and shell lengths fixed at the 25^{th}, 50^{th} and 75^{th} quantiles of the shell length distributions of the entire tested samples (Box et al., 1978). SL was fixed at the 25^{th}, 50^{th} and 75^{th} SL quantiles for each toxicity test by subtracting the value of that quantile from the initial SL value.

Behavioral Responses

Testing Conditions

Freshwater pulmonate snails, including *Physa acuta* and *Planorbella trivolvis*, have been observed to display a "crawl-out" response involving crawling above the

water's surface to temporarily avoid predation by crayfish (Alexander and Covich, 1991). Crawl-out behavior in these two species appeared to be triggered by chemical cues from the predator and from injured snails with no significant difference in behavior among individuals of differing size. In order to investigate if individuals of *P. acuta* show a similar crawl-out response to irritating conditions induced by the presence of dissolved $CdCl_2$, sub-samples of 30 adult snails each were placed in three one-liter replicate glass beakers containing 750 ml of media with concentrations ranging from 0, 10, 50, 100, 150, 200, 250, 300, 350, 400, 450, 600, 800, 1000, 1200, 1400, 1600 and 2000 μg $CdCl_2 \cdot L^{-1}$. The beakers were covered with a 1 mm nylon mesh preventing snail escape. During crawl-out testing, media in all chambers was continuously aerated and maintained at a constant temperature of 25 °C in a refrigerated incubator. Beakers were observed for the number of snails crawling above the water's surface, below the surface, or at the waterline or lying unattached on the bottom of the jar or crawling on the air stone and airline every hour for a period of 12 hours. Any snail observed to be above the medium surface was removed and its SL measured to the nearest 0.1 mm with a dial micrometer or with an ocular micrometer mounted in a dissecting microscope. The SL of all individuals remaining submerged was similarly measured at the end of the 12 h observation period.

Data Analysis

Alexander and Covich (1991) used arc-sine transformations of their data followed by ANOVA to determine differences in “crawl-out” behavior. In contrast, a maximum likelihood logistic regression analysis was employed in this study to

determine median crawl-out probability at each 1 h observation over a 12 h period at each tested concentration. Logistic regression analysis was utilized to calculate the probability (p) of individuals of *P. acuta* crawling above the waters surface (Hicks *et al.*, 2000). To determine the the probability of an individual crawling-out in a given interval without SL as a covariate the following generalized linear regression model with either the probit or logistic distribution was utilized: $\Gamma^{1}(p) = b_0 + b_1(Lconcentration+1)$. To determine the probability of of an individual crawling-out in a given interval the following regression model was utilized: $\Gamma^{1}(p) = b_0 + b_1(Lconcentration+1)+b_2\,(SL-SL^{0})+b_3[(Lconcentration+1)*(SL-SL^{0})]$.

These results provided a summary $CdCl_2$ concentration value (CO_{50}) for 50 % proportional crawl-out response, the concentration of toxicant that resulted in 50 % crawl-out response of an exposed population, adjusting for the confounding effects of individual size (Floyd, 2001). Values of CO_{50} were compared to acute LC_{50} values determined in prior mortality testing at the same $CdCl_2$ concentration levels, allowing estimation of the sensitivity of crawl-out response as a short-term measure of $CdCl_2$ toxicity relative to the more standard lethal concentration toxicity testing methodologies.

Results

Acute Percent Mortality

Raw percent sample mortality values on acute exposure to $CdCl_2$ concentrations ranging from 0-5000 μg $CdCl_2 \cdot L^{-1}$ were determined for exposure times of 24, 48, 72, 96, 120, 144, 168, and 192 h. There was an evident impact of exposure time on

mortality. In 192 h (8 day) exposures, concentrations below 1400 μg $CdCl_2 \cdot L^{-1}$ did not reach 50 % sample mortality, while 100 % sample mortalities were achieved at all concentrations ≥ 2000 μg $CdCl_2 \cdot L^{-1}$ (Table 2.1, Fig. 2.1). A concentration of 5000 μg $CdCl_2 \cdot L^{-1}$ resulted in 100 % sample mortality after 24 hours (1 day) (Table 2.1, Fig. 2.1).

Acute LC_{50} Analysis - Probit Regression

Probit analysis using maximum likelihood was conducted to determine the effect of concentration without shell length as a covariate on sample survival to allow comparisons with other published studies using this analytical method. This analysis revealed a significant relationship between sample mortality and $CdCl_2$ concentration at each tested exposure duration (24-192 hours) across which chi-square and p values ranged from chi-square = 476.20 ($p < 0.0001$) at 24 h to chi-square = 645.61($p < 0.0001$) at 120 h (Table 2.2). LC_{50} values decreased from 2960.49 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±66.79) at an exposure time of 24 h through 986.88 μg $CdCl_2 \cdot L^{-1}$ at 192 h (s.e. = ±22.50) (Table 2.3, Fig. 2.2 A).

Maximum likelihood probit analysis of the effect of $CdCl_2$ concentration with SL as a covariate allowed LC_{50} values to be calculated at three SL medians based on the 25^{th}, 50^{th} and 75^{th} SL quantiles. Significant effects for mortality and concentration

Table 2.1 Cumulative number actual mortality and percent mortality of specimens of *Physa acuta* on exposure to media with concentrations of cadmium chloride ranging from 0 - 5000 µg $CdCl_2 \cdot L^{-1}$ determined at 24 h intervals over a total exposure period of 192 h (8 d).

Exposure Concentration (µg $CdCl_2 \bullet L^{-1}$)	N	Exposure Time in Hours (h)															
		24 h		48 h		72 h		96 h		120 h		144 h		168 h		192 h	
		No. Dead	Percent Mortality	No. Dead	Percent Mortality	No. Dead	Percent Mortality	No. Dead	Percent Mortality	No. Dead	Percent Mortality	No. Dead	Percent Mortality	No. Dead	Percent Mortality	No. Dead	Percent Mortality
0	150	0	0	0	0	2	1.3	2	1.3	6	4.0	6	4.0	6	4.0	6	4.0
100	150	0	0	0	0	1	0.7	1	0.7	4	2.7	4	2.7	14	9.3	15	10
200	149	0	0	1	0.7	3	2	4	2.7	6	4.0	6	4.0	10	6.7	15	10.1
300	144	0	0	4	2.8	4	2.8	8	5.5	11	7.6	12	8.3	13	9	16	11.1
400	150	0	0	1	0.7	1	0.7	10	6.7	12	6.7	14	6.7	15	10	18	12
600	150	0	0	0	0	6	4	11	7.3	15	7.3	18	7.3	21	14	24	16
800	150	0	0	1	0.7	13	8.7	13	8.7	13	8.7	18	12.0	18	12	50	33.3
1000	149	0	0	7	4.7	12	8.1	20	13.4	26	17.4	26	17.4	39	26.2	57	38.3
1200	148	0	0	7	4.7	13	8.8	14	9.5	18	14.0	20	14.0	20	14	70	47.3
1400	150	0	0	13	8.7	13	8.7	29	19.3	31	19.3	39	26.0	48	32	77	51.3
1600	150	0	0	17	11.3	61	40.7	61	40.7	78	52.0	80	53.3	93	62	144	96
2000	150	27	18	150	100	150	100	150	100	150	100	150	100	150	100	150	100
3000	150	50	33.3	150	100	150	100	150	100	150	100	150	100	150	100	150	100
4000	150	131	87.3	150	100	150	100	150	100	150	100	150	100	150	100	150	100
5000	150	150	100	150	100	150	100	150	100	150	100	150	100	150	100	150	100

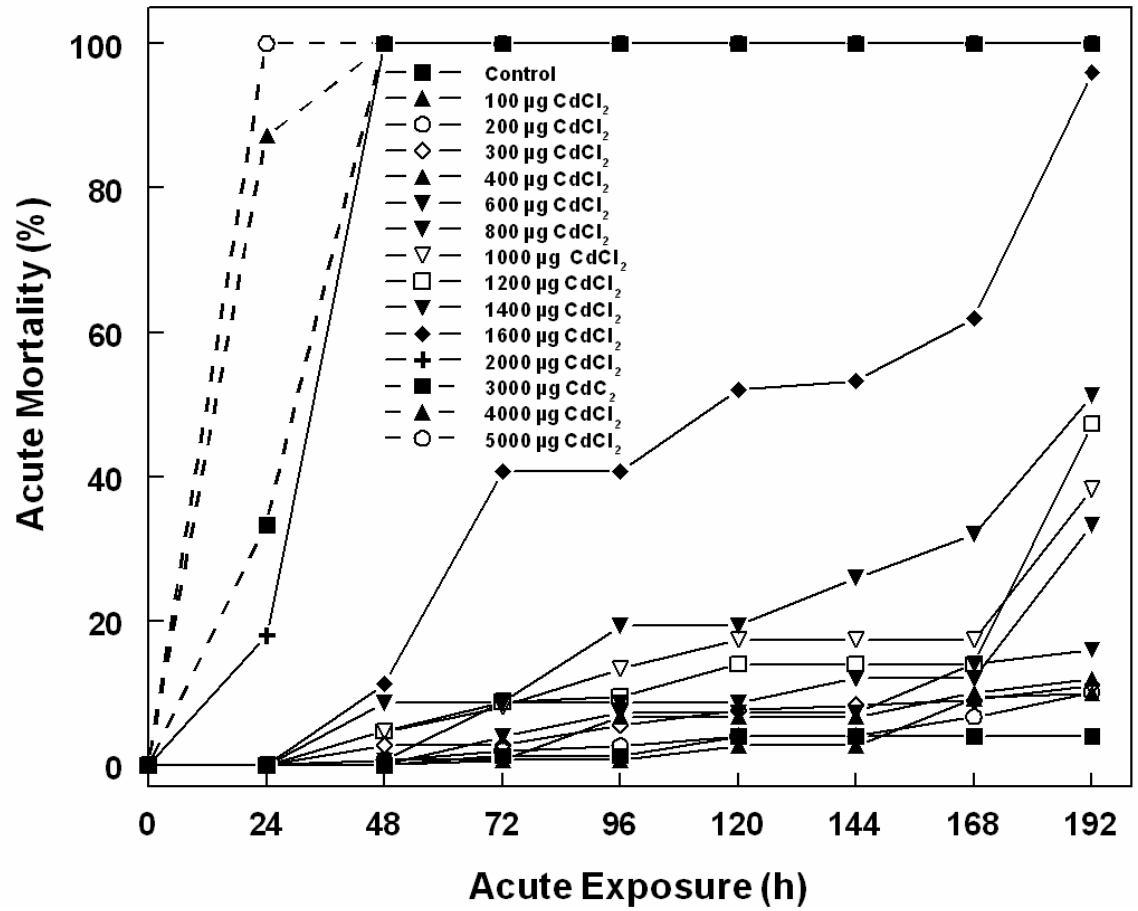


Fig. 2.1 The effect of acute exposure of samples of *Physa acuta* to various concentrations of cadmium chloride ($CdCl_2$) on cumulative percent sample mortality (vertical axis) at 24 h intervals during a total exposure period of 192 h (i.e., 8 days, horizontal axis) resulting from percent mortality. The results for each tested exposure concentrations are as indicated in the legend displayed in the center of the figure.

Table 2.2 Chi-square and probability (p) values for maximum likelihood probit regression analysis determinations of 50 % sample lethal concentration values (LC_{50}) for samples of *Physa acuta* exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 - 5000 µg $CdCl_2 \cdot L^{-1}$ over a total exposure period of 192 h (8 days). Chi-square values are provided for analysis carried out without shell length (SL) as a covariate to describe the probability of an individual dying in the given interval: $I^1(p) = b_0 + b_1(Lconcentration+1)$.The probability of of an individual dying in a given interval: $I^1(p) = b_0 + b_1(Lconcentration+1)+b_2\ (SL-SL^0)+b_3[(Lconcentration+1)*(SL-SL0)]$ with shell length as a covariate allowed determination of EC_{50} values for individuals in the 25th SL quantile (SL = 6.0 mm), 50th SL quantile (median SL = 7.1 mm) and the 75th SL quantile (SL = 8.3 mm).

Exposure Duration (hours)	Chi-Square SL not a Covariate	P	Chi-Square SL = 6.0 mm	p	Chi-Square Median SL = 7.1 mm	p	Chi-Square SL = 8.3 mm	P
24	476.20	<0.0001*	301.74	<0.0001*	425.68	<0.0001*	338.76	<0.0001*
48	508.75	<0.0001*	324.44	<0.0001*	487.34	<0.0001*	446.79	<0.0001*
72	591.74	<0.0001*	372.76	<0.0001*	563.58	<0.0001*	481.73	<0.0001*
96	597.50	<0.0001*	375.90	<0.0001*	577.57	<0.0001*	477.88	<0.0001*
120	645.61	<0.0001*	400.78	<0.0001*	640.27	<0.0001*	481.74	<0.0001*
144	638.15	<0.0001*	393.73	<0.0001*	632.5	<0.0001*	476.98	<0.0001*
168	631.95	<0.0001*	386.97	<0.0001*	624.22	<0.0001*	476.02	<0.0001*
192	619.32	<0.0001*	382.83	<0.0001*	593.47	<0.0001*	507.46	<0.0001*

(*) indicates a significant difference at $p<0.05$

Table 2.3 Acute 50 % sample mortality values (LC_{50}) estimated by maximum likelihood probit regression analysis for samples of *Physa acuta* (n = 150) exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0-5000 µg $CdCl_2 \cdot L^{-1}$ over a total exposure period of 192 h (8 days). LC_{50} values are provided for the entire sample based on analysis without shell length (SL) as a covariate, and, when analyzed with SL as a covariate, for individuals in the 25th SL quantile, for individuals in the 50th SL quantile and for individuals in the 75th SL quantile.

Sample without SL as a Covariate		
Exposure Time (h)	**LC_{50}**	**s.e.**
24	2960.49	±66.79
48	1785.34	±33.20
72	1631.25	±32.90
96	1572.86	±32.55
120	1444.18	±33.29
144	1385.30	±32.06
168	1318.17	±30.38
192	986.88	±22.50
With SL as a Covariate: 25th SL Quantile (SL = 6.0 mm)		
Exposure Time (h)	**LC_{50}**	**s.e.**
24	2610.69	±70.84
48	1743.62	±42.63
72	1588.37	±40.923
96	1504.57	±39.96
120	1357.58	±40.68
144	1295.68	±38.94
168	1220.66	±36.37
192	898.48	±25.34
With SL as a Covariate: 50th SL Quantile (Median SL = 7.1 mm)		
Exposure Time (h)	**LC_{50}**	**s.e.**
24	2839.03	±66.56
48	1777.54	±33.83
72	1621.38	±33.28
96	1563.96	±32.78
120	1440.47	±33.35
144	1381.22	±32.10
168	1312.52	±30.37
192	971.40	±21.96
With SL as a Covariate: 75th SL Quantile (SL = 8.3 mm)		
Exposure Time (h)	**LC_{50}**	**s.e.**
24	3172.99	±90.49
48	1814.23	±37.18
72	1658.79	±37.64
96	1628.26	±37.65
120	1525.93	±39.17
144	1470.16	±38.07
168	1409.90	±36.57
192	1061.70	±28.61

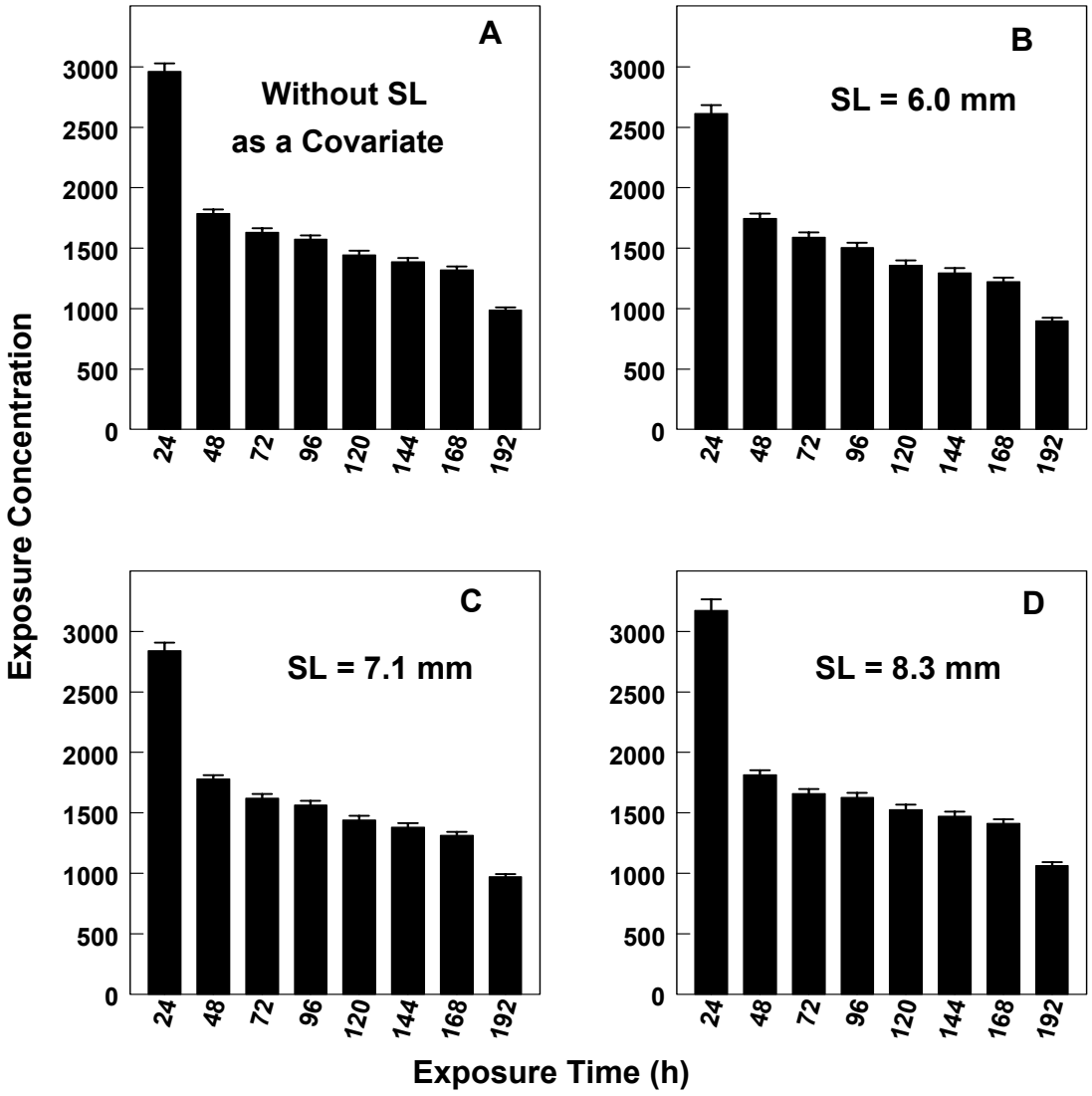


Fig. 2.2 Lethal concentration for 50 % mortality (LC_{50}) estimated by maximum likelihood probit regression for samples of *Physa acuta* (total sample size = 1940) with (A) LC_{50} values computed without SL as a covariate, (B-D) computed with SL as a covariate for (B) individuals in the 25th SL quantile. (C) individuals in the median 50th SL quantile and (D) individuals in the 75th SL quantile exposed to varying concentrations of cadmium chloride (μg $CdCl_2 \cdot L^{-1}$, horizontal axis) every 24 h over a total exposure time of 192 h (8 d). Error bars above histograms represent standard error of the LC_{50} value.

varied for each SL analysis; however effects of mortality and shell length and mortality and the interaction between concentration and shell length as a covariate remained constant over the three analyses. The effect of sample mortality and shell length was significant at 24 h and 120-168 h, with chi-square ranging from 4.88 (s.e. = ±0.0272) at 168 h to 8.62 (s.e. = ±0.0033) at 24 h. The effect of sample mortality and the interaction between concentration and shell length as a covariate was significant at 24 h and 120-144 h, with chi-square ranging from 4.23 (s.e. = ±0.0397) at 144 h to 10.96 (s.e. = ±0.0009) at 24 h. The first analysis to include SL as a covariate was based on the (25^{th} SL quantile of snails with the smallest shell length (SL = 6.0 mm). This analysis revealed a significant relationship between sample mortality and $CdCl_2$ concentration at each tested exposure duration (24-192 hours) across which chi-square and p values ranged from chi-square = 301.74 ($p < 0.0001$) at the 24 h exposure to chi-square = 400.78 ($p < 0.0001$) at the 120 h exposure (Table 2.2). LC_{50} values decreased from 2610.69 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±70.84) at an exposure time of 24 h through 898.48 μg $CdCl_2 \cdot L^{-1}$ at an exposure of 192 h (s.e. = ±25.34) (Table 2.3, Fig. 2.2 B).

Maximum likelihood probit analysis (n = 1940) in which SL was included as a covariate demonstrated that for an individual with a median SL of 7.1 mm, the 50^{th} SL quantile, there was a significant relationship between sample mortality and $CdCl_2$ concentration at each tested exposure duration (24-192 hours) across which chi-square and p values ranged from chi-square = 425.68 ($p < 0.0001$) at 24 h to chi-square = 640.27 ($p < 0.0001$) at 120 h (Table 2.2). LC_{50} values decreased from 2839.03 μg

$CdCl_2 \cdot L^{-1}$ (s.e. = ±66.56) at an exposure time of 24 h through 971.40 µg $CdCl_2 \cdot L^{-1}$ at 192 h (s.e. = ±21.96) (Table 2.3, Fig. 2.2 C).

For the 75th sample SL size (SL of 8.3 mm) there was a significant relationship between sample mortality and $CdCl_2$ concentration at each tested exposure duration (24-192 hours) across which chi-square and p values ranged from chi-square = 338.76 ($p < 0.0001$) at 24 h to chi-square = 481.74 ($p < 0.0001$) at 120 h (Table 2.2). LC_{50} values decreased from 3172.99 µg $CdCl_2 \cdot L^{-1}$ (s.e. = ±90.49) at an exposure time of 24 h through 1061.70 µg $CdCl_2 \cdot L^{-1}$ at 192 h (s.e. = ±28.61) (Table 2.3, Fig. 2.2 D).

The effect of SL was demonstrated through estimation of LC_{50} from maximum likelihood probit analysis, with the LC_{50} from the smallest shell length median 6.00 mm, demonstrating the lowest LC_{50} across the exposure period and the largest shell length median 8.3 mm, resulting in the highest LC_{50} across the exposure. Addition of SL as a covariate in maximum likelihood probit analysis resulted in LC_{50} values for the No SL covariate and median SL 7.1 mm having similar values across the exposure periods, with the least difference between estimated values occurring at 96-192 h. LC_{50} values were most similar at 48 h for all SL analyses (Fig 2.3).

Acute LC_{50} Analysis - Logistic Regression

Application of maximum likelihood logistic regression analysis without SL as a covariate to acute survivorship data revealed a significant relationship between mortality and $CdCl_2$ concentration at each tested exposure duration (24-192 hours)

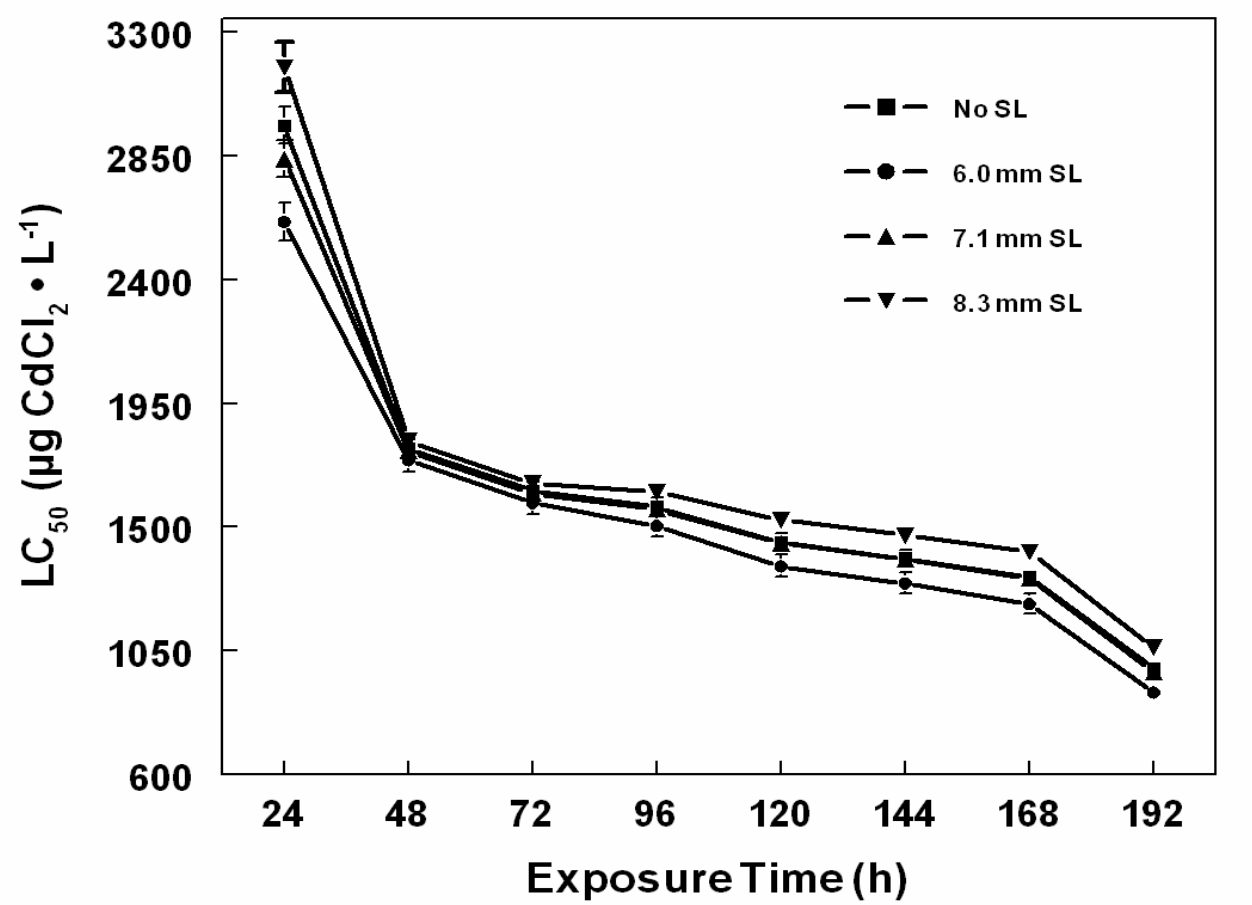


Fig.2 3 Comparison of acute LC_{50} values computed by maximum likelihood probit analysis for samples of *Physa acuta* at intervals of 24 h over a 192 h (eight d) period of exposure to varying concentrations of $CdCl_2$. The horizontal axis is exposure time in hours and the vertical axis, the estimated lethal concentration for 50 % sample mortality (LC_{50}) in µg $CdCl_2 \cdot L^{-1}$. Solid squares are LC_{50} values estimated without including shell length (SL) as a covariate. Solid circles, triangles and inverted triangles represent median LC_{50} values resulting from analysis with inclusion of SL as a covariate for individuals in the 25 th (SL = 6.0 mm), 50th (median SL = 7.1 mm) and 75th of the SL quantile (SL = 8.3), respectively. Vertical bars about LC_{50} values represent standard errors. Error bars are not apparent when they are less than the width of the symbol with which they are associated.

across which chi-square and p values ranged from chi-square = 234.38 ($p < 0.0001$) at an exposure of 48 h to chi-square = 453.86 ($p < 0.0001$) at an exposure of 192 h (Table 2.4). LC_{50} values decreased from 3006.22 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±59.61) at an exposure time of 24 h through 1018.18 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±22.16) at an exposure time of 192 h (Table 2.5, Fig. 2.4 A).

Maximum likelihood logistic regression analysis of the effect of $CdCl_2$ concentration with SL as a covariate allowed LC_{50} values to be computed at three SLmedians based on the 25th, 50th and 75th SL size class quantiles. Effects for mortality and concentration varied for each SL analysis; however effects of mortality and shell length and mortality and shell length as a covariate remained constant over the three analyses. The effect of sample mortality and shell length was significant at 24 and 120-168 h, with chi-square ranging from 3.97 (s.e. = ±0.0464) at 24 h to 8.25 (s.e. =±0.0041) at 120 h. The effect of mortality and the interaction between concentration and shell length as a covariate was significant at 120-168 h, with chi-square ranging from 5.00 (s.e. = ±0.0253) at 168 h to 6.79 (s.e. = ±0.0091) at 120 h (Table 2.4). The first analysis to include SL as a covariate was based on the snails with the smallest shell length with a median SL of 6.0 mm. Logistic regression analysis for this 6.0 mm SL group revealed a significant relationship between sample mortality and $CdCl_2$ concentration at each tested exposure duration (24-192 hours) across which chi-square and p values ranged from chi-square = 169.03 ($p < 0.0001$) at an exposure of 48 h to chi-square = 283.86 ($p < 0.0001$) at an exposure of 192 h (Table 2.4). LC_{50} values based on

Table 2.4. Chi-square and probability (p) values for maximum likelihood logistic regression analysis determinations of 50 % sample lethal concentration values (LC_{50}) for samples of *Physa acuta* exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 - 5000 µg $CdCl_2.L^{-1}$ over a total exposure period of 192 h (8 days). Chi-square values are provided for analysis carried out without shell length (SL) as a covariate to describe the probability of an individual dying in the given interval: $\Gamma^{1}(p) = b_0 + b_1(Lconcentration+1)$.The probability of of an individual dying in a given interval: $\Gamma^{1}(p) = b_0 + b_1(Lconcentration+1)+b_2\ (SL-SL^0)+b_3[(Lconcentration+1)*(SL-SL^0)]$with shell length as a covariate allowed determination of EC_{50} values for individuals in the 25th SL quantile (median SL = 6.0 mm), 50th SL quantile (median SL = 7.1 mm) and the 75th SL quantile (median SL = 8.3 mm).

Exposure Duration (hours)	Chi-Square SL not a Covariate	P	Chi-Square SL = 6.0 mm	P	Chi-Square Median SL = 7.1 mm	P	Chi-Square SL = 8.3 mm	P
24	328.23	<0.0001*	208.51	<0.0001*	303.93	<0.0001*	179.24	<0.0001*
48	234.38	<0.0001*	169.03	<0.0001*	245.89	<0.0001*	198.88	<0.0001*
72	325.20	<0.0001*	232.95	<0.0001*	325.69	<0.0001*	273.67	<0.0001*
96	356.28	<0.0001*	245.81	<0.0001*	346.95	<0.0001*	266.73	<0.0001*
120	420.45	<0.0001*	272.82	<0.0001*	401.36	<0.0001*	289.99	<0.0001*
144	430.58	<0.0001*	275.38	<0.0001*	411.07	<0.0001*	297.37	<0.0001*
168	435.24	<0.0001*	274.09	<0.0001*	413.97	<0.0001*	305.30	<0.0001*
192	453.86	<0.0001*	283.86	<0.0001*	420.11	<0.0001*	370.12	<0.0001*

* indicates a significant difference at $p<0.05$

Table 2.5. Acute 50 % sample mortality values (LC_{50}) estimated by maximum likelihood logistic regression analysis for samples of *Physa acuta* (n = 150) exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 - 5000 µg $CdCl_2 \cdot L^{-1}$ over a total exposure period of 192 h (8 days). LC_{50} values are provided for the entire sample based on analysis without SL as a covariate, and, when analyzed with SL as a covariate, for individuals in the 25th, 50th and 75th SL quantile.

Sample without SL as a Covariate		
Exposure Time (h)	**LC_{50}**	**s.e.**
24	3006.22	±59.61
48	1745.27	±23.54
72	1635.60	±26.64
96	1589.26	±27.51
120	1481.45	±29.76
144	1423.91	±29.35
168	1356.63	±28.25
192	1018.18	±22.16
With SL as a Covariate: 25th SL Quantile (SL = 6.0 mm)		
Exposure Time (h)	**LC_{50}**	**s.e.**
24	2625.18	±68.14
48	1700.30	±30.66
72	1569.60	±32.96
96	1499.32	±33.53
120	1380.15	±35.87
144	1321.15	±35.08
168	1248.00	±33.10
192	930.95	±24.44
With SL as a Covariate: 50th SL Quantile (Median SL = 7.1 mm)		
Exposure Time (h)	**LC_{50}**	**s.e.**
24	2850.26	±61.30
48	1737.65	±24.09
72	1620.19	±26.80
96	1577.21	±27.42
120	1480.36	±29.49
144	1423.64	±29.11
168	1355.74	±28.01
192	1008.50	±21.50
With SL as a Covariate: 75th SL Quantile (SL = 8.3 mm)		
Exposure Time (h)	**LC_{50}**	**s.e.**
24	3140.69	±70.79
48	1778.52	±29.15
72	1677.57	±30.99
96	1658.42	±32.04
120	1578.90	±34.77
144	1525.70	±34.64
168	1465.99	±33.87
192	1105.62	±29.01

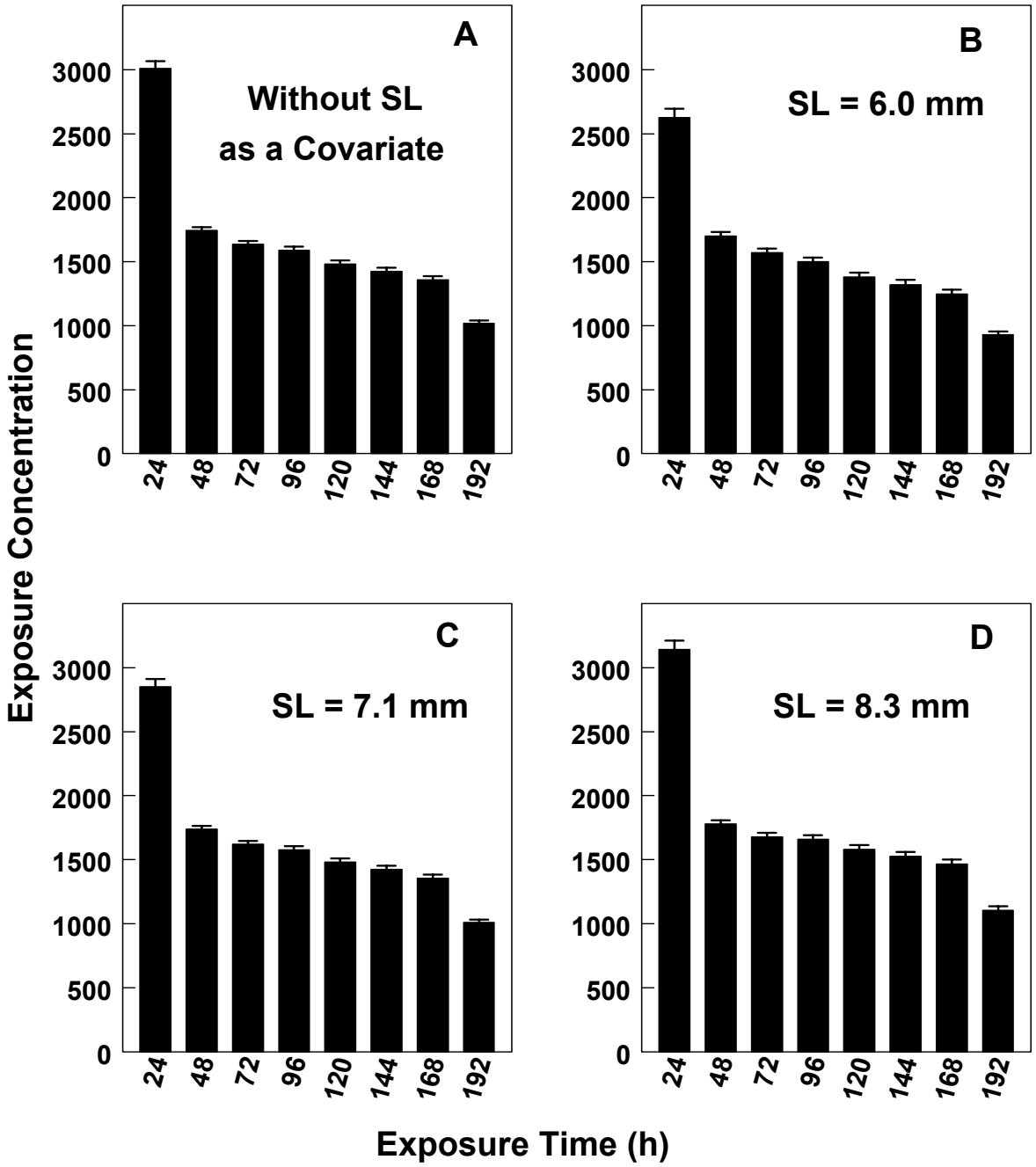


Fig. 2.4 Comparison of acute lethal concentration for 50 % sample mortality (LC_{50}) values computed by maximum likelihood logistic regression analysis for samples of *Physa acuta* with individuals in the sample computed without SL as a covariate (A), in the 25th (B), 50th (C) and 75th SL quantile (D), respectively, exposed to varying concentrations of $CdCl_2$ ranging from 0-5000 μg $CdCl_2 \cdot L^{-1}$ at 24 h intervals during a maximum exposure period of 192 h (8 d). The vertical axis is exposure concentration in μg $CdCl_2 \cdot L^{-1}$ and the horizontal axis exposure duration in hours. Vertical bars above histograms represent standard errors.

this analysis decreased from 2625.18 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±68.14) at an exposure of 24 h through 930.95 μg $CdCl_2 \cdot L^{-1}$ at an exposure of 192 h (s.e. = ±24.44) (Table 2.5, Fig. 2.4 B).

Maximum likelihood logistic regression analysis of acute survivorship data (n = 1940) in which SL was included as a covariate demonstrated that, for the median 50th SL quantile with a median SL of 7.1 mm, there was a significant relationship between sample mortality and $CdCl_2$ concentration at each tested exposure duration (24-192 hours) across which chi-square and p values ranged from chi-square = 245.89 ($p < 0.0001$) at an exposure of 48 h to chi-square = 420.11 ($p < 0.0001$) at an exposure of 192 h (Table 2.4). LC_{50} values decreased from 2850.26 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±61.30) at an exposure time of 24 h through 1008.50 μg $CdCl_2 \cdot L^{-1}$ at an exposure of 192 h (s.e. = ±21.50) (Table 2.5, Fig. 2.4 C).

At the size of the 75th SL quantile of 8.3 mm SL maximum likelihood logistic regression analysis revealed a significant relationship between sample mortality and $CdCl_2$ concentration at each tested exposure duration (24-192 hours) across which chi-square and p values ranged from chi-square = 179.24 ($p<0.0001$) at and exposure of 24 h to chi-square = 370.12 ($p < 0.0001$) at an exposure of 192 h (Table 2.4). LC_{50} values decreased from 3140.69 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±70.79) at an exposure time of 24 h through 1105.62 μg $CdCl_2 \cdot L^{-1}$ at 192 h (s.e. = ±29.01) (Table 2.5 Fig. 2.4 D).

As occurred in maximum likelihood probit analysis (see above), the effect of SL was demonstrated through estimation of LC_{50} from logistic analysis, with the LC_{50} from the smallest shell length, 6.00 mm, demonstrating the lowest LC_{50} across the exposure

period and the largest shell length, 8.3 mm, resulting in the highest LC_{50} across the exposure. Addition of SL as a covariate in logistic analysis resulted in LC_{50} values for the analysis without SL as a covariate and median SL 7.1 mm having similar values across the exposure periods, with the least difference between estimated values occurring at exposure periods of 48 - 192 h. The widest range of values for all SL analyses was at 24 h and was most similar at 48 h for all SL analyses (Figs. 2.5 and 2.6).

Acute Survival Probability Curves

Probability of survival was estimated from the discrete logistic failure time model (Hicks *et al*, 2000). Survival curves without SL as a covariate over an exposure period of 192 h indicated concentrations of 0-300 μg $CdCl_2 \cdot L^{-1}$ had the highest probability of survival, while concentrations of 2000-5000 μg $CdCl_2 \cdot L^{-1}$ had the lowest survival probability at 192 h. No decrease in probability of survival was seen for concentrations of 0-300 μg $CdCl_2 \cdot L^{-1}$ from 24-192 h. The survival curves demonstrated a decrease from 24-192 h for concentrations of 600-5000 μg $CdCl_2 \cdot L^{-1}$ (Fig. 2.7A-E).

Adding shell length as a covariate resulted in increased sensitivity for the survival curves estimated from the discrete logistic failure time model (Hicks *et al*., 2000). The ability to differentiate between concentrations increased after adding shell length as demonstrated by the difference between concentrations of 300-600 μg $CdCl_2 \cdot L^{-1}$ and between 800-1200 μg $CdCl_2 \cdot L^{-1}$. Survival curves for all three SL quantiles (25^{th}, 50^{th} and 75^{th}) over an exposure period of 192 h indicated concentrations

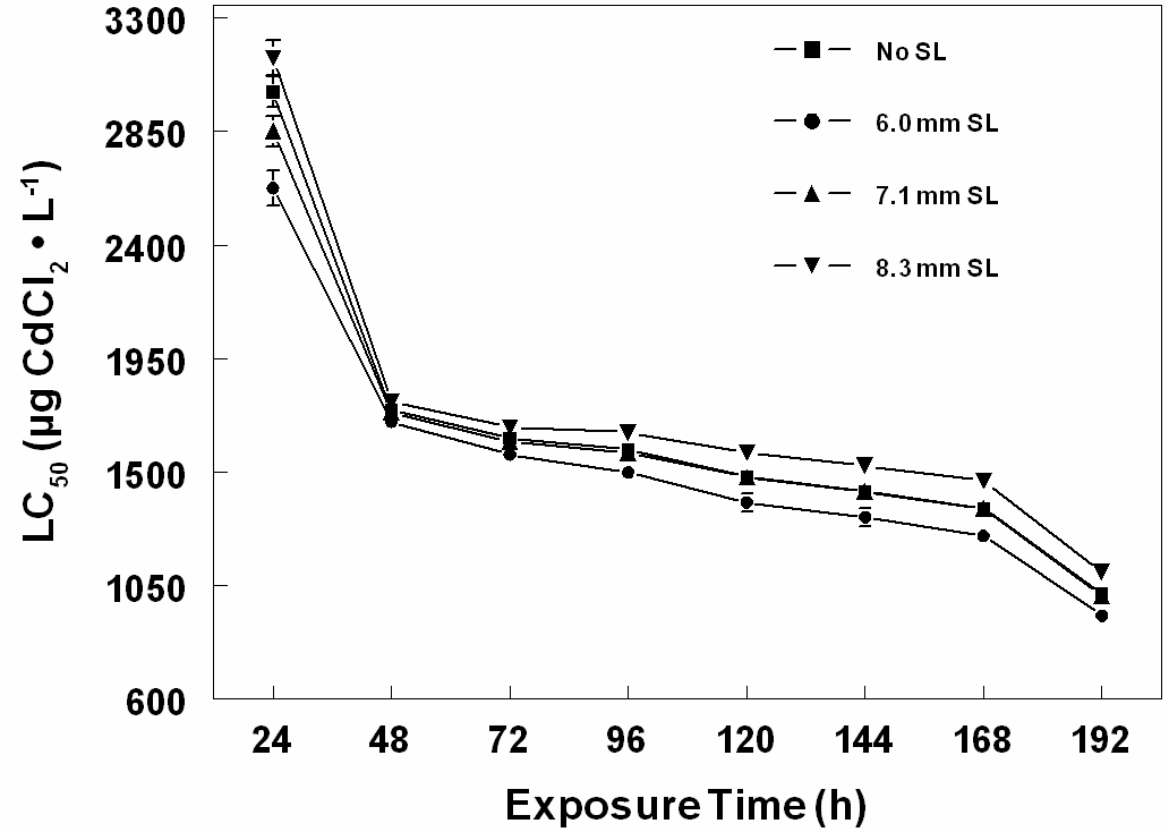


Fig. 2.5 Comparison of acute LC_{50} values computed by maximum likelihood logistic regression analysis for samples of *Physa acuta* at intervals of 24 h over a 192 h (eight d) period of exposure to varying concentrations of $CdCl_2$. The horizontal axis is exposure time in hours and the vertical axis, the estimated lethal concentration for 50 % sample mortality (LC_{50}) in µg $CdCl_2 \cdot L^{-1}$. Solid squares are LC_{50} values estimated without including shell length (SL) as a covariate. Solid circles, triangles and inverted triangles represent median LC_{50} values resulting from analysis with inclusion of SL as a covariate for individuals in the 25th (SL = 6.0 mm), 50^{th} (median SL = 7.1 mm) and 75th SL quantile (SL = 8.3), respectively. Vertical bars about LC_{50} values represent standard errors. Error bars are not apparent when they are less than the width of the symbol with which they are associated.

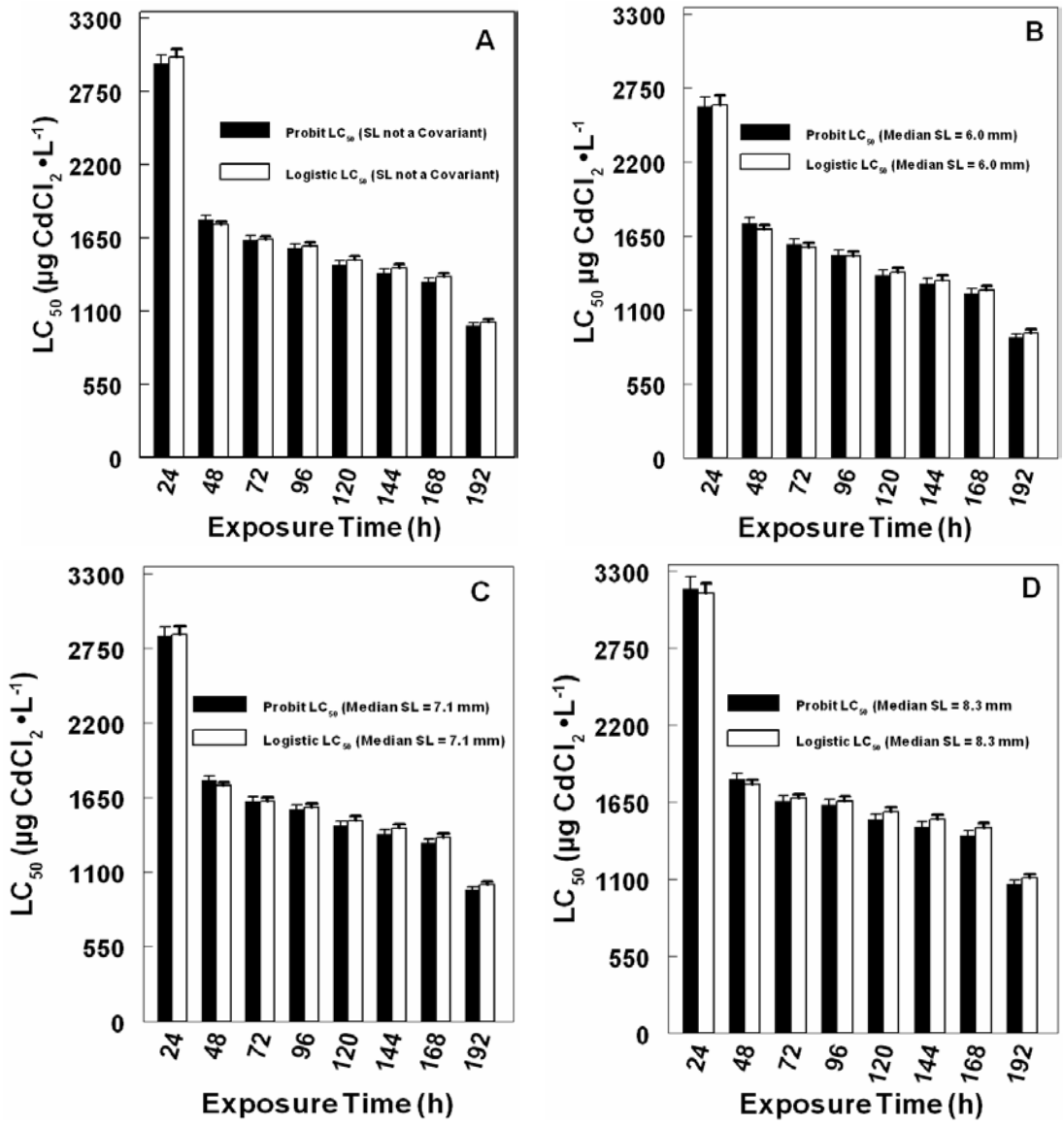


Fig.2.6 Comparison of acute LC_{50} values computed by maximum likelihood probit analysis (solid histograms) to those computed by maximum likelihood logistic regression (open histograms) for samples of *Physa acuta* for individuals without shell length as a covariate (A) and with of SL as a covariate for individuals in the 25th (SL =6.0 mm) (B), median 50th (SL = 7.1 mm) (C) and 75th SL quantile (SL = 8.3) (D), respectively, at intervals of 24 h over a 192 h (8 d) period of exposure to varying concentrations of $CdCl_2$. The horizontal axis is exposure time in hours and the vertical axis, the estimated LC_{50} in µg $CdCl_2 \cdot L^{-1}$. Vertical bars above histograms represent standard errors.

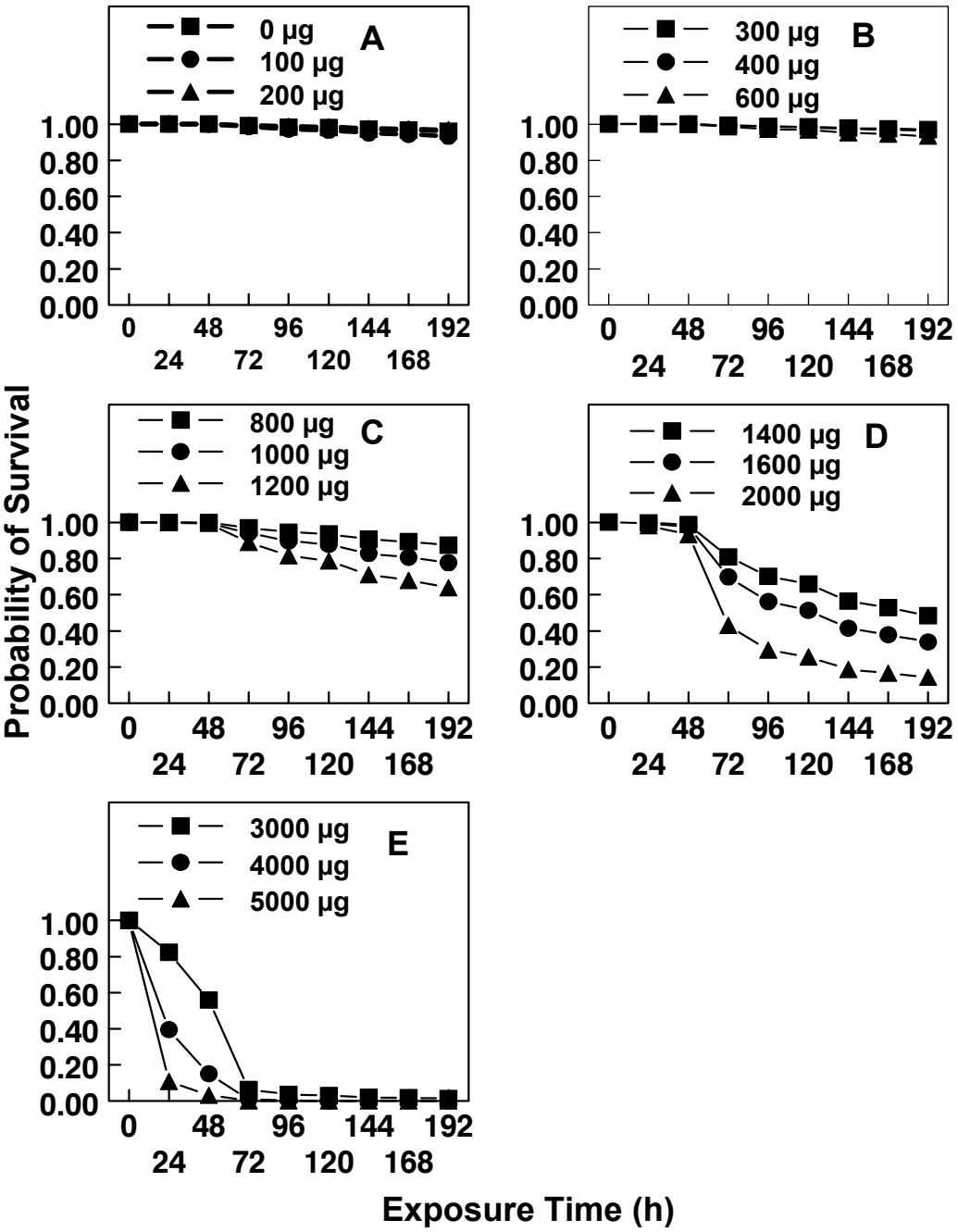


Fig. 2.7 Cumulative survival probability response for samples (n = 150) of *Physa acuta* without SL as a covariate exposed to media for 192 h with different concentrations of cadmium chloride ranging from (**A**) 0 (solid squares), 100 (solid circles), 200 (solid triangles), (**B**) 300 (solid squares), 400 (solid circles), 600 (solid triangles), (**C**) 800 (solid squares), 1000 (solid circles), 1200 (solid triangles), (**D**) 1400 (solid squares), 1600 solid circles), 2000 (solid triangles), (**E**) 3000 (solid squares), 4000 (solid circles), and 5000 (solid triangles) µg $CdCl_2 \cdot L^{-1}$.

of 0, 100 and 300 μg $CdCl_2 \cdot L^{-1}$ had the highest probability of survival, while concentrations of 2000-5000 μg $CdCl_2 \cdot L^{-1}$ had the lowest survival probability at 192 h. No decrease in probability of survival was seen for concentrations of 0-300 μg $CdCl_2 \cdot L^{-1}$ from 24-192 h. The survival curves demonstrated a decrease from 24-192 h for concentrations of 600-5000 μg $CdCl_2 \cdot L^{-1}$ (Fig. 2.8, 2.9, 2.10 A-E).

Acute Median Survival Time (LT_{50})

Median survival time was estimated from the (Hicks *et al*., 2000) logistic failure time model without SL as a covariate on exposure for 192 hours to concentrations of cadmium chloride ($CdCl_2$) ranging from 0-5000 μg $CdCl_2 \cdot L^{-1}$. Exposures to concentrations less than 1400 μg $CdCl_2 \cdot L^{-1}$ were excluded from DLFTM analysis because a 50 % mortality threshold was not reached in the sample group at this level (Figs. 2.11 A-D). DLFTM analysis revealed a significant relationship between sample mortality and $CdCl_2$ concentration at each tested exposure duration (192 hours) in which 50 % mortality was achieved across which chi-square and p values ranged from chi-square = 10.3170 ($p < 0.0013$). In this sample quantile LT_{50} values decreased from 183.13 h (s.e. = ±10.103) at a concentration of 1400 μg $CdCl_2 \cdot L^{-1}$ to 13.48 h (s.e. = ± 0.279) at a concentration of 5000 μg $CdCl_2 \cdot L^{-1}$ (Table 2.6, Fig. 2.11 A). All concentrations were significantly different from each other at $\alpha = 0.05$ based on the Scheffé pair-wise comparison.

Median survival time was estimated from the logistic failure time model (Hicks *et al*., 2000) with SL as a covariate allowing LT_{50} values to be estimated for the 25th SL

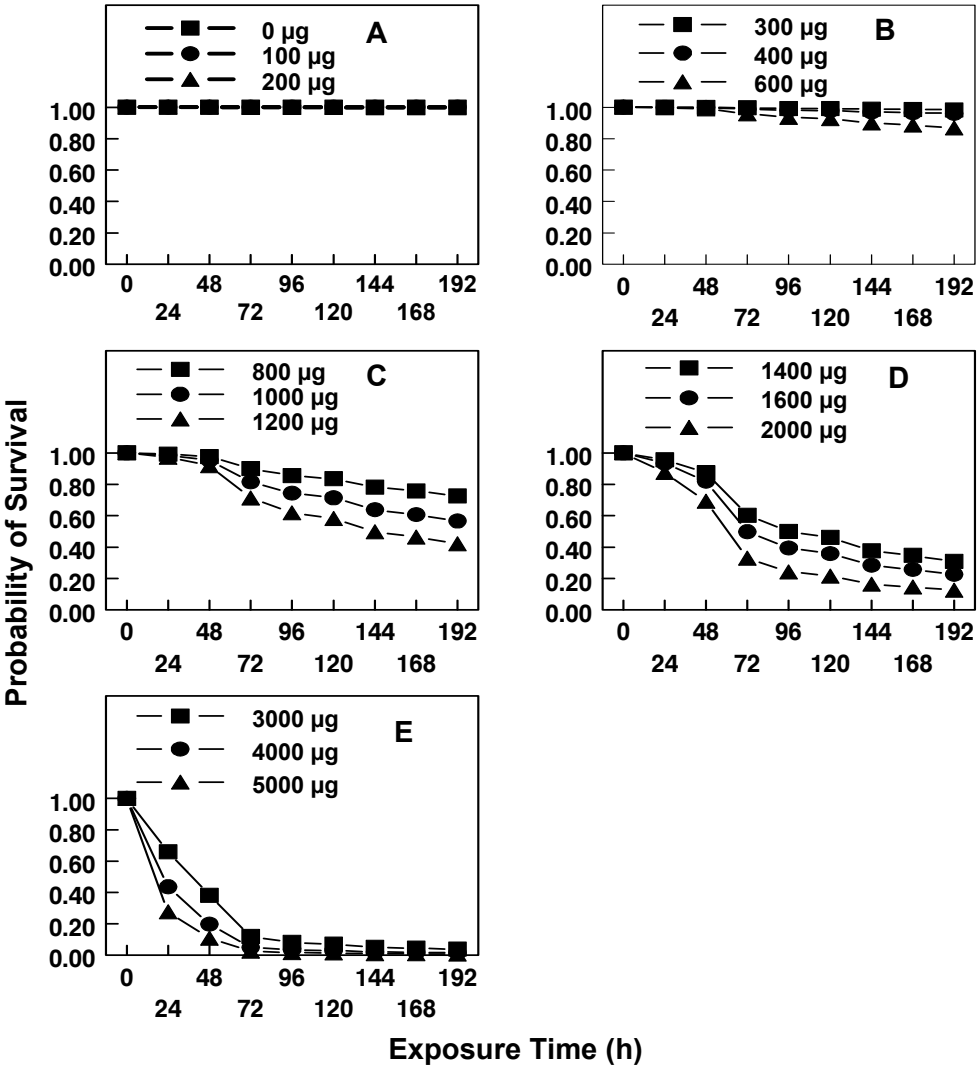


Fig. 2.8 Cumulative survival probability response for samples (n = 150) of *Physa acuta* with SL = 6.0 mm exposed to media for 192 h with different concentrations of cadmium chloride ranging from (**A**) 0 (solid squares), 100 (solid circles), 200 (solid triangles), (**B**) 300 (solid squares), 400 (solid circles), 600 (solid triangles), (**C**) 800 (solid squares), 1000 (solid circles), 1200 (solid triangles), (**D**) 1400 (solid squares), 1600 (solid circles), 2000 (solid triangles), (**E**) 3000 (solid squares), 4000 (solid circles), and 5000 (solid triangles) µg $CdCl_2 \cdot L^{-1}$.

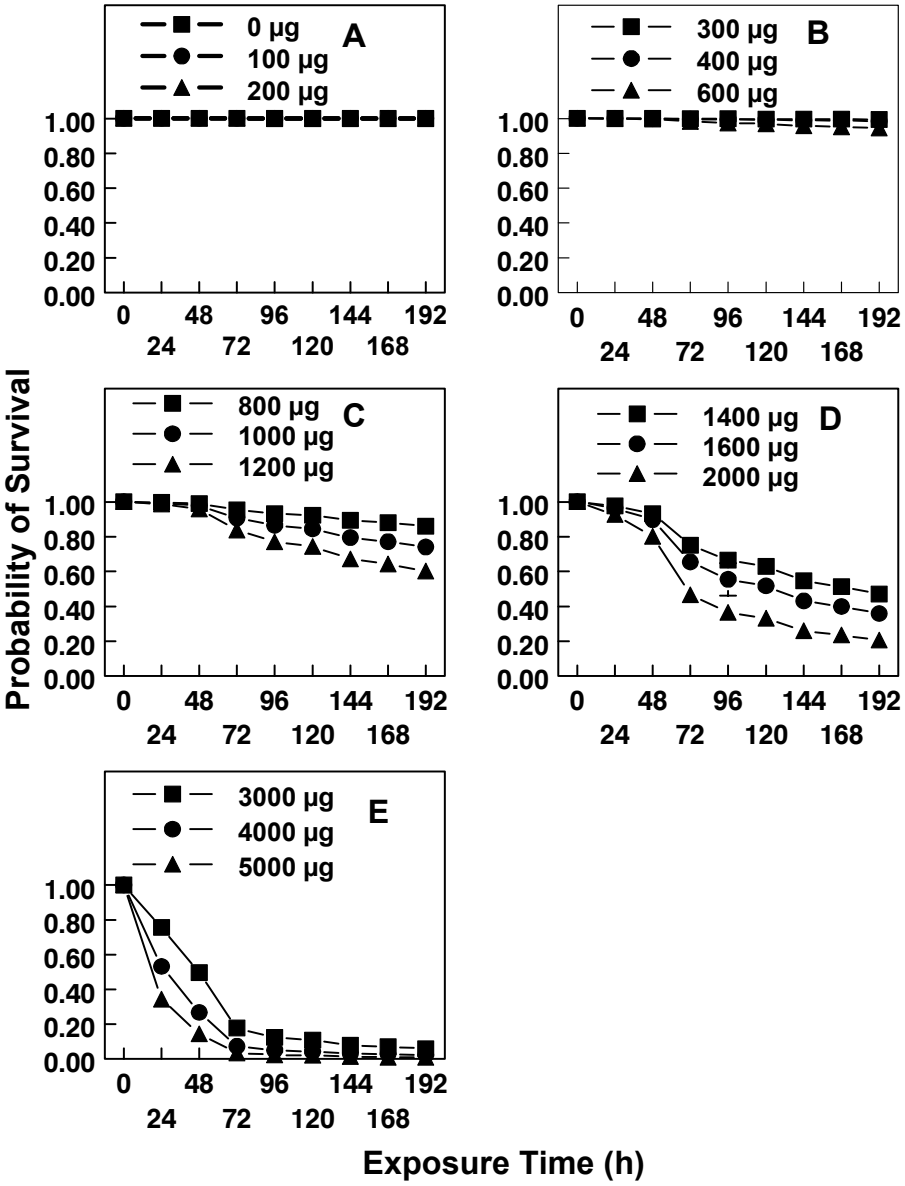


Fig.2.9 Cumulative survival probability response for samples (n = 150) of *Physa acuta* with median SL = 7.1 mm exposed to media for 192 h with different concentrations of cadmium chloride ranging from (**A**) 0 (solid squares), 100 (solid circles), 200 (solid triangles), (**B**) 300 (solid squares), 400 (solid circles), 600 (solid triangles), (**C**) 800 (solid squares), 1000 (solid circles), 1200 (solid triangles), (**D**) 1400 (solid squares), 1600 (solid circles), 2000 (solid triangles), (**E**) 3000 (solid squares), 4000 (solid circles), and 5000 (solid triangles) µg $CdCl_2 \cdot L^{-1}$.

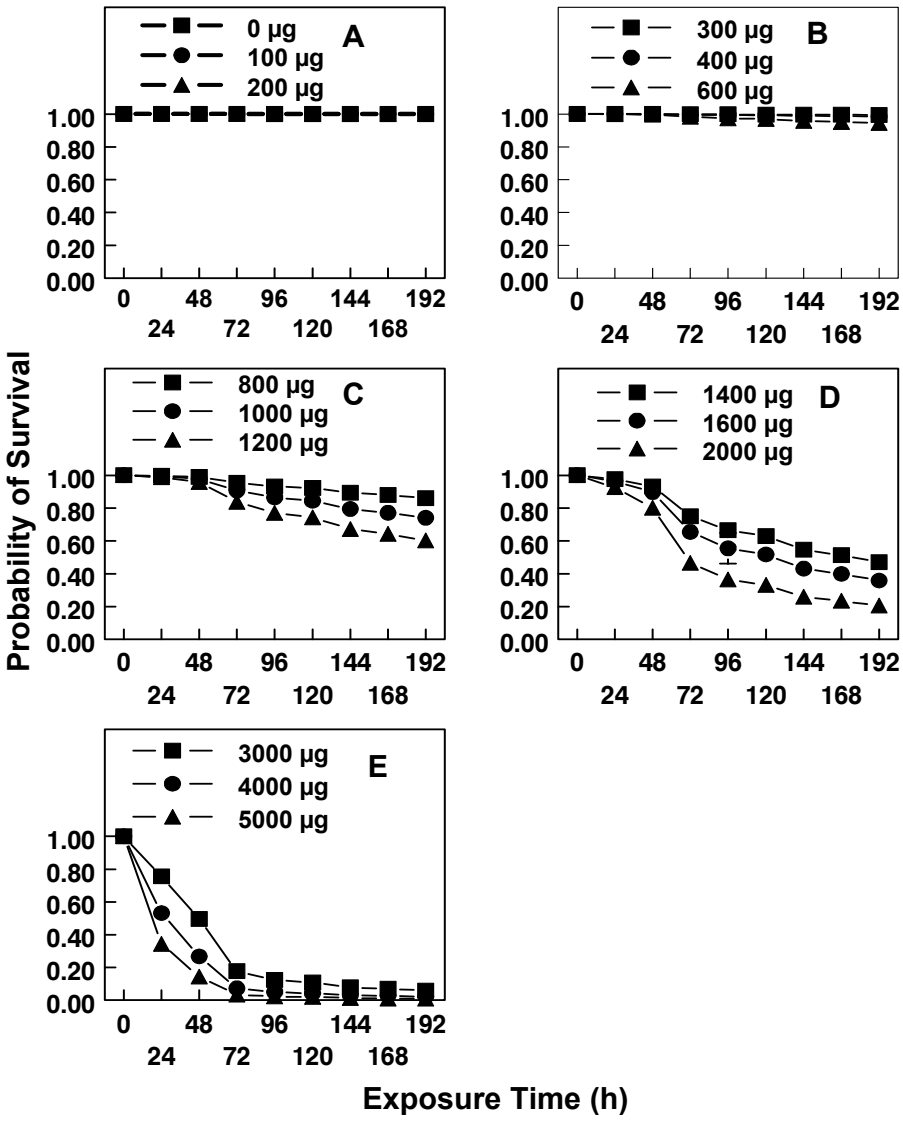
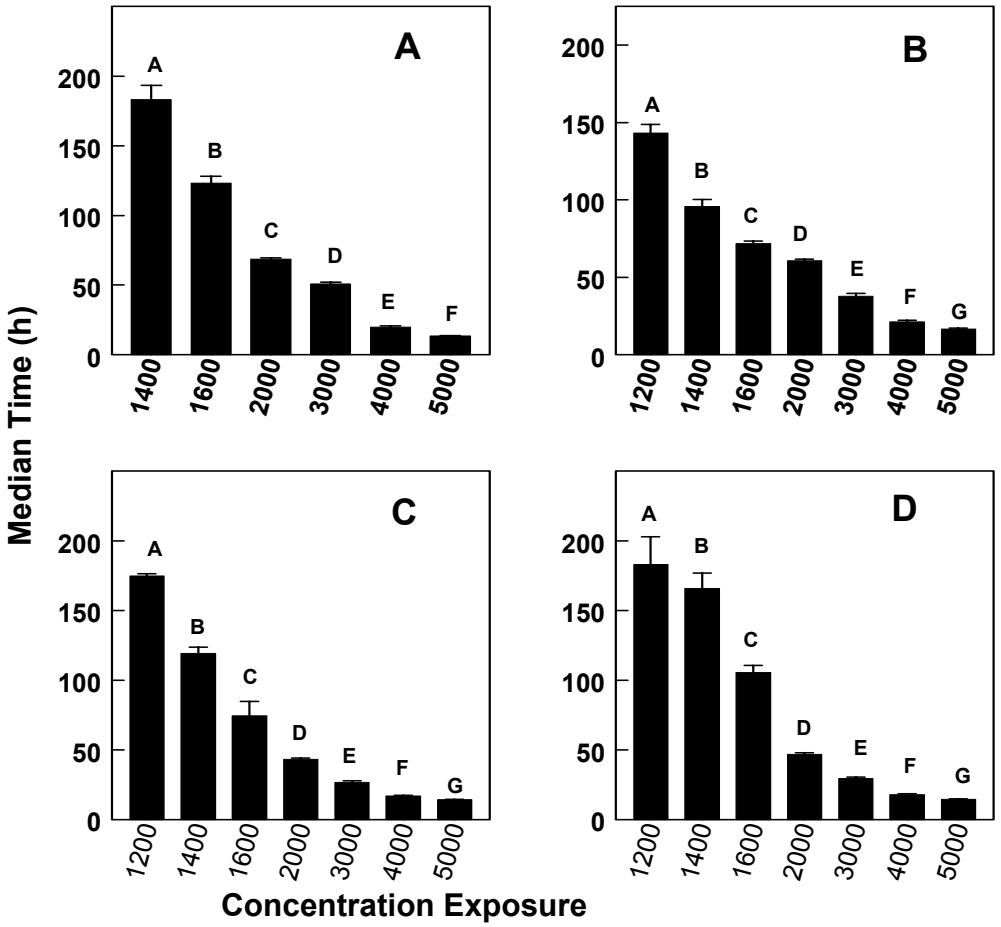


Fig. 2.10 Cumulative survival probability response for samples (n = 150) of *Physa acuta* with SL = 8.3 mm exposed to media for 192 h with different concentrations of cadmium chloride ranging from (**A**) 0 (solid squares), 100 (solid circles), 200 (solid triangles), (**B**) 300 (solid squares), 400 (solid circles), 600 (solid triangles), (**C**) 800 (solid squares), 1000 (solid circles), 1200 (solid triangles), (**D**) 1400 (solid squares), 1600 (solid circles), 2000 (solid triangles), (**E**) 3000 (solid squares), 4000 (solid circles), and 5000 (solid triangles) µg $CdCl_2 \cdot L^{-1}$.



Figs. 2.11 Comparison of acute LT_{50} values computed by discrete logistic failure time model analysis (DLFTM) for samples of *Physa acuta* for individuals without SL as a covariate (A) in the 25th (SL = 6.0 mm) (B), 50th (median SL = 7.1 mm) (C) and 75th SL quantile (SL = 8.3) (D), respectively.exposed to varying concentrations of $CdCl_2$ ranging from 1200-5000 μg $CdCl_2 \cdot L^{-1}$ for a maximum period of eight days. The horizontal axis is exposure concentration in μg $CdCl_2 \cdot L^{-1}$ and the vertical axis, the estimated median time in hours for theLT_{50}. Vertical bars above histograms represent standard errors. Different letters above histograms indicate that LT_{50} values are significantly different ($p < 0.05$).

Table 2.6 Acute 50 % sample mortality values (LT_{50}) estimated by discrete logistic failure time model analysis for samples of *Physa acuta* (n = 150) exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 - 5000 µg $CdCl_2 \cdot L^{-1}$ over a total exposure period of 192 h (8 d). LT_{50} values are provided for the entire sample based on analysis without SL as a covariate, and, when analyzed with SL as a covariate, for individuals in the 25th SL quantile, for individuals in the 50th SL quantile and for individuals in the 75th SL quantile.

Without SL as a Covariate		
Exposure Concentration (µg $CdCl_2 \cdot L^{-1}$)	**LT_{50} (h)**	**s.e.**
1400	183.13	±10.103
1600	123.28	±4.857
2000	68.62	±0.976
3000	50.81	±1.221
4000	19.82	±0.909
5000	13.48	±0.279
With SL as a Covariate: 25th SL Quantile (SL = 6.0 mm)		
Exposure Concentration (µg $CdCl_2 \cdot L^{-1}$)	**LT_{50} (h)**	**s.e.**
1200	143.20	±5.532
1400	95.76	±4.467
1600	71.86	±1.451
2000	60.65	±1.051
3000	37.77	±1.794
4000	21.28	±1.034
5000	16.57	±0.579
With SL as a Covariate: 50th SL Quantile (Median SL = 7.1 mm)		
Exposure Concentration (µg $CdCl_2 \cdot L^{-1}$)	**LT_{50} (h)**	**s.e.**
1200	174.67	±1.665
1400	119.11	±4.609
1600	74.33	±10.320
2000	43.22	±0.871
3000	26.53	±1.194
4000	16.91	±0.499
5000	14.14	±0.251
With SL as a Covariate: 75th SL Quantile (SL = 8.3 mm)		
Exposure Concentration (µg $CdCl_2 \cdot L^{-1}$)	**LT_{50} (h)**	**s.e.**
1200	182.81	±2.038
1400	165.66	±11.231
1600	105.44	±5.002
2000	46.78	±1.167
3000	29.34	±1.067
4000	17.90	±0.652
5000	14.51	±0.326

quantile (SL = 8.3 mm) on exposure for 192 hours to concentrations of cadmium

quantile (SL = 6.0 mm), median 50th SL quantile (median SL = 7.1 mm) and 75th SL chloride ($CdCl_2$) ranging from 0-5000 μg $CdCl_2 \cdot L^{-1}$. Exposures to concentrations less than 1200 μg $CdCl_2 \cdot L^{-1}$ were excluded from DLFTM analysis because a 50 % mortality threshold was not reached in any of the three sample SL quantile groups at these levels (Figs. 2.11 B-D). DLFTM analysis revealed a significant relationship between sample mortality and $CdCl_2$ concentration at each tested exposure duration in which 50 % mortality was achieved across which chi-square and p values ranged from chi-square = 681.86 ($p < 0.0001$) in the 75th SL quantile SL = 8.3 mm) through chi-square = 740.48 ($p < 0.0001$) in the 25th SL quantile (SL = 6.0 mm) to chi-square = 930.60 ($p < 0.0001$) in the 50th SL quantile (median SL = 7.1 mm). The effect shell length on sample mortality was significant over all three sample quantiles (chi-square = 8.79, $p < 0.0030$). A significant interaction between $CdCl_2$ and shell length was also constant over all three samples quantile SL (chi-square = 5.54, $p < 0.0186$).

For the 25th SL quantile (SL = 6.0 mm) survival was greatest in concentrations of 0, 100 and 200 μg $CdCl_2 \cdot L^{-1}$ and least at 2000-5000 μg $CdCl_2 \cdot L^{-1}$ (Figs. 2.8A, D and E). In this sample quantile LT_{50} values decreased from 143.20 h (s.e. = ± 5.532) at a concentration of 1200 μg $CdCl_2 \cdot L^{-1}$ to 16.57 h (s.e. = ± 0.579) at a concentration of 5000 μg $CdCl_2 \cdot L^{-1}$ (Table 2.6, Fig. 2.11 B). All concentrations were significantly different from each other at $\alpha = 0.05$ based on the Scheffé pair-wise comparison. Survival was greatest in the 50th quantile SL (median SL = 7.1 mm) over the 192 h exposure period at cadmium chloride ($CdCl_2$) concentrations of 0, 100 and 200 μg $CdCl_2 \cdot L^{-1}$ and least at concentrations of 2000-5000 μg $CdCl_2 \cdot L^{-1}$ (Fig. 2.11 C). The

LT_{50} values estimated by DLFTM for this group decreased from 174.67 h (s.e. = ± 1.665) at 1200 μg $CdCl_2 \cdot L^{-1}$ to 14.14 hours (s.e. = ± 0.251) at 5000 μg $CdCl_2 \cdot L^{-1}$ (Table 2.6, Fig 2.11 B). Based on the Scheffé pair-wise comparison test LT_{50} values were significantly different among all $CdCl_2$ concentrations at α=0.05 (Table 2.7). Survival for the 75th SL quantile (SL = 8.3 mm) over 192 h exposure period was greatest at 0, 100 and 200 μg $CdCl_2 \cdot L^{-1}$ and least at 2000-5000 μg $CdCl_2 \cdot L^{-1}$ (Fig. 2.11D). As estimated by DLFTM, LT_{50} values in this size group decreased from 182.81 h (s.e. = ± 2.038) at a concentration of 1200 μg $CdCl_2 \cdot L^{-1}$ to 14.51 h (s.e. = ±0.326) at a concentration of 5000 μg $CdCl_2 \cdot L^{-1}$ (Table 2.6, Fig 2.11 D). Values of LT_{50} at 1400-2000 μg $CdCl_2 \cdot L^{-1}$ were significantly different from each other at α = 0.05 based on the Scheffé pair-wise comparison test (Fig. 2.11 D).

The DLFTM analysis revealed a significant effect of size on survivorship. Across exposure concentrations of 1200-5000 μg $CdCl_2 \cdot L^{-1}$, LT_{50} values were smallest in the 25th SL quantile (SL = 6.0 mm) and 75th SL qunatile (SL = 8.3 mm). Estimated LT_{50} values for all three sample SL quantile groups became increasing dissimilar with reduction in exposure concentration from 5000 to 1400 $CdCl_2 \cdot L^{-1}$ indicating that impacts of individual size on survivorship were reduced at increasingly more toxic $CdCl_2$ concentrations (Fig 2.11 A-D).

Chronic Percent Sample Mortality

Sample mortalities of individuals of *Physa acuta* were recorded at 24 h intervals through 672 h (28 d) of exposure to concentrations of cadmium chloride ($CdCl_2$)

Table 2.7 Chi-square and probability (p) values for maximum likelihood probit regression analysis determinations of chronic 50 % lethal concentration values (LC_{50}) for samples of *Physa acuta* exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 - 2000 µg $CdCl_2 \cdot L^{-1}$ over a total exposure period of 672 h (28 days). Chi-square values are provided for analysis carried out without shell length (SL) as a covariate to describe the probability of an individual dying in the given interval: $I^{-1}(p) = b_0 + b_1(Lconcentration+1)$. and the probability of of an individual dying in a given interval: $I^{-1}(p) = b_0 + b_1(Lconcentration+1)+b_2(SL-SL^0)+b_3[(Lconcentration+1)*(SL-SL^0)]$ with shell length as a covariate allowing determination of EC_{50} values for individuals in the 25th SL quantile (SL = 5.6 mm), median 50th SL quantile (median SL = 6.7 mm) and the 75th SL quantile (SL = 7.9 mm).

Exposure Duration (hours)	Chi-Square SL not a Covariate	P	Chi-Square SL = 5.6 mm	p	Chi-Square SL = 6.7 mm	P	Chi-Square SL = 7.9 mm	p
24	35.60	<0.0001*	31.22	<0.0001*	22.63	<0.0001*	11.38	0.0004*
48	201.93	<0.0001*	262.60	<0.0001*	241.43	<0.0001*	212.99	<0.0001*
72	208.47	<0.0001*	203.16	<0.0001*	193.62	<0.0001*	83.83	<0.0001*
96	263.01	<0.0001*	245.98	<0.0001*	220.64	<0.0001*	108.79	<0.0001*
120	288.43	<0.0001*	265.09	<0.0001*	253.69	<0.0001*	133.62	<0.0001*
144	320.87	<0.0001*	284.59	<0.0001*	287.28	<0.0001*	164.43	<0.0001*
168	324.52	<0.0001*	280.35	<0.0001*	301.00	<0.0001*	170.78	<0.0001*
192	353.60	<0.0001*	300.98	<0.0001*	350.36	<0.0001*	206.50	<0.0001*
216	426.90	<0.0001*	343.65	<0.0001*	426.60	<0.0001*	266.23	<0.0001*
240	403.86	<0.0001*	308.24	<0.0001*	437.52	<0.0001*	283.23	<0.0001*
264	488.14	<0.0001*	347.32	<0.0001*	518.95	<0.0001*	345.54	<0.0001*
288	518.48	<0.0001*	330.14	<0.0001*	573.74	<0.0001*	405.00	<0.0001*
312	535.58	<0.0001*	321.36	<0.0001*	611.57	<0.0001*	452.35	<0.0001*
336	531.49	<0.0001*	297.62	<0.0001*	592.81	<0.0001*	482.55	<0.0001*
360	528.83	<0.0001*	261.60	<0.0001*	565.95	<0.0001*	511.94	<0.0001*
384	518.06	<0.0001*	219.23	<0.0001*	514.48	<0.0001*	523.15	<0.0001*
408	495.25	<0.0001*	218.12	<0.0001*	472.25	<0.0001*	504.07	<0.0001*
432	436.89	<0.0001*	165.43	<0.0001*	410.51	<0.0001*	479.08	<0.0001*
456	394.26	<0.0001*	146.90	<0.0001*	347.91	<0.0001*	446.08	<0.0001*
480	374.39	<0.0001*	127.22	<0.0001*	307.74	<0.0001*	424.77	<0.0001*
504	353.87	<0.0001*	115.76	<0.0001*	282.16	<0.0001*	407.07	<0.0001*
528	327.66	<0.0001*	81.84	<0.0001*	248.59	<0.0001*	385.39	<0.0001*
552	283.95	<0.0001*	75.39	<0.0001*	205.32	<0.0001*	339.90	<0.0001*
576	263.64	<0.0001*	62.61	<0.0001*	182.58	<0.0001*	308.72	<0.0001*
600	216.22	<0.0001*	58.53	<0.0001*	146.71	<0.0001*	251.76	<0.0001*
624	200.29	<0.0001*	59.67	<0.0001*	138.15	<0.0001*	233.39	<0.0001*
648	187.23	<0.0001*	62.87	<0.0001*	131.28	<0.0001*	218.30	<0.0001*
672	91.87	<0.0001*	26.69	<0.0001*	60.04	<0.0001*	95.38	<0.0001*

* indicates a significant difference at $p < 0.005$

ranging from 0-2000 $CdCl_2 \cdot L^{-1}$. Fifty percent sample mortality was exceeded in concentrations ranging from 10-2000 $CdCl_2 \cdot L^{-1}$, with 100 % sample mortality being recorded at 800-2000 µg $CdCl_2 \cdot L^{-1}$ during the 672 h exposure period (Fig. 2.12). Fifty percent sample mortality was achieved after 504 h (21 d) at 250-450 $CdCl_2 \cdot L^{-1}$, at 336 h (14 d) at 600-1600 µg $CdCl_2 \cdot L^{-1}$, and at 168 h (7 d) at 2000 µg $CdCl_2 \cdot L^{-1}$ (Fig.2.12). Less than 100 % sample mortality within the 672 h exposure period was recorded in exposures ranging from 5-200 $CdCl_2 \cdot L^{-1}$ (Fig. 2.12).

Chronic LC_{50} Analysis-Probit Regression

Most aquatic toxicity studies estimated LC_{50} without size as a covariate (U.S. EPA, 2000). Therefore, in order, to allow direct comparison with toxicological $CdCl_2$ data published for other aquatic species, chronic LC_{50} values were initially determined for *P. acuta* in this study. Maximum likelihood probit analysis without shell length as a size covariate revealed a significant relationship between mortality and $CdCl_2$ concentration in samples of *P. acuta* at each tested exposure duration (i.e, 24-672 h) across which chi-square and p values ranged from chi-square = 35.60 ($p < 0.0001$) at 24 h to chi-square = 535.58 ($p < 0.0001$) at 312 h (Table 2.7). Estimated LC_{50} values for 24, 72, 96 and 120 h exposure were removed from further analysis because they fell beyond the maximum exposure concentration of 2000 µg $CdCl_2 \cdot L^{-1}$. Estimated LC_{50} values decreased from 1928.99 µg $CdCl_2 \cdot L^{-1}$ (s.e. = ±108.88) at an exposure time of 144 h through 79.28 µg $CdCl_2 \cdot L^{-1}$ (s.e. = ±10.58) at 672 h (Table 2.8, Fig. 2.13 A).

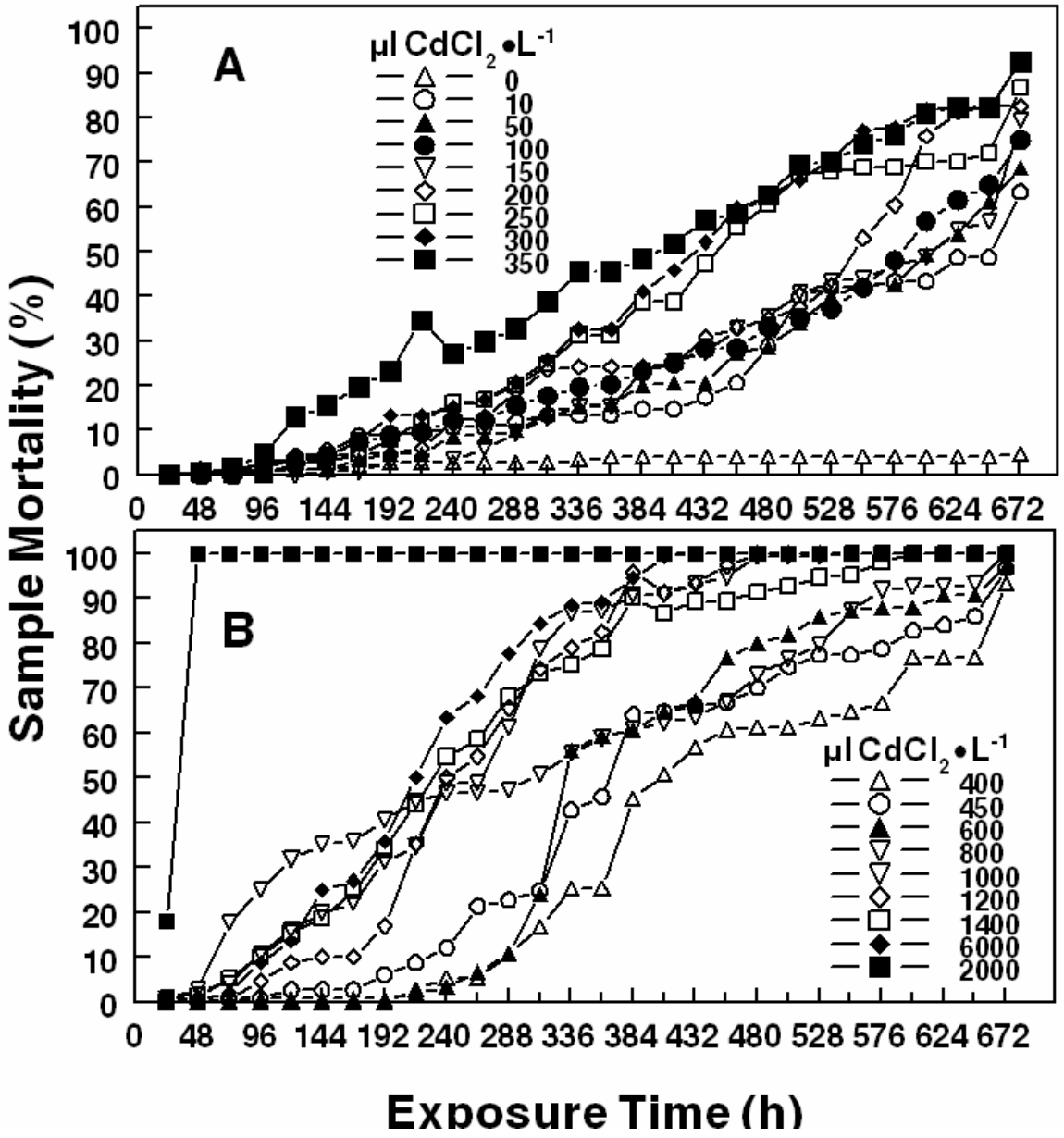


Fig. 2.12 The effect of chronic exposure of samples of *Physa acuta* to various concentrations of cadmium chloride as A) 0-350 µg $CdCl_2 \cdot L^{-1}$ and B) 400-200 µg $CdCl_2 \cdot L^{-1}$ as percent mortality (vertical axis) at 24 h intervals during a total exposure period of 672 h (i.e., 28 d, horizontal axis).

Table 2.8 Chronic 50 % sample mortality values (LC_{50}) estimated by maximum likelihood probit regression analysis for samples of *Physa acuta* (n = 150) exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 - 2000 µg $CdCl_2 \cdot L^{-1}$ over a total exposure period of 672 h (28 days). LC_{50} values are provided for analyses of the entire sample without SL included as a covariate, and with inclusion of SL as a covariate for individuals in the 25th SL quantile (SL = 5.6 mm), median 50th SL quantile (median SL = 6.7 mm) and 75th SL quantile (SL = 7.9 mm).

Exposure Time (h)	LC_{50} SL not a Covariate	s.e.	LC_{50} SL = 5.6 mm	s.e.	LC_{50} SL = 6.7 mm	s.e.	LC_{50} SL = 7.9 mm	s.e.
24	2695.20	±196.86	2686.69	±229.02	2714.20	±243.21	2733.53	±358.17
48	1864.89	±40.18	1638.23	±33.84	1749.67	±21.20	1808.83	±25.03
72	2074.73	±88.56	1654.32	±69.80	1971.96	±79.81	2378.29	±168.46
96	2164.79	±3118.68	1586.87	±84.87	1932.38	±86.06	2274.99	±148.22
120	2144.11	±133.66	1417.03	±80.68	1836.72	±84.20	2242.01	±144.97
144	1928.99	±108.88	1294.01	±68.81	1664.50	±65.73	1988.74	±100.70
168	1863.97	±108.43	1214.70	±66.23	1607.53	±65.37	1959.35	±101.74
192	1588.73	±85.19	978.64	±47.29	1392.00	±50.36	1768.38	±78.78
216	1278.39	±55.12	811.57	±33.95	1182.84	±35.37	1507.43	±49.54
240	1178.07	±52.69	710.51	±30.97	1106.71	±34.07	1446.88	±47.23
264	943.73	±34.47	591.03	±22.82	937.80	±26.06	1245.00	±33.98
288	780.46	±26.37	481.66	±18.65	814.11	±22.25	1107.26	±28.20
312	630.41	±20.142	369.95	±14.44	674.78	±18.49	977.65	±24.78
336	474.32	±15.03	272.43	±11.54	517.06	±14.30	801.54	±21.27
360	405.06	±12.98	230.28	±10.99	451.97	±12.46	710.72	±19.01
384	348.75	±11.34	193.12	±10.67	399.29	±10.86	636.56	±16.87
408	318.49	±10.82	183.00	±9.86	357.75	±9.71	578.28	±16.06
432	269.78	±10.36	145.29	±10.96	307.35	±9.21	511.32	±15.13
456	233.98	±10.04	129.40	±10.73	264.60	±8.41	447.19	±14.09
480	215.22	±9.60	116.71	±10.84	244.32	±7.96	417.09	±13.09
504	200.26	±9.35	109.19	±11.02	224.86	±7.83	381.98	±12.32
528	187.00	±9.27	86.44	±12.54	207.26	±8.30	363.91	±12.03
552	163.08	±9.37	80.72	±12.33	179.37	±8.28	316.30	±11.30
576	155.13	±9.17	74.16	±12.92	167.93	±8.39	294.60	±10.66
600	132.49	±9.29	73.65	±12.79	141.89	±8.68	237.41	±9.72
624	123.80	±9.46	75.16	±12.63	133.39	±8.86	216.15	±9.65
648	116.20	±9.61	77.85	±12.23	127.35	±8.91	199.53	±9.50
672	79.28	±10.58	51.94	±15.67	82.56	±11.70	121.71	±10.74

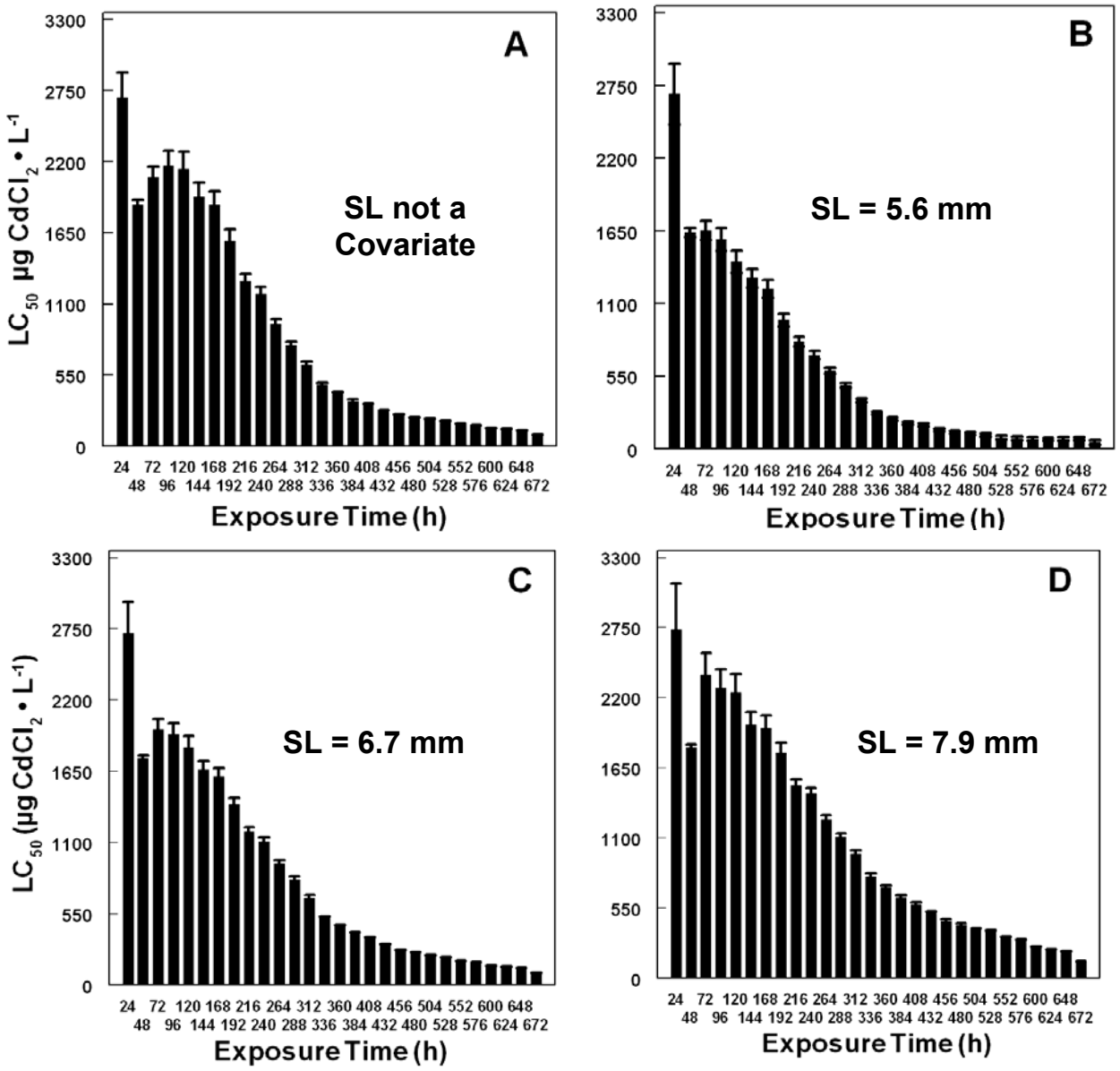


Fig.2.13 Lethal concentration for 50 % mortality (LC_{50}) estimated by maximum likelihood probit regression for samples of *Physa acuta* (total sample size = 1940) computed (A) without shell length (SL) as a covariate and (B-D) with SL as a covariate for individuals in the (B) 25th SL quantile (SL = 5.6 mm), (C) 50th SL quantile (median SL = 6.7 mm) and (D) 75th SL quantile (SL = 7.9 mm) exposed to varying concentrations of cadmium chloride (µg $CdCl_2 \cdot L^{-1}$, horizontal axis) every 24 h over a total exposure time of 672 h (i.e., 28 d). Error bars above histograms represent standard error of the LC_{50} value. In some cases, standard errors are so small that they are not readily differentiated from the top of the histogram bar.

Following the procedure for determination of acute LC_{50} values described above, maximum likelihood probit analysis was used to determine LC_{50} values for chronic exposures to 10-2000 μg $CdCl_2 \cdot L^{-1}$ for the 25th SL quantile (SL = 5.6 mm), median 50th SL quantile (median SL = 6.7 mm) and 75th SL quantile (SL = 7.9 mm). Values of LC_{50} estimated at 24 h for all three quantiles (n = 2081) and for exposures of 72, 96 and 120 h for the median SL = 7.9 mm quantile were eliminated from further analysis because they extended beyond the maximum tested concentration of 2000 μg $CdCl_2 \cdot L^{-1}$ (Table 2.8). Effect of SL on mortality and the interaction between concentration and SL were consistent across all three quantile groups across all quantile groups at exposure periods of 24 h and 72 h, there was no significant effect of SL on mortality. There was a significant effect ($\alpha = 0.05$) of SL on mortality for all quantile groups from 48 h, and 96 h to 672 h (Table 2.9). Significant chi-square values ranged from 4.13 ($p = 0.0421$) at 672 h to 132.48 ($p < 0.0001$) at 48 h. There was no significant interaction between concentration and SL on sample mortality for 24 h, 72 h and for 624-672 h. Significant chi-square values ranged from 6.55 ($p=0.0105$) at 600 h to 65.79 ($p < 0.0001$) at 288 h (Table 2.9).

For the 25th SL quantile (SL = 5.6 mm) maximum likelihood probit analysis revealed a significant relationship between mortality and $CdCl_2$ concentration at each tested exposure duration (i.e., 24-672 hours) across which chi-square and p values ranged from 26.69 ($p < 0.0001$) at the 672 h exposure to 347.32 ($p < 0.0001$) at the 264 h exposure (Table 2.7). LC_{50} values decreased from 1654.32 μg $CdCl_2 \cdot L^{-1}$ (s.e. = 69.80)

Table 2.9. Effect of SL and the interaction between concentration and SL on 50 % sample chronic mortality (LC_{50}) response chi-square and probability (p) values estimated by probit regression analysis determinations for samples of *Physa acuta* exposed to media with concentrations of cadmium chloride ranging from 0 - 2000 µg $CdCl_2 \cdot L^{-1}$ over a total exposure period of 672 h. The probability of of an individual dying in a given interval: $\Gamma^1(p) = b_0 + b_1(Lconcentration+1)+b_2\,(SL-L^0) + b_3[(Lconcentration+1)*(SL-SL^0)]$ with SL as a covariate allowing determination of SL and interaction chi-square values for individuals in the 25th SL quantile, 50th SL quantile and the 75th SL quantile. Probability values are consistent across all SL calculations.

Exposure Duration (h)	SL Effect on LC_{50} Chi-square	P	Concentration-SL Interaction Effect on LC_{50} Chi-square	P
24	1.33	0.02491	1.18	0.2783
48	132.48	<0.0001*	126.03	<0.0001*
72	0.02	0.9018	0.28	0.05940
96	6.10	0.0130*	3.45	0.0634
120	13.32	0.0003*	7.72	0.0055*
144	23.82	<0.0001*	15.79	<0.0001*
168	24.41	<0.0001*	15.67	<0.0001*
192	34.92	<0.0001*	22.21	<0.0001*
216	52.59	<0.0001*	35.82	<0.0001*
240	64.84	<0.0001*	44.52	<0.0001*
264	74.34	<0.0001*	51.31	<0.0001*
288	93.43	<0.0001*	65.79	<0.0001*
312	91.15	<0.0001*	59.10	<0.0001*
336	73.43	<0.0001*	40.52	<0.0001*
360	81.14	<0.0001*	45.47	<0.0001*
384	94.99	<0.0001*	55.52	<0.0001*
408	68.34	<0.0001*	33.47	<0.0001*
432	70.03	<0.0001*	36.32	<0.0001*
456	51.88	<0.0001*	22.88	<0.0001*
480	53.03	<0.0001*	23.68	<0.0001*
504	46.90	<0.0001*	20.44	<0.0001*
528	55.57	<0.0001*	29.06	<0.0001*
552	38.44	<0.0001*	17.07	<0.0001*
576	35.23	<0.0001*	16.61	<0.0001*
600	17.91	<0.0001*	6.55	0.0105*
624	11.77	0.0006*	3.38	0.0659
648	6.88	0.0087*	1.06	0.3025
672	4.13	0.0421*	1.59	0.2073

*indicates a significant difference at $p<0.05$

at an exposure time of 72 h through 51.94 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±15.67) at 672 h (Table 2.8, Fig. 2.13 B). For the median 50th SL quantile (median SL = 6.7 mm) there was a significant relationship between sample mortality and $CdCl_2$ concentration at each tested exposure duration (24-672 hours) across which chi-square and p values ranged from chi-square = 22.63 ($p < 0.0001$) at 24 h to 611.57 ($p < 0.0001$) at 312 h (Table 2.7). LC_{50} values decreased from 1971.96 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±79.81) at an exposure time of 72 h to 82.56 μg $CdCl_2 \cdot L^{-1}$ at 672 h (s.e. = ±11.70) (Table 2.8, Fig. 2.13 C). For the 75th SL quantile (SL = 7.9 mm) the relationship between mortality and $CdCl_2$ concentration was significant ($\alpha = 0.05$) at each tested exposure duration (24-672 hours) across which chi-square and p values ranged from chi-square = 11.38,($p < 0.0004$) at 24 h to chi-square = 523.15, ($p < 0.0001$) at 384 h (Table 2.7). LC_{50} values decreased from 1988.74 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±100.70) at an exposure time of 144 h to 121.71 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±10.74) at 672 h (Table 2.8, Fig. 2.13 D). The LC_{50} values of the median 50^{th} and 75th SL quartiles at 48 h were lower than those recorded at 72 h due to recording of 100 % mortality at 2000 μg $CdCl_2 \cdot L^{-1}$ and near zero mortality at all other tested concentrations (10-1600 μg $CdCl_2 \cdot L^{-1}$) (Fig. 2.13).

Addition of SL as a covariate to maximum likelihood probit analysis indicated that LC_{50} values for exposure to $CdCl_2$ increased with size measured as SL in *P. acuta*. The LC_{50} values estimated without SL as a covariate tended to be similar to those of the 75th SL quantile (SL =7.9 mm) over exposure periods ranging from 24-168 h, thereafter declining and approximating those of the median 50^{th} SL quantile across exposure times

ranging from 216-672 h. Differences between the LC_{50} values of the three SL quantile groups, and of these groups relative to that estimated for the sample as a whole without SL as a covariate, tended to decline with increasing exposure time, such that recorded differences were minimal at the maximum 672 h exposure period (Fig 2.14).

Chronic LC_{50} Analysis-Logistic Regression

Maximum likelihood logistic regression analysis without SL as a covariate revealed a significant relationship between sample mortality and $CdCl_2$ concentration at each tested exposure duration (24-672 hours) across which chi-square and p values ranged from chi-square = 25.32 ($p < 0.0001$) at an exposure of 24 h to chi-square = 467.56 ($p < 0.0001$) at an exposure of 312 h (Table 2.10). The LC_{50} estimate at an exposure of 24 h was eliminated from further analyses because it fell outside the maximum concentration of tested of 2000 μg $CdCl_2 \cdot L^{-1}$ (Table 2.11). Estimates of LC_{50} values increased from 1779.68 (s.e. = ±20.18) to 1953.80 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±95.04) across exposure times ranging from 48-120 h. Thereafter, LC_{50} values declined with further increase in exposure duration reaching a minimum value of 92.07 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±9.82) at the maximum exposure of 672 h (Table 2.11, Fig. 2.15 A).

Logistic regression analysis of the effect of $CdCl_2$ concentration with SL as a covariate was used to determine LC_{50} values for chronic exposures to 10-2000 μg $CdCl_2 \cdot L^{-1}$ for the 25th SL quantile (SL = 5.6 mm), 0th SL quantile (median SL = 6.7 mm) and 75th SL quantile (SL = 7.9 mm). The LC_{50} estimate for 24 h resulted in LC_{50} values for all three groups that were not included in further analyses because they fell outside the maximum concentration tested (i.e., 2000 μg $CdCl_2 \cdot L^{-1}$) as were the LC_{50}

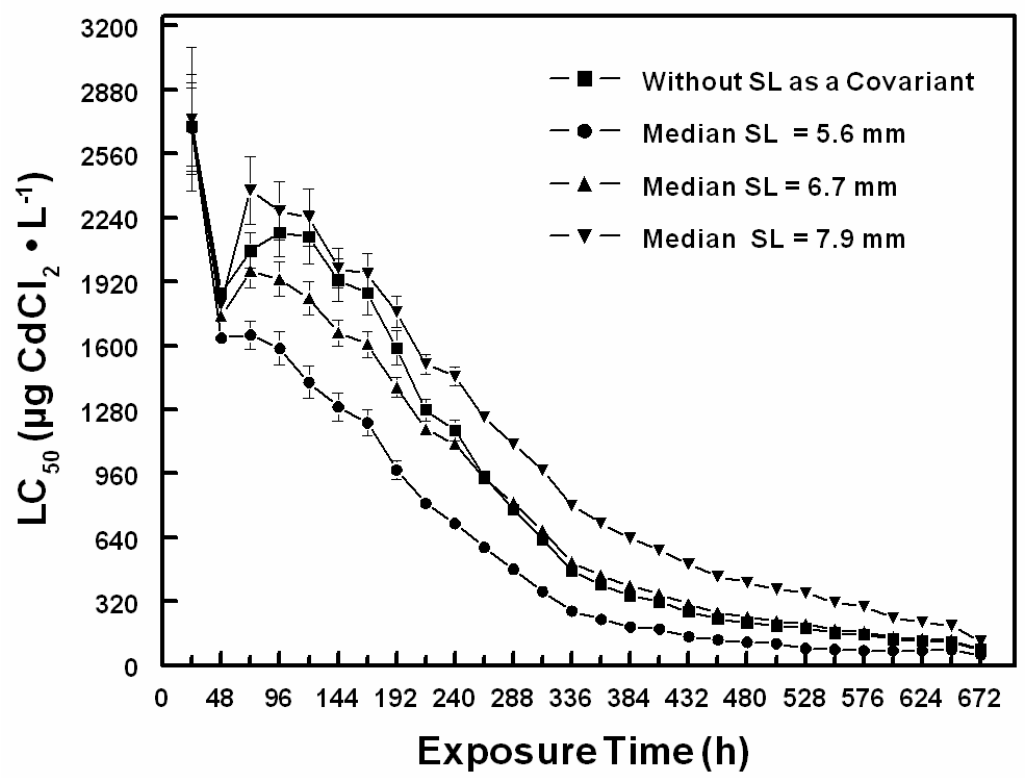


Fig. 2.14. Comparison of chronic LC_{50} values computed by maximum likelihood probit regression analysis for samples of *Physa acuta* at intervals of 24 h over a 672 h (28 d) period of exposure to varying concentrations of cadmium chloride ($CdCl_2$). The horizontal axis is exposure time in hours and the vertical axis, the estimated lethal concentration for 50 % sample mortality (LC_{50}) in µg $CdCl_2 \cdot L^{-1}$. Solid squares are LC_{50} values estimated without including shell length (SL) as a covariate. Solid circles, triangles and inverted triangles represent median LC_{50} values resulting from analysis with inclusion of SL as a covariate for individuals in the 25th (SL = 5.6 mm), 50th (median SL = 6.7 mm) and 75th SL quantile (SL = 7.9), respectively. Vertical bars about LC_{50} values represent standard errors. Error bars are not apparent when they are less than the width of the symbol with which they are associated.

Table 2.10 Chi-square and probability (p) values for maximum likelihood logistic regression analysis determinations of chronic 50 % sample lethal concentration values (LC_{50}) for samples of *Physa acuta* exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 - 2000 μg $CdCl_2 \cdot L^{-1}$ over a total exposure period of 672 h (28 days). Chi-square values are provided for analysis carried out without shell length (SL) as a covariate to describe the probability of an individual dying in the given interval: $\Gamma^1(p) = b_0 + b_1(Lconcentration+1)$. The probability of of an individual dying in the given interval: $\Gamma^1(p) = b_0 + b_1(Lconcentration+1)+b_2\ (SL-SL^0)+b_3[(Lconcentration+1)*(SL-SL^0)]$ with shell length as a covariate allowing determination of EC_{50} values for individuals in the 25th SL quantile (SL = 5.6 mm), median 50th SL quantile (median SL = 6.7 mm) and the 75th SL quantile (SL = 7.9 mm).

Exposure Duration (hours)	Chi-Square SL not a Covariate	P	Chi-Square SL = 5.6 mm	p	Chi-Square Median SL = 6.7 mm	p	Chi-Square SL = 7.9 mm	P
24	25.32	<0.0001*	21.30	<0.0001*	17.48	<0.0001*	10.61	= 0.0011*
48	174.04	<0.0001*	169.73	<0.0001*	160.74	<0.0001*	148.82	<0.0001*
72	169.40	<0.0001*	144.92	<0.0001*	146.87	<0.0001*	58.57	<0.0001*
96	216.60	<0.0001*	213.16	<0.0001*	171.19	<0.0001*	91.75	<0.0001*
120	250.97	<0.0001*	247.06	<0.0001*	203.03	<0.0001*	121.08	<0.0001*
144	279.48	<0.0001*	265.01	<0.0001*	232.97	<0.0001*	148.93	<0.0001*
168	287.66	<0.0001*	265.36	<0.0001*	248.68	<0.0001*	155.53	<0.0001*
192	317.09	<0.0001*	283.95	<0.0001*	291.02	<0.0001*	186.56	<0.0001*
216	377.34	<0.0001*	317.30	<0.0001*	349.55	<0.0001*	235.13	<0.0001*
240	363.49	<0.0001*	288.91	<0.0001*	364.37	<0.0001*	250.72	<0.0001*
264	430.98	<0.0001*	315.25	<0.0001*	427.84	<0.0001*	300.16	<0.0001*
288	455.58	<0.0001*	297.74	<0.0001*	468.02	<0.0001*	345.70	<0.0001*
312	467.56	<0.0001*	286.13	<0.0001*	493.47	<0.0001*	381.85	<0.0001*
336	459.89	<0.0001*	265.01	<0.0001*	481.45	<0.0001*	410.57	<0.0001*
360	453.82	<0.0001*	236.89	<0.0001*	459.82	<0.0001*	429.36	<0.0001*
384	442.14	<0.0001*	203.59	<0.0001*	425.22	<0.0001*	434.29	<0.0001*
408	423.60	<0.0001*	207.58	<0.0001*	401.43	<0.0001*	423.00	<0.0001*
432	379.11	<0.0001*	163.66	<0.0001*	356.13	<0.0001*	407.41	<0.0001*
456	344.85	<0.0001*	149.29	<0.0001*	316.19	<0.0001*	386.55	<0.0001*
480	329.14	<0.0001*	131.63	<0.0001*	285.53	<0.0001*	368.21	<0.0001*
504	313.15	<0.0001*	119.93	<0.0001*	263.60	<0.0001*	351.66	<0.0001*
528	294.08	<0.0001*	87.75	<0.0001*	234.93	<0.0001*	332.58	<0.0001*
552	259.49	<0.0001*	83.02	<0.0001*	198.64	<0.0001*	301.51	<0.0001*
576	245.55	<0.0001*	71.11	<0.0001*	178.55	<0.0001*	279.45	<0.0001*
600	207.22	<0.0001*	67.40	<0.0001*	146.12	<0.0001*	231.92	<0.0001*
624	193.01	<0.0001*	68.89	<0.0001*	139.50	<0.0001*	217.24	<0.0001*
648	180.92	<0.0001*	71.61	<0.0001*	133.40	<0.0001*	203.61	<0.0001*
672	96.49	<0.0001*	29.97	<0.0001*	63.42	<0.0001*	97.16	<0.0001*

* indicates p values are significant a $\alpha = 0.05$.

Table 2.11 Chronic 50 % sample mortality values (LC_{50}) estimated by maximum likelihood logistic regression analysis for samples of *Physa acuta* (n = 150) exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 - 2000 µg $CdCl_2 \cdot L^{-1}$ over a total exposure period of 672 h (28 days). LC_{50} values are provided for analysis of the entire sample without shell length (SL) as a covariate, and with SL as a covariate for individuals in the 25th SL quantile (SL = 5.6 mm), 50th SL quantile (median SL = 6.7 mm) and 75th SL quantile (SL = 7.9 mm).

Exposure Time (h)	LC_{50} SL not a Covariate	s.e.	LC_{50} Med. SL = 5.6 mm	s.e.	LC_{50} Med. SL = 6.7 mm	s.e.	LC_{50} Med. SL = 7.9 mm	s.e
24	2333.61	±99.81	2370.76	±129.12	2374.87	±132.17	2377.53	±178.86
48	1779.68	±20.18	1669.66	±29.94	1758.75	±19.18	1805.62	±24.42
72	1866.78	±48.61	1594.10	±48.73	1815.37	±47.68	2056.90	±101.12
96	1936.51	±75.58	1537.45	±64.67	1800.58	±57.21	2016.45	±91.55
120	1953.80	±95.04	1400.06	±67.63	1741.52	±60.30	2004.52	±92.97
144	1804.00	±81.79	1296.29	±60.42	1618.61	±50.00	1850.40	±69.31
168	1761.37	±84.73	1221.76	±59.98	1574.55	±51.96	1838.96	±73.72
192	1543.96	±71.88	997.16	±46.43	1396.51	±43.55	1706.08	±61.71
216	1273.30	±49.51	829.82	±34.27	1207.46	±32.97	1497.82	±41.62
240	1179.58	±48.91	726.37	±31.59	1134.48	±32.79	1447.63	±41.25
264	956.17	±33.52	601.90	±23.61	965.18	±26.55	1262.70	±31.59
288	793.98	±26.49	489.67	±19.16	839.17	±23.22	1129.01	±27.13
312	640.04	±20.73	374.00	±14.51	694.34	±19.59	999.92	±24.61
336	475.48	±15.38	273.50	±11.12	520.59	±14.84	809.98	±21.76
360	404.70	±13.11	232.82	±10.49	453.27	±12.74	715.09	±19.48
384	348.09	±11.32	197.10	±10.18	399.66	±11.02	638.53	±17.27
408	317.42	±10.63	186.49	±9.44	357.73	±9.80	579.11	±16.46
432	269.13	±9.99	151.41	±10.28	305.94	±9.08	507.46	±15.44
456	235.12	±9.55	134.45	±10.13	263.72	±8.24	443.16	±14.32
480	217.61	±9.03	122.03	±10.14	243.97	±7.70	413.16	±13.19
504	203.95	±8.71	115.53	±10.24	225.78	±7.41	378.87	±12.33
528	190.78	±8.68	95.29	±11.80	209.81	±7.80	361.12	±12.01
552	168.53	±8.71	89.91	±11.48	182.15	±7.63	313.15	±11.10
576	160.40	±8.53	83.87	±11.98	170.79	±7.70	291.76	±10.43
600	139.82	±8.57	84.94	±11.66	147.09	±7.78	236.46	±9.25
624	131.99	±8.75	86.60	±11.50	139.30	±7.96	215.90	±9.10
648	125.33	±8.91	88.74	±11.13	134.07	±7.99	201.35	±8.93
672	92.07	±9.82	63.54	±15.49	93.52	±10.88	131.32	±9.74

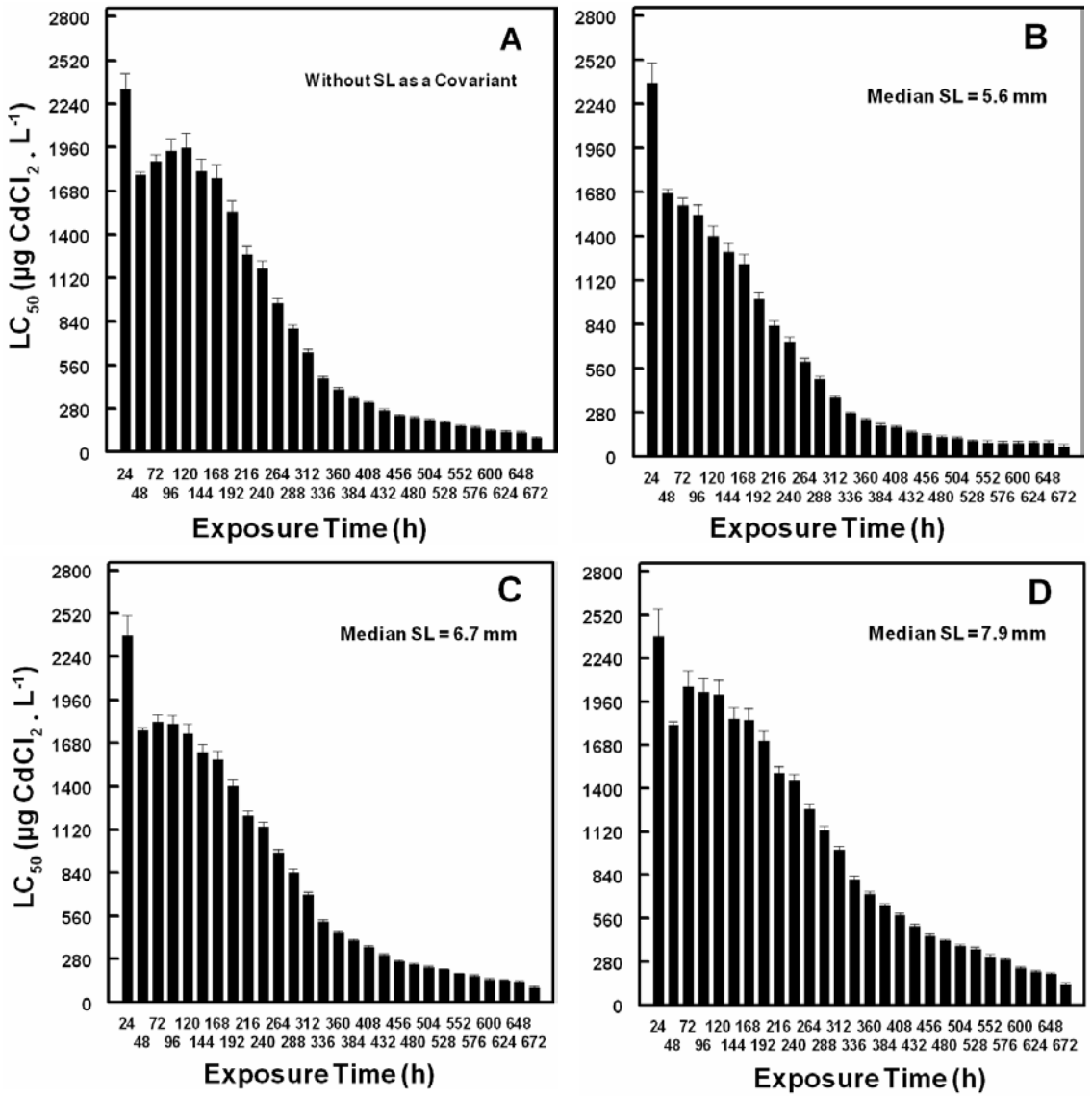


Fig. 2.15 Comparison of chronic lethal concentration for 50 % sample mortality (LC_{50}) values computed by maximum likelihood logistic regression analysis for samples of *Physa acuta* for individuals in the entire sample computed without SL as a covariate (A), and in the 25th (SL = 5.6 mm) (B), 50th (median SL = 6.7 mm) (C) and 75th SL quantile (SL = 7.9) (D), respectively exposed to varying concentrations of cadmium chloride ranging from 0-2000 µg $CdCl_2 \cdot L^{-1}$ at 24 h intervals during a maximum exposure period of 672 h (28 d). The vertical axis is exposure concentration in µg $CdCl_2 \cdot L^{-1}$ and the horizontal axis exposure duration in hours. Vertical bars above histograms represent standard errors. Error bars are not apparent when they are less than the width of the symbol with which they are associated.

and the interaction between $CdCl_2$ concentration and SL were significant at all tested exposure periods with the exceptions of no SL effects at 24, 72, 648, 672 h ($p < 0.05$) (Table 2.12). Significant chi-square values for the effect of SL on mortality ranged from 6.59 ($p = 0.0103$) at 624 h to 108.12 ($p < 0.0001$) at 48 h. The interaction between concentration and SL was not significant for 24 h, 72 h, or 600-672 h. Significant chi-square values ranged from 8.68 ($p = 0.0032$) at h 96 h to 104.30 ($p < 0.0001$) at 48 h (Table 2.12).

For all three SL quantile groups, estimated LC_{50} values initially increased somewhat from 48 to 72-120 h hours of exposure depending on quantile group (Table 2.11, Figs. 12.15 B, C and D). Thereafter, LC_{50} values declined with further increase in exposure duration reaching minimum values at the maximum exposure of 672 h (Table 2.11, Fig. 2.15 B, C and D). Logistic regression analysis (n = 2081) for the 25th SL quantile (SL = 5.6 mm) revealed a significant relationship between mortality and $CdCl_2$ concentration at each tested exposure duration (24-672 h) across which chi-square and p values ranged from chi-square = 21.30 ($p < 0.0001$) at an exposure of 24 h to chi-square = 317.30 ($p < 0.0001$) at 216 h (Table 2.10). LC_{50} values based on this analysis decreased from 1669.66 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±29.94) at an exposure of 48 h through 63.54 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±15.49) at 672 h (Table 2.11, Fig. 2.15 B). There was also a significant relationship between mortality and $CdCl_2$ concentration for the median 50th SL quantile (median SL = 6.7 mm) at each tested exposure duration (24-672 hours) across which chi-square and p-values ranged from chi-square = 17.48 ($p < 0.0001$) at an

Table 2.12 Effect of shell length (SL) and the interaction between concentration and SLon 50 % sample chronic mortality (LC_{50}) response chi-square and probability (p) values estimated by maximum likelihood probit regression analysis determinations for samples of *Physa acuta* exposed to media with concentrations of cadmium chloride ranging from 0 - 2000 µg $CdCl_2 \cdot L^{-1}$ over a total exposure period of 672 h. The probability of of an individual dying in a given interval: $\Gamma^{1}(p) = b_0 + b_1(Lconcentration+1)+b_2 (SL-SL^0)+b_3[(Lconcentration+1)*(SL-SL^0)]$with shell length as a covariate allowed determination of SL and interaction chi-square values for individuals in the 25th (SL = 5.6 mm), 50th (SL = 6.7 mm)and the 75th (SL=7.9 mm) SL quantiles. Probability values are consistent across all SL calculations.

Exposure Duration (h)	**SL Effect on LC_{50}**	**P**	**Concentration-SL Effect on LC_{50}**	**P**
24	1.86	0.02491	1.75	0.2783
48	108.12	<0.0001*	104.30	<0.0001*
72	0.29	0.9018	0.09	0.05940
96	6.10	0.0130*	3.45	0.0634
120	13.32	0.0003*	19.51	0.0055*
144	23.82	<0.0001*	15.79	<0.0001*
168	24.41	<0.0001*	15.67	<0.0001*
192	34.92	<0.0001*	22.21	<0.0001*
216	52.59	<0.0001*	35.82	<0.0001*
240	64.84	<0.0001*	44.52	<0.0001*
264	74.34	<0.0001*	51.31	<0.0001*
288	93.43	<0.0001*	65.79	<0.0001*
312	91.15	<0.0001*	59.10	<0.0001*
336	73.43	<0.0001*	40.52	<0.0001*
360	81.14	<0.0001*	45.47	<0.0001*
384	94.99	<0.0001*	55.52	<0.0001*
408	68.34	<0.0001*	33.47	<0.0001*
432	70.03	<0.0001*	36.32	<0.0001*
456	51.88	<0.0001*	22.88	<0.0001*
480	53.03	<0.0001*	23.68	<0.0001*
504	46.90	<0.0001*	20.44	<0.0001*
528	55.57	<0.0001*	29.06	<0.0001*
552	38.44	<0.0001*	17.07	<0.0001*
576	35.23	<0.0001*	16.61	<0.0001*
600	17.91	<0.0001*	6.55	0.0105*
624	11.77	0.0006*	3.38	0.0659
648	6.88	0.0087*	1.06	0.3025
672	4.13	0.0421*	1.59	0.2073

*indicates a significant difference at p<0.05

exposure of 24 h to chi-square = 493.47 ($p < 0.0001$) at an exposure of 312 h (Table 2.10). Based on this analysis, LC_{50} values decreased from 1815.37 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±47.68) at an exposure time of 72 h through 93.52 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±10.88) at 672 h (Table 2.11, Fig. 2.15 C). A significant relationship between mortality and $CdCl_2$ concentration at each tested exposure duration (24-672 hours) was recorded in the 75th SL quantile (median SL = 7.9 mm) across which chi-square and p values ranged from chi-square = 10.61 ($p < 0.0011$) at an exposure of 24 h to chi-square = 434.29 ($p < 0.0001$) at an exposure of 384 h (Table 2.12). LC_{50} values decreased from 1850.40 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±69.31) at an exposure time of 144 h through 131.32 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±9.74) at 672 h (Table 2.11, Fig. 2.15 D).

Inclusion of SL in the logistic analysis revealed that individual size had significant impact on mortality and resulting LC_{50} estimates. The smallest individuals of *P. acuta* in the 25th SL quantile (SL = 5.6 mm) were the most sensitive to $CdCl_2$ exposure associated with recording of the lowest LC_{50} values for this group across all tested exposure periods (24-672 h) (Fig 2.16). In contrast the largest individuals (SL = 7.9 mm) in the 75th SL quantile where the least sensitive to $CdCl_2$ exposure as reflected by this group having the greatest LC_{50} values across the entire 24-672 h range of exposure periods (Fig 2.16). Estimates of LC_{50} for the 50th SL quantile (median SL = 6.7 mm) were intermediate between those of the 25th and 75th SL quantile groups suggesting that increasing size confers increasing resistance to the lethal effects of $CdCl_2$ exposure in this species. As was recorded using probit analysis (Fig.2.7), logistic regression analysis without inclusion of SL as a size covariate led to estimates of LC_{50}

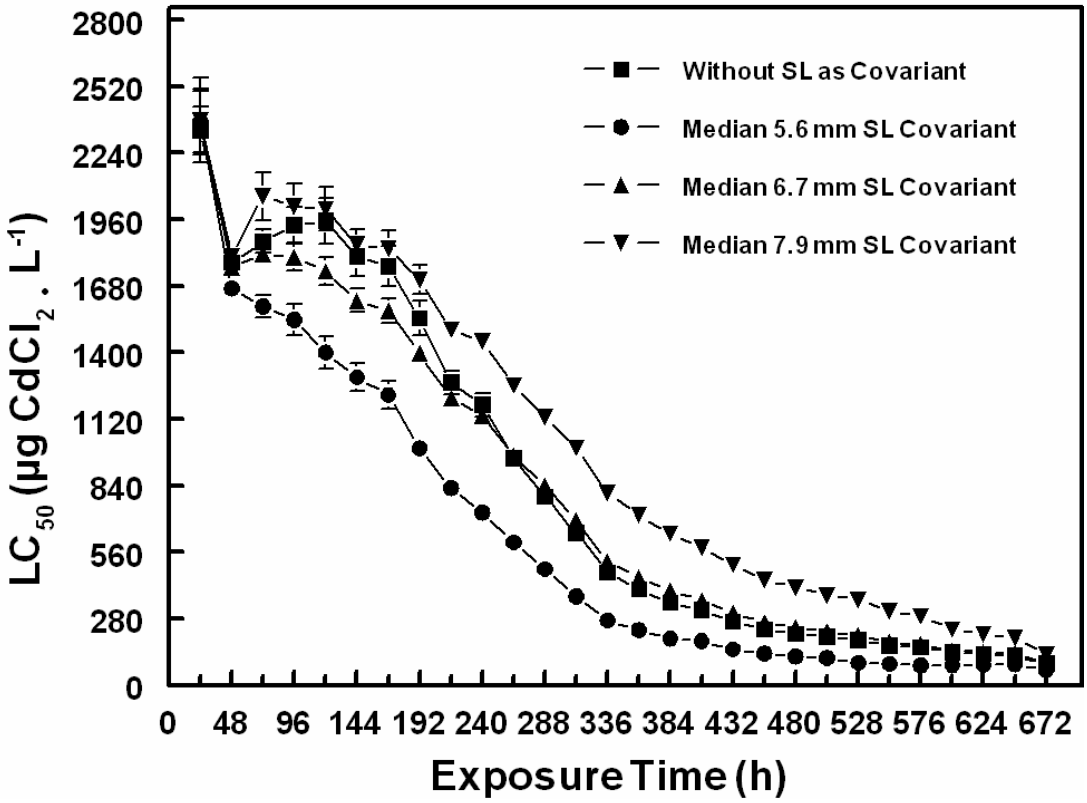


Fig. 2.16. Comparison of chronic LC_{50} values computed by maximum likelihood logistic regression analysis for samples of *Physa acuta* at intervals of 24 h over a 672 h (28 d) period of exposure to varying concentrations of $CdCl_2$. The horizontal axis is exposure time in hours and the vertical axis, the estimated lethal concentration for 50 % sample mortality (LC_{50}) in µg $CdCl_2 \cdot L^{-1}$. Solid squares are LC_{50} values estimated without including shell length (SL) as a covariate. Solid circles, triangles and inverted triangles represent median LC_{50} values resulting from analysis with inclusion of SL as a covariate for individuals in the 25th (SL = 5.6 mm), 50th (median SL = 6.7 mm) and 75th SL quantile (SL = 7.9), respectively. Vertical bars about LC_{50} values represent standard errors. Error bars are not apparent when they are less than the width of the symbol with which they are associated.

values being relatively high, approaching those of the 75th SL quantile in the initial 24-192 h of exposure, thereafter declining between 192-288 h of exposure to a point where they remained essentially similar to those of the median 50th SL quantile group over the remaining range of exposure periods from 288 to 672 h (Fig. 2.16 and Fig. 2.17).

Chronic Median Survival Time (LT_{50})

All concentrations less than 400 μg $CdCl_2 \cdot L^{-1}$ were excluded from the chronic discrete logistic failure time model (Hicks *et al*., 2000) analysis of estimated median lethal time for 50 % sample mortality versus concentration because the 50 % sample mortality (LT_{50}) required for this analysis was not achieved in these samples within the 672 h exposure time. This analysis revealed a significant relationship between LT_{50} and $CdCl_2$ concentration in two of the three quantile groups including the 25th SL guantile (SL = 5.6 mm) and median 50th SL quantile (median SL = 6.7 mm), across which chi-square and p values ranged from chi-square = 7.23 (p = 0.01) to chi-square = 23.38 (p <0.0001). In contrast, significant correlation did not occur between LT_{50} and $CdCl_2$ concentration in the 75th SL quantile (SL = 7.9 mm) (chi-square = 1.4, p =0.24). However, there was a significant interaction between concentration and SL on survivorship for all three quantile size classes (chi-square = 6.46, p = 0.01).

For an individual from the 25th SL quantile with a SL of 5.6 mm, DLFTM analysis resulted in estimated LT_{50} values that decreased from 313.75 h (s.e. = ± 5.331) at a concentration of 400 μg $CdCl_2 \cdot L^{-1}$ to 99.80 h (s.e. = ± 2.977) at a concentration of 2000 resulted in estimated LT_{50} values that decreased from 313.75 h (s.e. = ± 5.331) at

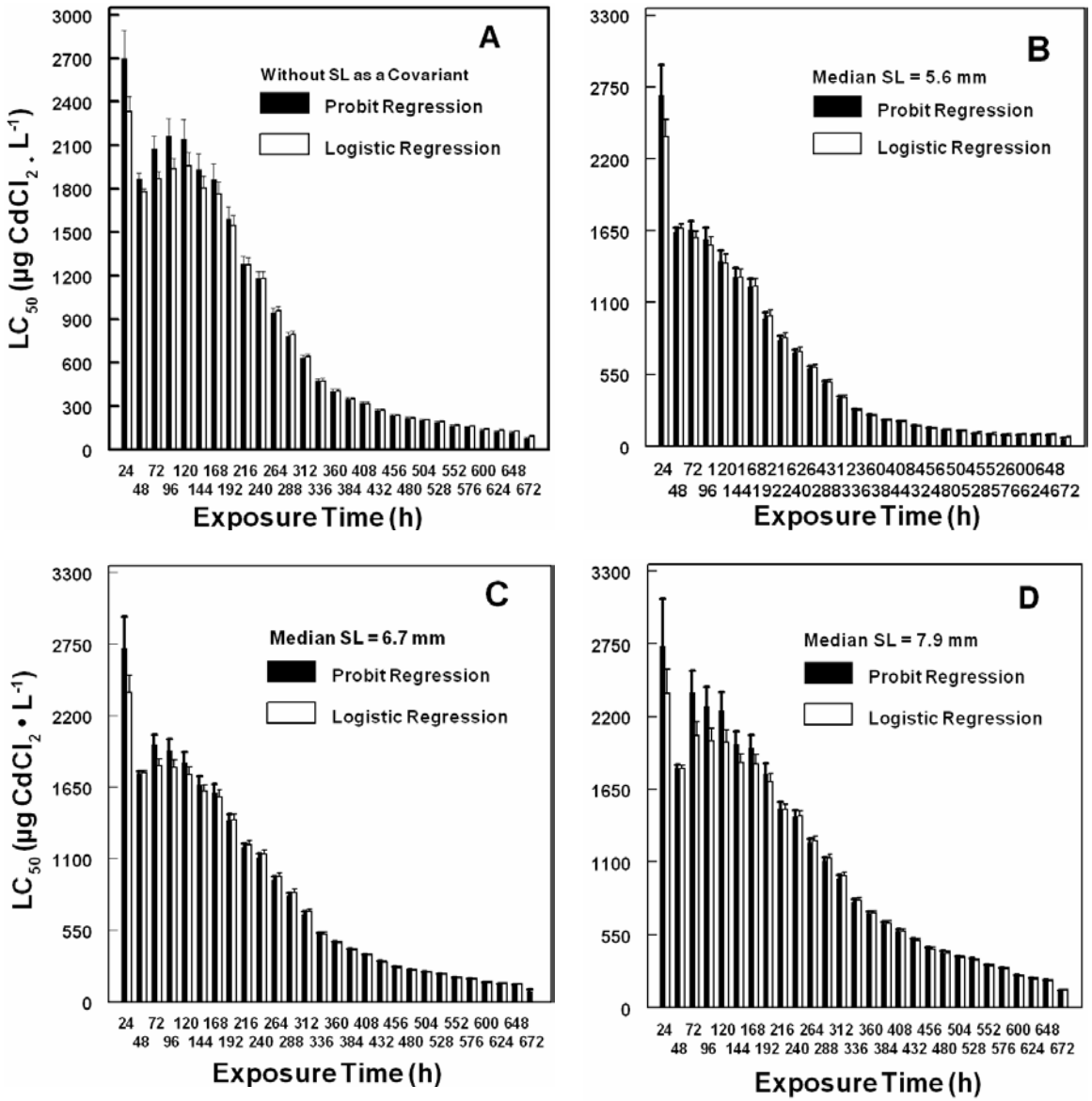


Fig.2.17 Comparison of chronic LC_{50} values computed by maximum likelihood probit analysis (solid histograms) to those computed by maximum likelihood logistic regression (open histograms) for samples of *Physa acuta* for individuals without shell length as a covariate (A) and with inclusion of SL as a covariate for individuals in the 25th (SL = 5.6 mm)(B), 50th (median SL = 6.7 mm) (C) and 75th SL quantile (SL = 7.9) (D), respectively, at intervals of 24 h over a 672 h (28 d) period of exposure to varying concentrations of $CdCl_2$.
The horizontal axis is exposure time in hours and the vertical axis, the estimated lethal concentration for the LC_{50} in µg $CdCl_2 \cdot L^{-1}$.
Vertical bars above histograms represent standard errors.
Error bars are not apparent when they are less than the width of the symbol with which they are associated.

a concentration of 400 μg $CdCl_2 \cdot L^{-1}$ to 99.80 h (s.e. = ± 2.977) at a concentration of 2000 μg $CdCl_2 \cdot L^{-1}$ (Table 2.13, Fig. 2.18 A). All tested concentration LT_{50} values were significantly different from each other at $\alpha = 0.05$ based on the Scheffé pair-wise comparison (Fig. 2.18 B). For an individual from the 50th SL quantile with a median SL of 6.7 mm, DLFTM analysis yielded LT_{50} values that decreased from 395.31 h (s.e. = ±4.978) at a concentration of 400 μg $CdCl_2 \cdot L^{-1}$ to 111.24 h (s.e. = ± 3.688) at a concentration of 2000 μg $CdCl_2 \cdot L^{-1}$ (Table 2.13, Fig. 2.18 B). For this median quantile, Scheffé pair-wise comparisons of LT_{50} values at different $CdCl_2$ concentrations were all significantly different from each other at $\alpha = 0.05$ (Fig. 2.18 B). For an individual from the 75th SL quantile with a SL of 7.9 mm, DLFTM analysis yielded estimated LT_{50} values that decreased from 471.39 (s.e. = ± 7.068) at a concentration of 400 μg $CdCl_2 \cdot L^{-1}$ to 131.05 h (s.e. = ± 6.872) at a concentration of 2000 μg $CdCl_2 \cdot L^{-1}$ (Table 2.13, Fig. 2.18 C). Scheffé pair-wise comparison testing indicated that the LT_{50} values for this 75th SL quantile were significantly different from each other at $\alpha = 0.05$ across all tested dosages from 400-2000 μg $CdCl_2 \cdot L^{-1}$ (Fig. 2.18 D).

A size effect was demonstrated through estimated LT_{50} values, the lowest values were recorded for the lowest SL quantile (SL = 5.6 mm) across all tested $CdCl_2$ concentrations (i.e., 400-2000 μg $CdCl_2 \cdot L^{-1}$) while highest LT_{50} values were recorded in the largest sample quantile (median SL = 7.9 mm). The LT_{50} values of the median 50th SL quantile were intermediate between those of the 25th and 75th SL quantiles (Table 2.13). Estimated LT_{50} values for all three quantile groups were most different at the

Table 2.13 Chronic 50 % sample mortality values (LT_{50}) estimated by discrete logistic failure time model regression analysis for samples of *Physa acuta* (n=150) exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 - 2000 µg $CdCl_2 \cdot L^{-1}$ over a total exposure period of 672 h (28 days). LC_{50} values are provided for analysis of the entire sample without shell length (SL) as a covariate, and for analysis with SL as a covariate for individuals in the l25th of the SL quantile (SL = 5.6 mm), 50th SL quantile (median SL = 6.7 mm) and 75th SL quantile (SL = 7.9 mm).

25th SL Quantile (SL =5.6 mm)		
Concentration (µg ·$CdCl_2$ L^{-1})	**LT_{50}**	**s.e.**
400	313.75	±5.331
450	300.48	±4.875
600	269.40	±4.350
800	234.32	±5.187
1000	194.47	±7.627
1200	161.47	±7.056
1400	133.82	±5.218
1600	117.11	±4.209
2000	99.80	±2.977
Median 50th SL Quantile (Median SL =6.7 mm)		
Concentration (µg ·$CdCl_2$ L^{-1})	**LT_{50}**	**s.e.**
400	395.31	±4.978
450	379.10	±5.582
600	327.79	±5.607
800	284.74	±3.790
1000	252.51	±3.558
1200	219.29	±4.848
1400	181.87	±4.780
1600	149.22	±5.931
2000	111.24	±3.688
75th SL Quantile (SL = 7.9 mm)		
Concentration (µg ·$CdCl_2$ L^{-1})	**LT_{50}**	**s.e.**
400	471.39	±7.068
450	455.35	±6.617
600	412.82	±6.328
800	354.63	±8.240
1000	302.21	±4.876
1200	267.67	±3.797
1400	235.74	±4.767
1600	196.81	±8.252
2000	131.05	±6.872

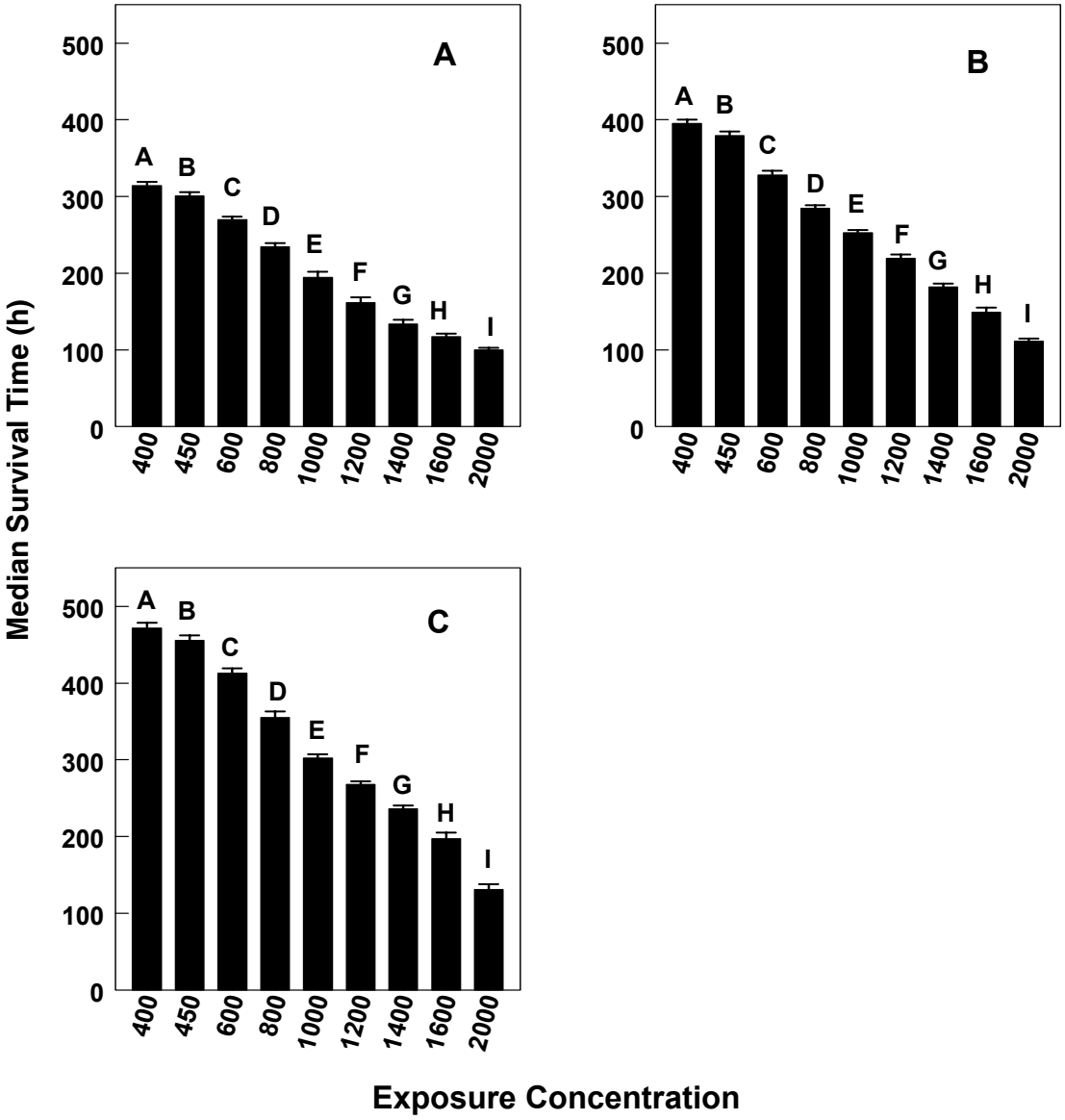


Fig. 2.18 Comparison of chronic LT_{50} values computed by discrete logistic failure time model (DLFTM) analysis for samples of *Physa acuta* with inclusion of SL as a covariate for individuals in the 25th (SL = 5.6 mm)(B), 50th(median SL = 6.7 mm) (C) and 75th SL quantile (SL = 7.9) (D), respectively, exposed to varying concentrations of $CdCl_2$ ranging from 400-2000 µg $CdCl_2 \cdot L^{-1}$ for a maximum period of 28 d. The horizontal axis is exposure concentration in µg $CdCl_2 \cdot L^{-1}$ and the vertical axis, the estimated median time in hours for LT_{50}. Vertical bars above histograms represent standard errors. Different letters above histograms indicate that LT_{50} values are significantly different using ($p < 0.05$).

lowest tested concentration of 400 μg $CdCl_2 \cdot L^{-1}$ and least different from each other at the highest tested concentration of 2000 μg $CdCl_2 \cdot L^{-1}$ (Table 2.13).

Percent Sample Crawl-out Response

Sample crawl-out responses were determined for individuals of *P. acuta* subjected to acute 1-12 h $CdCl_2$ exposures at concentrations ranging from 0-2000 μg $CdCl_2 \cdot L^{-1}$. The time at which 50 % sample crawl-out was achieved was negatively correlated with $CdCl_2$ concentration. At 12 h, samples exposed to less than 300 μg $CdCl_2 \cdot L^{-1}$ did not achieve 50 % sample crawl-out. In addition, 100 % sample crawl-out response was not achieved after 12 h exposures at any tested concentration (300-2000 μg $CdCl_2 \cdot L^{-1}$) (Table 2.14). At a concentration of 300 μg $CdCl_2 \cdot L^{-1}$, sample crawl-out reached 50 % at 6 h, while at 350 and 400 μg $CdCl_2 \cdot L^{-1}$ 50 % sample crawl-out was exceeded at 5 h. Fifty-percent crawl-out response was recorded at 9 h for 450 μg $CdCl_2 \cdot L^{-1}$, 6 h for 600 and 800 μg $CdCl_2 \cdot L^{-1}$, and at 8 h for 1000 μg $CdCl_2 \cdot L^{-1}$. At a concentration of 1200 μg $CdCl_2 \cdot L^{-1}$ a 50 % crawl-out response was achieved within 3 h. Exposure to 1400-2000 μg $CdCl_2 \cdot L^{-1}$ resulted in 50 % sample crawl-out response within 2 h (Table 2.14, Fig. 2.19).

Crawl-out Probability

Logistic regression analysis was utilized to calculate the probability (p) of individuals of *P. acuta* crawling above the water's surface (i.e., crawl-out response) in each tested concentration of $CdCl_2$ (0-2000 μg $CdCl_2 \cdot L^{-1}$), utilizing the generalized

Table 2.14 Percent crawl-out of specimens of *Physa acuta* on exposure to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 - 2000 µg $CdCl_2 \cdot L^{-1}$ determined at 1 h intervals over a total exposure period of 12 h.

Exposure Concentration (µg $CdCl_2L^{-1}$)	N	1 h		2 h		3 h		4 h		5 h		6 h	
		No. Crawl-out	Percent Crawl-out	No. Crawl-out	Percent Crawl-out	No. Crawl-out	Percent Crawl-Out	No. Crawl-out	Percent Crawl-out	No. Crawl-out	Percent Crawl-out	No. Crawl-out	Percent Crawl-out
0	90	4	4.44	7	7.78	8	8.89	14	15.56	17	18.89	20	22.22
10	90	4	4.44	5	5.56	7	7.78	13	14.44	15	16.67	16	17.78
50	90	11	12.22	14	15.56	17	18.89	20	22.22	23	25.56	25	27.78
100	90	6	6.67	8	8.89	9	10.00	12	13.33	15	16.67	19	21.11
150	90	5	5.56	10	11.11	12	13.33	13	14.44	15	16.67	16	17.78
200	90	3	3.33	5	5.56	6	6.67	8	8.89	9	10.00	11	12.22
250	90	4	4.44	6	6.67	10	11.11	14	15.56	19	21.11	26	28.89
300	90	23	25.56	32	35.56	37	41.11	41	45.56	43	47.78	45	50.00
350	90	19	21.11	31	34.44	37	41.11	42	46.67	48	53.33	52	57.78
400	90	18	20.00	25	27.78	35	38.89	40	44.44	51	56.67	61	67.78
450	90	14	15.56	17	18.89	21	23.33	28	31.11	33	36.67	37	41.11
600	90	25	27.78	30	33.33	34	37.78	38	42.22	44	48.89	49	54.44
800	90	19	21.11	28	31.11	36	40.00	41	45.56	43	47.78	45	50.00
1000	90	12	13.33	22	24.44	31	34.44	34	37.78	35	38.89	37	41.11
1200	90	32	35.56	41	45.56	50	55.56	54	60.00	56	62.22	57	63.33
1400	90	37	41.11	48	53.33	56	62.22	60	66.67	60	66.67	61	67.78
1600	90	32	35.56	52	57.78	56	62.22	64	71.11	65	72.22	70	77.78
2000	90	34	37.78	47	52.22	55	61.11	60	66.67	60	66.67	60	66.67

Table 2.14 continued.

Exposure Concentration (µg $CdCl_2 \cdot L^{-1}$)	N	7 h		8 h		9 h		10 h		11 h		12 h	
		No. Crawl-out	Percent Crawl-out	No. Crawl-out	Percent Crawl-out	No. Crawl-out	Percent Crawl-out	No. Crawl-out	Percent Crawl-out	No. Crawl-out	Percent Crawl-out	No. Crawl-out	Percent Crawl-out
0	90	21	23.33	22	24.44	25	27.78	28	31.11	28	31.11	29	32.22
10	90	17	18.89	19	21.11	20	22.22	24	26.67	24	26.67	25	27.78
50	90	31	34.44	34	37.78	35	38.89	39	43.33	39	43.33	39	43.33
100	90	20	22.22	23	25.56	26	28.89	27	30.00	28	31.11	30	33.33
150	90	16	17.78	16	17.78	17	18.89	20	22.22	21	23.33	21	23.33
200	90	12	13.33	17	18.89	21	23.33	24	26.67	27	30.00	28	31.11
250	90	32	35.56	35	38.89	35	38.89	36	40.00	39	43.33	39	43.33
300	90	48	53.33	54	60.00	57	63.33	60	66.67	65	72.22	66	73.33
350	90	56	62.22	57	63.33	59	65.56	61	67.78	63	70.00	63	70.00
400	90	64	71.11	65	72.22	66	73.33	66	73.33	67	74.44	69	76.67
450	90	41	45.56	44	48.89	46	51.11	48	53.33	50	55.56	54	60.00
600	90	50	55.56	50	55.56	50	55.56	50	55.56	51	56.67	56	62.23
800	90	47	52.22	48	53.33	49	54.44	50	55.56	50	55.56	51	56.67
1000	90	41	45.56	45	50.00	47	52.22	47	52.22	47	52.22	47	52.22
1200	90	59	65.56	59	65.56	60	66.67	60	66.67	60	66.67	60	56.67
1400	90	62	68.89	62	68.89	64	71.11	64	71.11	64	71.11	64	71.11
1600	90	70	77.78	70	77.78	70	77.78	70	77.78	70	77.78	70	77.78
2000	90	60	66.67	60	66.67	60	66.67	60	66.67	60	66.67	60	66.67

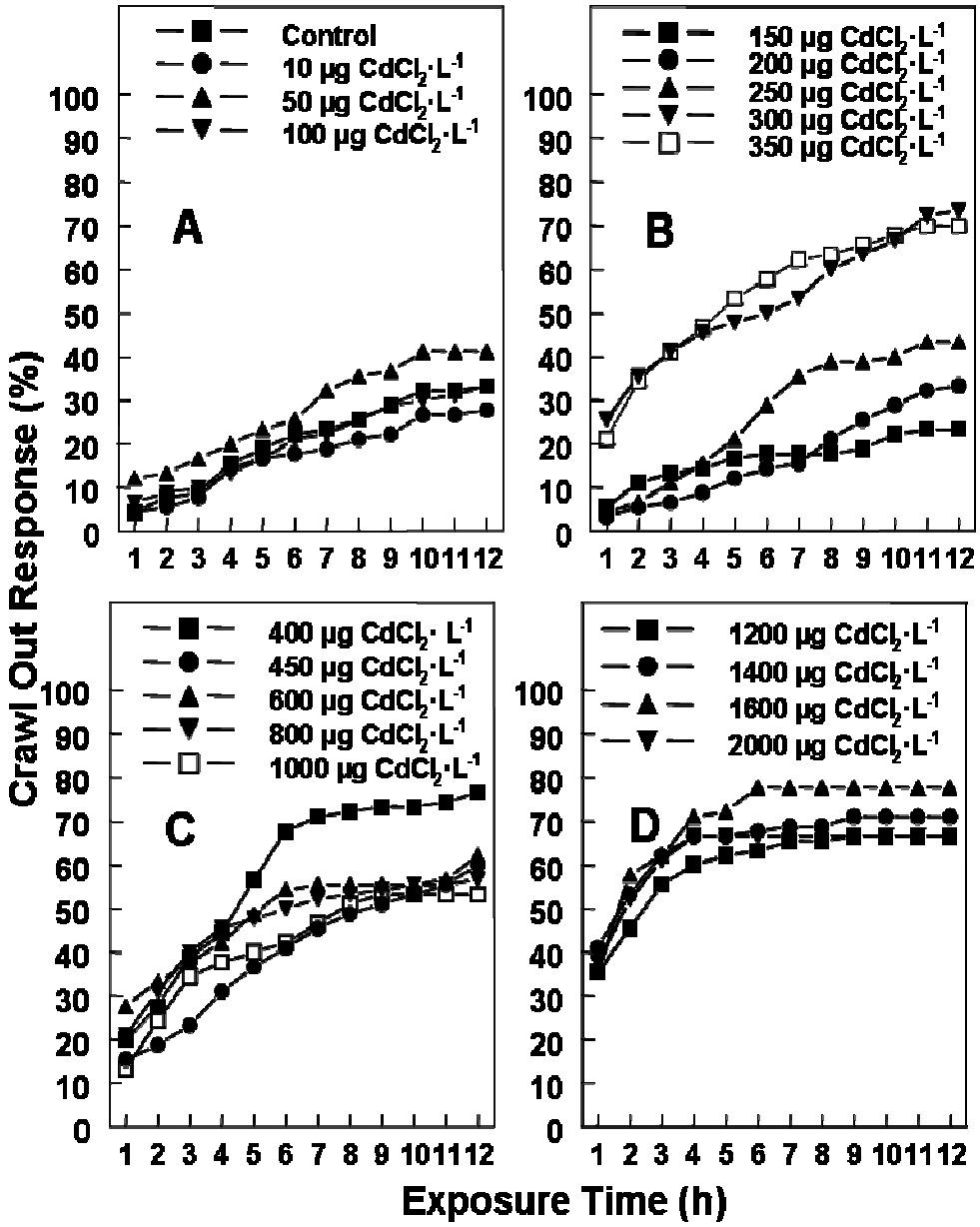


Fig. 2.19 Cumulative percent sample crawl out response (i.e., crawling out of the medium) for samples (n = 90) of *Physa acuta* exposed to media for 12 h with different concentrations of $CdCl_2$ ranging from (**A**) 0 (solid squares), 10 (solid circles), 50 (solid triangles) 100 (solid inverted triangles), (**B**) 150 (solid squares), 200 (solid circles), 250 (solid triangles) 300 (solid inverted triangles), 350 (open squares), (**C**) 400 (solid squares), 450 (solid circles), 600 (solid triangles) 800 (solid inverted triangles), 1000 (open squares), (**D**) 1200 (solid squares), 1400 (solid circles), 1600 (solid triangles) and 2000 µg $CdCl_2 \cdot L^{-1}$ (solid inverted triangles).

linear regression model. To determine the the probability of an individual crawling-out in a given interval without SL as a covariate the following generalized linear regression model with either the probit or logistic distribution is utilized: $\Gamma^{1}(p) = b_0 + b_1(Lconcentration+1)$. To determine the the probability of an individual crawling-out in a given interval with SL as a covariate the following generalized linear regression model with either the probit or logistic distribution is utilized: $\Gamma^{1}(p) = b_0 + b_1(Lconcentration+1)+b_2\ (SL-SL^{0})+b_3[(Lconcentration+1)*(SL-SL^{0})]$.

Probability of crawl-out was estimated both without shell length (SL) as a covariate and with shell length as a covariate. Including SL as a covariate allowed probability of crawl-out to be estimated for individuals falling within the 25th SL quantile, (SL= 6.7 mm), 50th SL quantile (median SL = 7.9 mm) and 75th SL quantile (SL = 9.1 mm). Scheffé multiple comparison tests revealed no significant differences in crawl-out responses between the three replicate samples tested at each $CdCl_2$ concentration ($p < 0.5$) allowing results from the replicates to be combined into a single data set (n = 1260) for further statistical analyses.

When assessed for the entire sample without SL as a covariate, logistic regression analysis revealed a significant effect of $CdCl_2$ concentration on crawl-out response (chi-square = 76.3, $p <0.0001$). The probability of crawl-out after 12 h exposure varied from 0.42 (s.e. $= \pm 0.0178$) at a concentration of 0 μg $CdCl_2 \cdot L^{-1}$ to 0.78 of the sample (s.e. $= \pm 0.0247$) at a concentration of 2000 μg $CdCl_2 \cdot L^{-1}$ (Table 2.15, Fig.2.20 A). Probability values at all tested concentrations proved to be significantly different from each other ($p <0.05$) based on Scheffé multiple comparison testing.

Table 2.15 Maximum likelihood logistic regression analysis determinations of probability of crawl-out response values for samples of *Physa acuta* exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 - 2000 µg $CdCl_2 \cdot L^{-1}$ over a total exposure period of 12 h. Probability values are provided for the entire sample without shell length (SL) as a covariate, for individuals in the 25th SL quanilte (SL = 6.7 mm), for individuals in the 50th SL quanitle (median SL = 7.9 mm) and for individuals in the 75th SL range (SL = 9.1 mm) based on analysis with SL as a covariate.

Exposure Concentration (µg $CdCl_2 \cdot L^{-1}$)	Probability SL not a Covariate	s.e.	Probability SL 6.7 mm	s.e.	Probability SL 7.9 mm	s.e.	Probability SL 9.1 mm	s.e.
0	0.42	±0.0178	0.46	±0.0224	0.43	±0.0183	0.39	±0.0199
10	0.43	±0.0177	0.46	±0.0221	0.43	±0.0181	0.39	±0.0196
50	0.43	±0.0171	0.47	±0.0215	0.44	±0.0176	0.40	±0.0190
100	0.44	±0.0164	0.48	±0.0207	0.45	±0.0168	0.42	±0.0185
150	0.45	±0.0158	0.49	±0.0199	0.46	±0.0161	0.43	±0.0176
200	0.46	±0.0152	0.50	±0.0192	0.47	±0.0154	0.44	±0.0167
250	0.47	±0.0146	0.50	±0.0181	0.48	±0.0148	0.45	±0.0160
300	0.48	±0.0141	0.51	±0.0175	0.49	±0.0142	0.47	±0.0157
350	0.49	±0.0136	0.52	±0.0169	0.50	±0.0137	0.48	±0.0152
400	0.50	±0.0132	0.53	±0.0164	0.51	±0.0134	0.49	±0.0149
450	0.51	±0.0130	0.54	±0.0159	0.53	±0.0133	0.50	±0.0147
600	0.54	±0.0128	0.59	±0.0148	0.56	±0.0130	0.54	±0.0155
800	0.58	±0.0137	0.60	±0.0153	0.60	±0.0140	0.59	±0.0182
1000	0.62	±0.0156	0.63	±0.0167	0.65	±0.0161	0.64	±0.0218
1200	0.66	±0.0179	0.66	±0.0188	0.69	±0.0183	0.68	±0.0236
1400	0.69	±0.0202	0.69	±0.0188	0.72	±0.0200	0.72	±0.0276
1600	0.73	±0.0222	0.72	±0.0235	0.76	±0.0217	0.76	±0.0295
2000	0.78	±0.0247	0.78	±0.0272	0.82	±0.0231	0.83	±0.0307

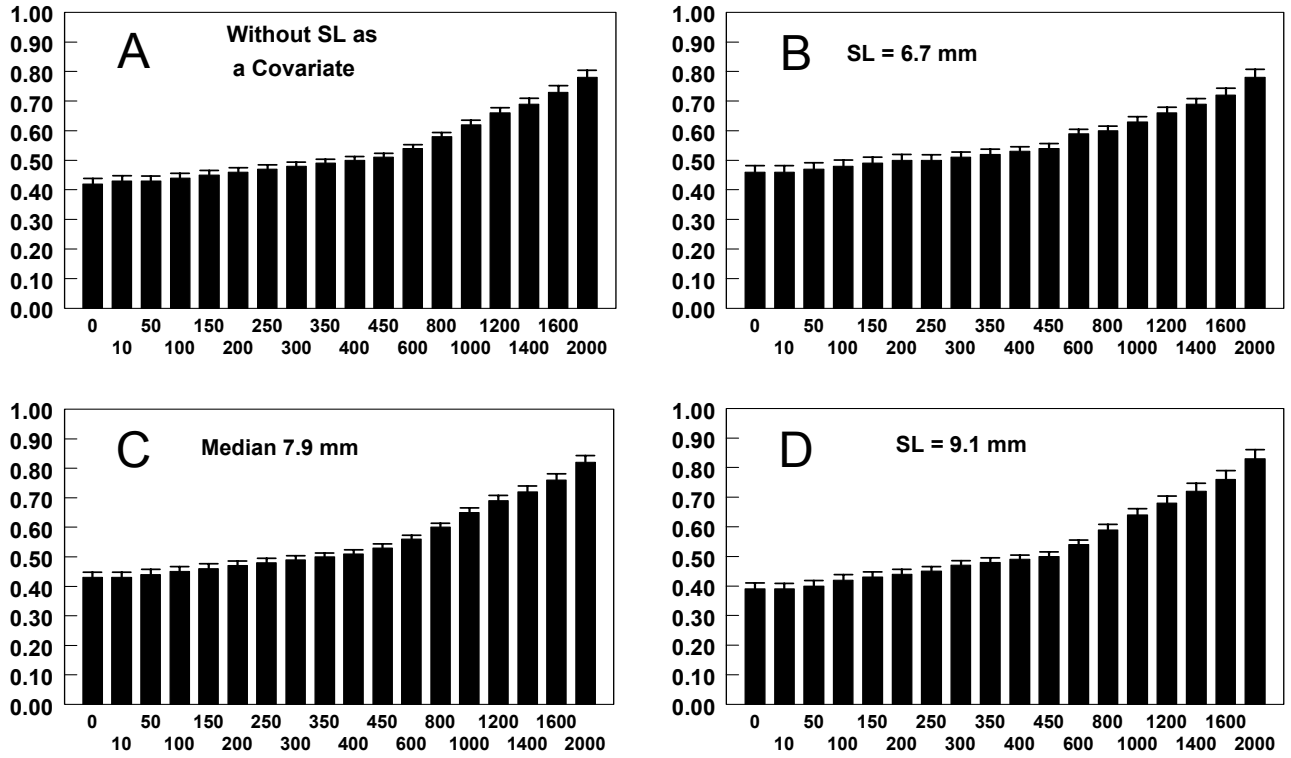


Fig.2.20 Probability of sample crawl-out response (i.e., crawling out of the medium) computed by maximum likelihood logistic regression analysis for samples (n = 90) of *Physa acuta* without shell length (SL) as a covariate and B-D) crawl -out probabilities determined by analysis with inclusion of SL as a covariate for B) individuals in the 25th (SL = 6.7 mm), C) 50th (median SL = 7.9 mm) and D) and 75th (SL=9.1 mm) SL quantile exposed to media for 12 h with different concentrations of cadmium chloride ($CdCl_2$) ranging from 0-2000 µg $CdCl_2 \cdot L^{-1}$. The horizontal axis is exposure time in hours and the vertical axis, the probability of crawl-out response. Vertical bars above histograms represent standard errors.

Logistic regression analysis revealed a significant relationship between probability of crawl-out and $CdCl_2$ concentration in all three quantile groups. For the 25th SL quantile (SL = 6.7 mm) chi-square = 41.6 ($p < 0.0001$). For the 50th SL quantile (median SL = 7.9 mm) chi-square = 74.8 ($p < 0.0001$) and for the 75th SL quantile (SL = 9.1 mm) chi-square = 61.4 ($p < 0.0001$). Individual SL was also significantly correlated with crawl-out probability (chi-square = 9.2, $p = 0.0024$) and a significant interaction occurred between $CdCl_2$ concentration and SL (chi-square = 7.4, $p = 0.007$).

In all three SL quantile groups, probability of crawl-out response increased exponentially with $CdCl_2$ concentration. The probability of crawl-out for the 25th SL quantile (SL = 6.7 mm) increased from 0.46 (s.e. = ± 0.0224) at a concentration of 0 μg $CdCl_2 \cdot L^{-1}$ to 0.78 (s.e. = ± 0.0272) at a concentration of 2000 μg $CdCl_2 \cdot L^{-1}$ (Table 2.15, Fig. 2.20 B). For the 50th SL quantile (median SL = 7.9 mm) crawl-out probability ranged from 0.43 (s.e. = ± 0.0183) at 0 μg $CdCl_2 \cdot L^{-1}$, to 0.82 (s.e. = ± 0.0231) at a concentration of 2000 $CdCl_2 \cdot L^{-1}$ (Table 2.15, Fig.2.20 C). For the 75th SL quantile (SL = 9.1 mm), it ranged from 0.39 (s.e. = ± 0.0199) at 0 μg $CdCl_2 \cdot L^{-1}$ to 0.83 (s.e. = ± 0.0307) at a concentration of 2000 μg $CdCl_2 \cdot L^{-1}$ (Table 2.15, Fig.2.20 D). The significant interaction between $CdCl_2$ concentration and SL appeared to result from the tendency of the 75th sample SL quantile to have lower probabilities of crawl-out than individuals with small SL at lower $CdCl_2$ concentrations (< 250 μg $CdCl_2 \cdot L^{-1}$), but higher probabilities of crawl-out than individuals with smaller SL at the highest $CdCl_2$ concentrations (> 1200 μg $CdCl_2 \cdot L^{-1}$) (Table 2.15).

The 50 % Sample Crawl-out Response Effect Concentration (CO_{50})

Maximum likelihood probit and logistic regression analysis were utilized to determine the 50 % sample crawl-out effect concentration of $CdCl_2$ (CO_{50}), defined as the $CdCl_2$ concentration at which 50 % sample crawl-out response was observed. Analyses were completed with and without shell length as a covariate. Probit regression analysis using the maximum likelihood method was utilized to determine CO_{50} values for the entire sample at each hour of exposure to $CdCl_2$ concentrations ranging from 0-2000 μg $CdCl_2 \cdot L^{-1}$ at hourly intervals over a 12 h exposure period without including SL as a covariate. This analysis indicated that there was a significant relationship between crawl-out response and $CdCl_2$ concentration at each tested exposure duration from 1-5 h across which chi-square and p values ranged from chi-square = 73.43 ($p < 0.0001$) at an exposure period of 1 h to chi-square = 141.64 ($p < 0.0001$) at an exposure of 5 h (Table 2.16). The effect of $CdCl_2$ concentration on CO_{50} was not significant ($p < 0.05$) at exposures ranging from 6-12 h suggesting that maximum crawl-out responses had been attained across all tested concentrations over this time period (Table 2.16). With the exclusion of the CO_{50} at the 1 h exposure period which, at 3435.81 μg $CdCl_2 \cdot L^{-1}$ fell outside the highest concentration tested (i.e., 2000 μg $CdCl_2 \cdot L^{-1}$), CO_{50} values decreased with increasing duration of exposure from 1613.67 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±186.21) at 2 h exposure to 295.43 μg $CdCl_2 \cdot L^{-1}$ (s.e. =±45.86) at 12 h exposure (Table 2.17, Fig. 2.21 A).

Table 2.16 Sub-lethal 50 % sample crawl-out (CO_{50}) response chi-square and probability (p) values estimated by maximum likelihood probit regression analysis determinations for samples of *Physa acuta* exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 - 2000 µg $CdCl_2 \cdot L^{-1}$ over a total exposure period of 12 h. Chi-square values are provided for analysis carried out without shell length (SL) as a covariate to describe the probability of an individual crawling-out in the given interval: $\Gamma^1(p) = b_0 + b_1(Lconcentration+1)$. The probability of of an individual crawling-out in a given interval: $\Gamma^1(p) = b_0 + b_1(Lconcentration+1)+b_2\ (SL-SL^0)+b_3[(Lconcentration+1)*(SL-SL^0)]$ with shell length as a covariate allowed determination of EC_{50} values for individuals in the 25th SL quantile (SL = 6.7 mm), 0th SL quantile (median SL = 8.7 mm) and the 75th SL quantile (SL = 11.1 mm).

Exposure Duration (h)	**CO_{50} Without SL Covariate**	**p**	**CO_{50} SL =6.7 mm**	**P**	**CO_{50} SL =7.9 mm**	**P**	**CO_{50} SL =9.1 mm**	**P**
1	73.43	<0.0001*	45.1	<0.0001*	66.4	<0.0001*	47.4	<0.0001*
2	107.29	0.0001*	61.7	<0.0001*	95.5	<0.0001*	70.7	<0.0001*
3	131.30	0.002*	68.2	<0.0001*	109.0	<0.0001*	82.7	<0.0001*
4	141.64	<0.0001*	70.8	<0.0001*	112.3	<0.0001*	84.5	<0.0001*
5	118.46	<0.0001*	54.8	<0.0001*	91.3	<0.0001*	71.6	<0.0001*
6	104.91	0.07	45.2	<0.0001*	80.5	<0.0001*	66.4	<0.0001*
7	94.36	0.10	46.0	<0.0001*	78.0	<0.0001*	102.3	<0.0001*
8	77.72	0.12	39.7	<0.0001*	66.9	<0.0001*	88.0	<0.0001*
9	71.38	0.12	38.1	<0.0001*	63.5	<0.0001*	83.3	<0.0001*
10	58.77	0.07	30.5	<0.0001*	52.2	<0.0001*	69.9	<0.0001*
11	48.89	0.22	25.8	<0.0001*	43.3	<0.0001*	57.4	<0.0001*
12	42.38	0.47	18.0	<0.0001*	26.4	<0.0001*	32.1	<0.0001*

*indicates a significant difference at $p<0.05$

Table 2.17 Sub-lethal 50 % sample crawl-out responses (CO_{50}) estimated by maximum likelihood probit regression analysis determinations of crawl-out response values for samples of *Physa acuta* exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 - 2000 µg $CdCl_2 \cdot L^{-1}$ over a total exposure period of 12 h. Probability values are provided for the entire sample without shell length (SL) as a covariate, for individuals in the 25th SL quantile (SL = 6.7 mm), for individuals in the 50th SL quantile (median SL = 7.9 mm) and for individuals in the 75th SL quantile (SL = 9.1 mm) based on analysis with SL as a covariate.

Exposure Duration (h)	CO_{50} Without SL Covariate	s.e.	CO_{50} SL 6.7 mm	s.e.	CO_{50} SL 7.9 mm	s.e.	CO_{50} SL 9.1 mm	s.e.
1	3435.81	±720.18	3493.78	±925.10	3520.55	±791.09	3541.71	±979.11
2	1613.67	±186.21	1653.43	±247.95	1732.06	±222.29	1795.98	±286.00
3	1088.32	±89.79	1044.94	±114.72	1167.33	±110.91	1270.13	±151.48
4	853.2	±60.08	759.03	±72.03	910.04	±74.43	1047.60	±109.93
5	730.27	±53.60	600.96	±63.83	773.93	±65.99	936.07	±101.15
6	612.67	±46.35	476.43	±58.84	636.29	±54.92	782.48	±79.13
7	534.8	±42.81	428.52	±58.72	570.59	±50.12	694.53	±56.55
8	479.23	±43.26	382.25	±59.91	516.43	±49.56	636.01	±55.51
9	429.95	±41.96	347.31	±58.70	473.12	±47.61	587.38	±52.85
10	386.51	±43.67	309.51	±62.73	438.56	±50.05	556.85	±55.04
11	340.37	±45.17	271.97	±64.75	387.37	±51.34	495.99	±55.27
12	295.43	±45.86	276.40	±78.47	349.97	±63.07	421.39	±66.57

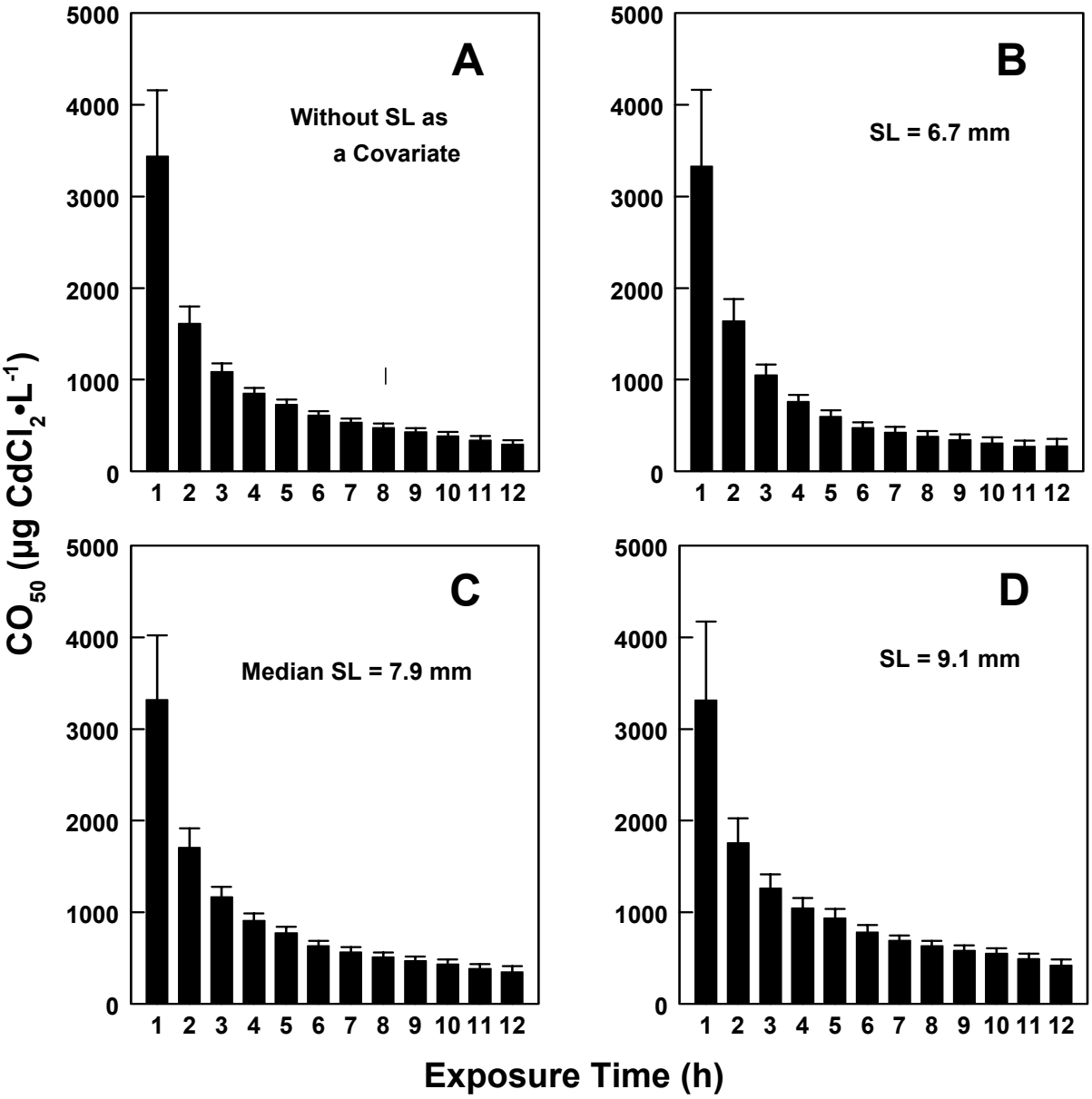


Fig.2.21 Estimated concentrations for 50 % sample crawl-out response (CO_{50}) (i.e., crawling out of the medium) computed by maximum likelihood probit regression analysis for samples (n = 90) of *Physa acuta* A) without inclusion of SL as acovariate and with inclusion of SL for B) individuals in the 25th (SL = 6.7mm), C) 50th (median SL = 7.9 mm) and D) 75th (SL=9.1 mm) SLquantile exposed to media for 12 h with different concentrations of cadmium chloride ($CdCl_2$) ranging from 0-2000 µg $CdCl_2 \cdot L^{-1}$. The horizontal axis is exposure time in hours and the vertical axis, 50 % sample crawl-out response (CO_{50}). shell length (SL) as a covariate. B-D) crawl-out CO_{50} values determined by analysis. Vertical bars above Histograms represent standard errors.

Maximum likelihood probit regression analysis with SL as a covariate revealed a significant effect of SL on crawl-out response that was constant across the 25th, 50th and 75th SL quantile groups using the same sample population. For all three groups a significant relationship between SL and crawl-out response ($p < 0.05$) was demonstrated for observation intervals of 2-12 h. Chi-square values for the effect of SL on crawl-out response ranged from 4.3 ($p = 0.0380$) at an exposure of 12 h to 27.3 ($p < 0.0001$) at an exposure of 7 h (Table 2.18). Maximum likelihood probit regression analysis also revealed a significant interaction between $CdCl_2$ concentration and SL at exposure times ranging from 3-12 h, chi-square range = 4.0 ($p = 0.047$) at an exposure of 4 h to 27.3 ($p < 0.0001$) at an exposure of 7 h (Table 2.18). Because there was a significant impact of shell size on crawl-out response on exposure to $CdCl_2$, maximum likelihood probit analysis was carried out for the three SL quantile groups including the 25th SL quantile, the median 50th SL quantile and the 75th SL quantile. For the 25th SL quantile (SL = 6.7 mm), maximum likelihood probit analysis revealed a significant relationship between crawl-out response and $CdCl_2$ concentration at each tested exposure duration (1-12 hours) across which chi-square and p values ranged from chi-square = 18.0 ($p < 0.0001$) at an exposure of 12 h to chi-square = 70.8 ($p < 0.0001$) at 4 h (Table 2.16). With the exception of the CO_{50} value for 1 h exposure which, at 3493.8 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±925.10), fell outside the highest concentration tested (i.e., 2000 μg $CdCl_2 \cdot L^{-1}$), CO_{50} values for the 25th SL quantile group decreased with increasing time of exposure from 1653.4 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±247.95) at an exposure time of 2 h through 276.4 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±78.47) at an exposure of 12 h

Table 2.18 Effect of shell length (SL) and the interaction between concentration and SL on sub-lethal 50 % sample crawl-out (CO_{50}) response chi-square and probability (p) values were estimated by maximum likelihood probit regression analysis determinations for samples of *Physa acuta* exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 - 2000 µg $CdCl_2 \cdot L^{-1}$ over a total exposure period of 12 h. The probability of of an individual crawling-out in a given interval: $\Gamma^1(p) = b_0 + b_1(Lconcentration+1)+b_2\ (SL-SL^0)+b_3[(Lconcentration+1)*(SL-SL^0)]$ with shell length as a covariate allow determination of EC_{50} values for individuals in the 25th SL quantile (median SL = 6.7 mm), median 50th SL quantile (median SL = 7.9 mm) and the 75th SL quantile (median SL = 9.1 mm). Probability values are consistent across all SL calculations.

Exposure Duration (h)	**SL Effect on CO_{50} Chi-square**	**p**	**Concentration-SL Interaction Effect on CO_{50} Chi-square**	**p**
1	3.5	0.0624	2.1	0.1443
2	4.8	0.0287*	3.2	0.0721
3	6.6	0.0103*	4.4	0.0355*
4	6.4	0.0113*	4.0	0.0462*
5	6.9	0.0089*	4.1	0.0434*
6	7.9	0.0050*	5.0	0.0255*
7	27.3	<0.0001*	27.3	<0.0001*
8	23.2	<0.0001*	23.2	<0.0001*
9	21.1	<0.0001*	21.1	<0.0001*
10	19.6	<0.0001*	19.6	<0.0001*
11	15.0	<0.0001*	15	0.0001*
12	4.3	0.0380*	4.3	0.0380*

*indicates a significant difference at $p<0.05$

(Table 2.17, Fig. 2.21 B). There was also a significant relationship between crawl-out response and SL in the 50th SL quantile (median SL = 7.9 mm) at each tested exposure duration (1-12 hours) across which chi-square and p values ranged from chi-square = 26.4 ($p < 0.0001$) at an exposure of 12 h to chi- square = 112.3 ($p < 0.0001$) at 4 h (Table 2.16). Removing the CO_{50} value for 1 h exposure (3520.5 $CdCl_2 \cdot L^{-1}$) (s.e. = ±791.09) which fell outside the highest concentration tested (i.e., 2000 μg $CdCl_2 \cdot L^{-1}$), crawl-out response concentration CO_{50} values for the median 50th SL quantile decreased from 1732.1 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±222.29) at an exposure time of 2 h through 349.97 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±63.07) at 12 h (Table 2.17, Fig. 2.21 C).

There was a significant relationship between CO_{50} and $CdCl_2$ concentration among the 75th SL quantile (SL = 9.1 mm) at each tested exposure duration (1-12 hours) across which chi-square and p values ranged from chi-square = 32.1 ($p < 0.0001$) after an exposure of 12 h to chi-square = 102.3 ($p < 0.0001$) after 7 h (Table 2.16). Removing the CO_{50} value for 1 h exposure 3541 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±979.11), which fell outside at fell outside the highest concentration tested (i.e., 2000 μg $CdCl_2 \cdot L^{-1}$), CO_{50} values decreased from 1795.98 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±286.00) at an exposure time of 2 h through 421.39 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±66.57) at an exposure of 12 h (Table 2.17, Fig. 2.21 D).

Values of CO_{50} in response to $CdCl_2$ exposure increased with increasing shell length in individuals of *P. acuta* suggesting that larger individuals were much less sensitive to the irritating effects of $CdCl_2$ than smaller individuals. Thus, individuals in the 25th SL quantile (SL = 6.7 mm) had a CO_{50} of 276.12 μg $CdCl_2 \cdot L^{-1}$ after an exposure period of 12 h, which was only 65.6 % that of the CO_{50} of the 75th SL quantile (SL = 9.1 mm) at 421.95 μg $CdCl_2 \cdot L^{-1}$ (Fig 2.21).

In addition to the maximum likelihood probit regression anlysis, logistic regression analysis was utilized to determine CO_{50} values for the entire sample at each hour of exposure to $CdCl_2$ concentrations ranging from 0-2000 μg $CdCl_2 \cdot L^{-1}$ at hourly intervals over a 12 h exposure period without including SL as a covariate. This analysis revealed a significant relationship between crawl-out response and $CdCl_2$ concentration at each tested exposure duration from 1-5 h across which chi-square and p valuesranged from chi-square = 70.06 ($p < 0.0001$) at an exposure period of 1 h to chi-square = 112.76 ($p = 0.01$) at 5 h (Table 2.19). The effect of $CdCl_2$ concentration on CO_{50} was not significant ($p < 0.05$) at exposures ranging from 6-12 h suggesting that maximum crawl-out responses had been attained across all tested concentrations over this time period (Table 2.19). Excluding the CO_{50} (i.e., effective concentration for estimated 50 % sample crawl-out response) for the 1 h exposure period which at 3253.93 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±645.09) fell outside the highest concentration tested (i.e., 2000 μg $CdCl_2 \cdot L^{-1}$), crawl-out CO_{50} values decreased with increasing duration of exposure from 1601.28 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±178.61) at the 2 h exposure period to 294.81 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±45.22) at the 12 h exposure period (Table 2.20, Fig. 2.22 A).

In contrast to analysis without SL as a covariate, logistic regression analysis with inclusion of SL as a covariate revealed a significant relationship between SL and crawl-out response ($p < 0.05$) at all tested observation periods (1-12 h). Chi-square values for the effect of SL on crawl-out response ranged from 3.9 ($p = 0.0476$) at 1 h exposure to 26.1 ($p < 0.0001$) at 7 h exposure (Table 2.21). Logistic regression analysis also revealed a significant interaction between $CdCl_2$ concentration and SL at exposure times of 1h and from 3-12 h with chi-square values ranging from 2.6 ($p = 0.0111$) at 1 h exposure to 26.0 ($p < 0.0001$) at the 7 h (Table 2.21).

Table 2.19 Sub-lethal 50 % sample crawl-out (CO_{50}) response chi-square and probability (p) values estimated by maximum likelihood logistic regression analysis determinations for samples of *Physa acuta* exposed to media with concentrations of cadmium chloride ranging from 0 - 2000 µg $CdCl_2 \cdot L^{-1}$ over a total exposure period of 12 h. Probability values are provided for the entire sample without shell length (SL) as a covariate, for individuals in the 25th SL quantile (SL = 6.7 mm), for individuals in the 50th SL qauntile (median SL = 7.9 mm) and for individuals in the 75th SL quantile (SL = 9.1 mm) based on analysis with SL as a covariate.

Exposure Duration (h)	**CO_{50} Without SL Covariate**	**P**	**CO_{50} SL 6.7 mm**	**p**	**CO_{50} SL 7.9 mm**	**P**	**CO_{50} SL 9.1 mm**	**p**
1	70.06	<0.0001*	44.4	<0.0001*	63.7	<0.0001*	45.8	<0.0001*
2	102.04	0.002*	60.7	<0.0001*	91.5	<0.0001*	67.8	<0.0001*
3	123.98	0.001*	66.5	<0.0001*	104.0	<0.0001*	78.3	<0.0001*
4	133.49	0.004*	68.9	<0.0001*	107.1	<0.0001*	79.5	<0.0001*
5	112.76	0.01*	53.3	<0.0001*	88.0	<0.0001*	78.7	<0.0001*
6	100.55	0.07	44.1	<0.0001*	77.8	<0.0001*	62.9	<0.0001*
7	90.94	0.10	45.2	<0.0001*	75.5	<0.0001*	97.4	<0.0001*
8	75.49	0.12	39.1	<0.0001*	65.1	<0.0001*	84.5	<0.0001*
9	69.57	0.12	37.6	<0.0001*	61.9	<0.0001*	80.2	<0.0001*
10	57.60	0.07	30.2	<0.0001*	51.1	<0.0001*	67.7	<0.0001*
11	48.16	0.22	25.6	<0.0001*	42.5	<0.0001*	56.0	<0.0001*
12	41.91	0.47	17.9	<0.0001*	26.3	<0.0001*	31.8	<0.0001*

* indicates a significant difference at $p<0.05$

Table 2.20 Sub-lethal 50 % sample crawl-out responses estimated by maximum likelihood logistic regression analysis determinations of and probability (p) of crawl-out response values for samples of *Physa acuta* exposed to media with concentrations of cadmium chloride ranging from 0 - 2000 µg $CdCl_2 \cdot L^{-1}$ over a total exposure period of 12 h. Chi-square values are provided for analysis carried out without shell length (SL) as a covariate to describe the probability of an individual crawling-out in the given interval: $\Gamma^1(p) = b_0 + b_1(Lconcentration+1)$. The probability of of an individual crawling-out in a given interval: $\Gamma^1(p) = b_0 + b_1(Lconcentration+1)+b_2 (SL-SL^0)+b_3[(Lconcentration+1)*(SL-SL^0)]$ with shell length as a covariate allowing determination of EC_{50} values for individuals in the 25th SL quantile (median SL = 6.7 mm), median 50th SL quantile (median SL = 7.9 mm) and the 75th SL quantile (median SL = 9.1 mm).

Exposure Duration (h)	CO_{50} Without SL Covariate	s.e.	CO_{50} SL 6.7 mm	s.e.	CO_{50} SL 7.9 mm	s.e.	CO_{50} SL 9.1 mm	S.e.
1	3253.93	±645.09	3327.91	±832.11	3320.66	±704.13	3315.11	±856.62
2	1601.28	±178.61	1642.86	±237.66	1707.20	±210.06	1758.40	±266.49
3	1091.84	±89.30	1049.98	±114.39	1167.96	±108.93	1264.94	±146.95
4	855.80	±60.64	762.31	±72.79	912.64	±74.43	1047.96	±108.98
5	730.23	±54.34	601.27	±64.40	774.80	±66.33	936.15	±100.95
6	610.64	±46.88	475.83	±58.92	635.65	±55.33	782.00	±79.65
7	531.76	±43.05	427.81	±58.58	568.79	±50.50	691.97	±57.39
8	476.40	±43.28	381.37	±59.70	514.26	±49.79	632.90	±56.18
9	426.99	±41.76	346.77	±58.31	471.00	±47.64	584.04	±53.27
10	384.60	±43.34	309.46	±62.35	436.82	±50.01	553.77	±55.35
11	339.02	±44.68	271.75	±64.35	385.69	±51.15	49309	±55.36
12	294.81	±45.22	276.12	±77.74	349.07	±62.49	419.95	±66.214

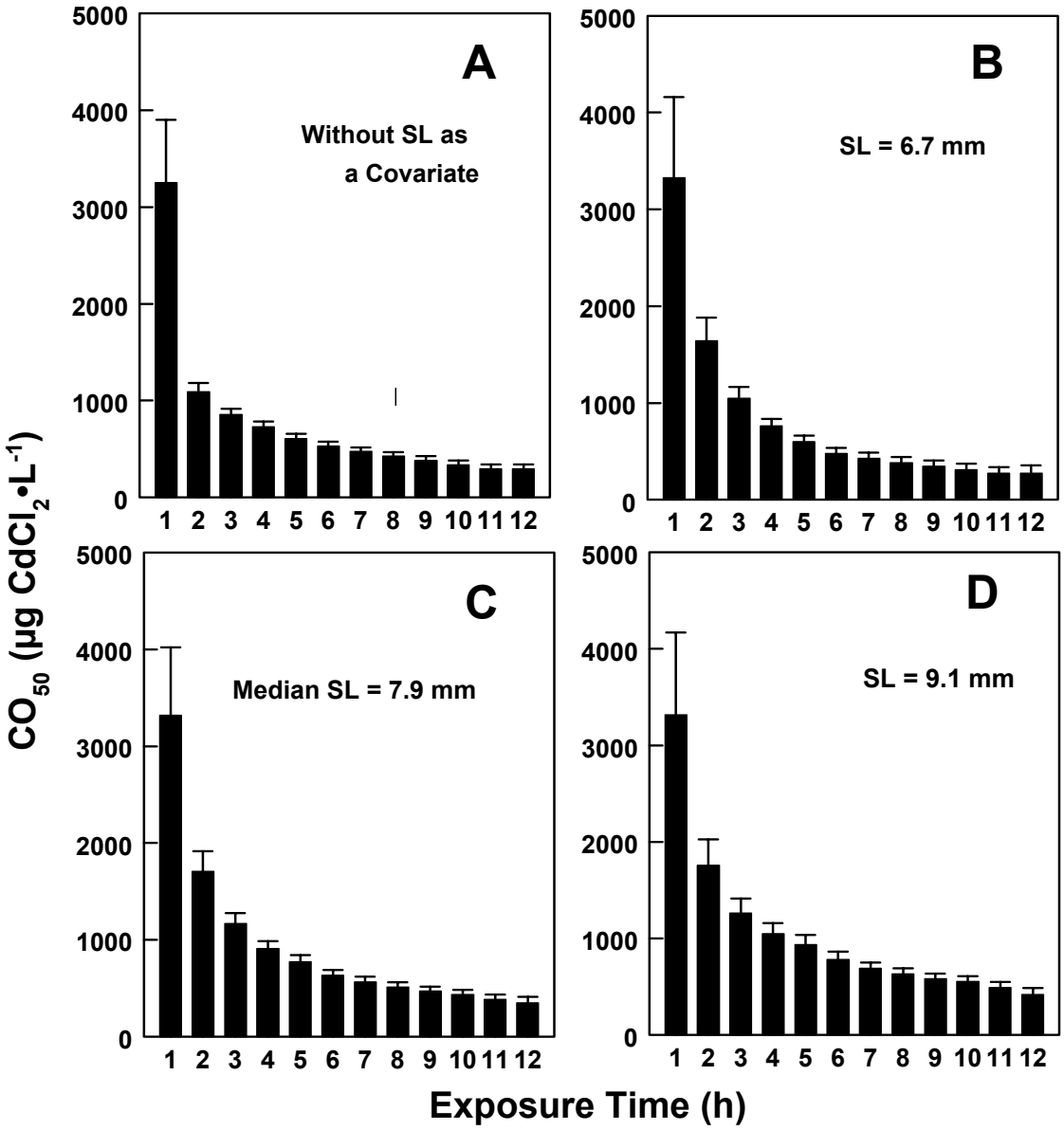


Fig.2.22 Estimated concentrations for 50 % sample crawl-out response (CO_{50}) (i.e., crawling out of the medium) computed by maximum likelihood logistic regression analysis for samples (n = 90) of *Physa acuta* A) without shell length (SL) as a covariate, and B-D) with inclusion of SL as a covariate for B) individuals in the 25th (SL = 6.7 mm), C) 50th (median SL = 7.9 mm) and D) 75th SL quantile (SL = 9.1) exposed to media for 12 h with different concentrations of cadmium chloride ($CdCl_2$) ranging from 0-2000 µg $CdCl_2 \cdot L^{-1}$. The horizontal axis is exposure time in hours and the vertical axis, 50 % sample crawl-out response (CO_{50}). Verticalbars above histograms represent standard errors.

Table 2.21 Effect of shell length (SL) and the interaction between concentration and SL on sub-lethal 50 % sample crawl-out (CO_{50}) response chi-square and probability (p) values estimated by maximum likelihood logistic regression analysis determinations for samples of *Physa acuta* exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 - 2000 µg $CdCl_2 \cdot L^{-1}$ over a total exposure period of 12 h. Chi-square values are provided for analysis carried out without shell length (SL) as a covariate to describe the probability of an individual crawling-out in the given interval: $\Gamma^1(p) = b_0 + b_1(Lconcentration+1)$. The probability of of an individual crawling-out in a given interval: $\Gamma^1(p) = b_0 + b_1(Lconcentration+1)+b_2\ (SL-SL^0)+b_3[(Lconcentration+1)*SL-SL^0]$ with shell length as a covariate allowing determination of EC_{50} values for individuals in the 25th SL quantile (SL = 6.7 mm), 50th SL quantile (median SL = 7.9 mm) and the 75th SL quantile (SL = 9.1 mm). Probability values are consistent across all SL calculations.

Exposure Duration (h)	SL Effect on CO_{50} Chi-square	p	Concentration-SL Interaction Effect on CO_{50} Chi-square	P
1	3.9	0.0476*	2.6	0.01103*
2	5.2	0.0225*	3.7	0.0554*
3	7.0	0.0084*	4.8	0.0281*
4	6.5	0.0111*	4.1	0.0433*
5	6.7	0.0095*	4.1	0.0436*
6	7.4	0.0066*	4.7	0.0303*
7	26.1	<0.0001*	26.0	<0.0001*
8	22.2	<0.0001*	22.2	<0.0001*
9	20.3	<0.0001*	20.3	<0.0001*
10	18.9	<0.0001*	18.9	<0.0001*
11	14.6	<0.0001*	14.6	0.0001*
12	4.3	0.0391*	4.2	0.0394*

*indicates a significant difference at $p<0.05$

Because logistic analysis revealed a significant impact of shell size on crawl-out response, logistic analysis was carried out for three sample SL quantile groups including individuals with SL's in the 25th SL quantile (SL = 6.7 mm), 50th SL quantile (SL = 7.9 mm) and the 75th SL quantile (SL = 9.1). The 25th SL quantile (SL = 6.7 mm) logistic regression analysis revealed a significant relationship between crawl-out response and $CdCl_2$ concentration at each tested exposure duration (1-12 hours) across which chi-square and p values ranged from chi-square = 17.9 ($p < 0.0001$) at an exposure of 12 h to chi-square = 68.9 ($p < 0.0001$) at an exposure of 4 h. (Table 2.19). Removing the CO_{50} value for the 1 h exposure (3327.91 μg $CdCl_2 \cdot L^{-1}$, s.e. = ±832.91), which fell outside the highest concentration tested (i.e., 2000 μg $CdCl_2 \cdot L^{-1}$), CO_{50} crawl-out values based on this analysis decreased from 1642.86 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±237.66) at an exposure of 2 h through 276.12 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±77.74) at an exposure of 12 h (Table 2.20, Fig. 2.22 B). For the 50th quantile SL (median SL = 7.9 mm), logistic regression analysis revealed a significant relationship between crawl-out response and $CdCl_2$ concentration at each tested exposure duration (1-12 hours) across which chi-square and p values ranged from chi-square = 26.3 ($p < 0.0001$) at an exposure of 12 h to chi-square = 107.1 ($p < 0.0001$) at an exposure of 4 h (Table 2.19). Removing the CO_{50} value for 1 h exposure (3320.66 μg $CdCl_2 \cdot L^{-1}$, s.e. = ±704.13), which fell outside the highest concentration tested (i.e., 2000 μg $CdCl_2 \cdot L^{-1}$), CO_{50} crawl-out values based on logistic regression analysis decreased from 1707.20 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±210.06) at an exposure time of 2 h to 349.07 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±62.49) at an exposure of 12 h (Table 2.20, Fig. 2.22 C). For the 75th quantile SL (SL = 9.1 mm), logistic regression analysis revealed a significant relationship between crawl-out response and $CdCl_2$ concentration at each tested exposure duration (1-12 hours) across which chi-square and p values ranged from chi-square = 31.8 ($p < 0.0001$) at an exposure of 12 h to chi-square = 97.4 ($p < 0.0001$) at an

exposure of 7 h. (Table 2.19). Removing the CO_{50} value for 1 h exposure (3315.11 μg $CdCl_2 \cdot L^{-1}$, s.e. = ±856.62), which fell outside the highest concentration tested (i.e., 2000 μg $CdCl_2 \cdot L^{-1}$), CO_{50} values decreased from 1758.40 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±266.49) at an exposure time of 2 h through 419.95 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±66.21) at an exposure time of 12 h (Table 2.20, Fig. 2.22 D).

As occurred with probit analysis, CO_{50} values in response to $CdCl_2$ exposure resulting from logistic regression analysis increased with increasing shell length in individuals of *P. acuta*, suggesting that larger individuals are less sensitive to the irritating effects of $CdCl_2$ than are smaller individuals. Thus, individuals in the 25th SL quantile SL had a CO_{50} of 276.1 μg $CdCl_2 \cdot L^{-1}$, which was only 65.7 % that of the CO_{50} of the 75th SL quantile at 419.9 μg $CdCl_2 \cdot L^{-1}$ (Table 2.20, Fig 2.23).

Discussion

Comparison of Acute LC_{50} Values for Probit and Logistic Regression Analyses

Both maximum likelihood probit and logistic regression demonstrated a significant effect of concentration on mortality for all observation periods for the analysis with no SL as a covariate. Both analyses without SL as a covariate also resulted in a concentration-response effect ranging from the highest LC_{50} at 24 h to the lowest LC_{50} at 192 h (Fig. 2.6A-D). The probit and logistic regression analyses with the addition of SL as a covariate also showed a significant effect of concentration across all observation periods (24-192 h). There was a significant SL effect across all median SL analyses for 24 h and 120-168 h. A significant concentration-SL interaction was demonstrated 120-144 h. The LC_{50} demonstrated a similar decrease over the period 24-192 h (Fig.2.6 A-D).

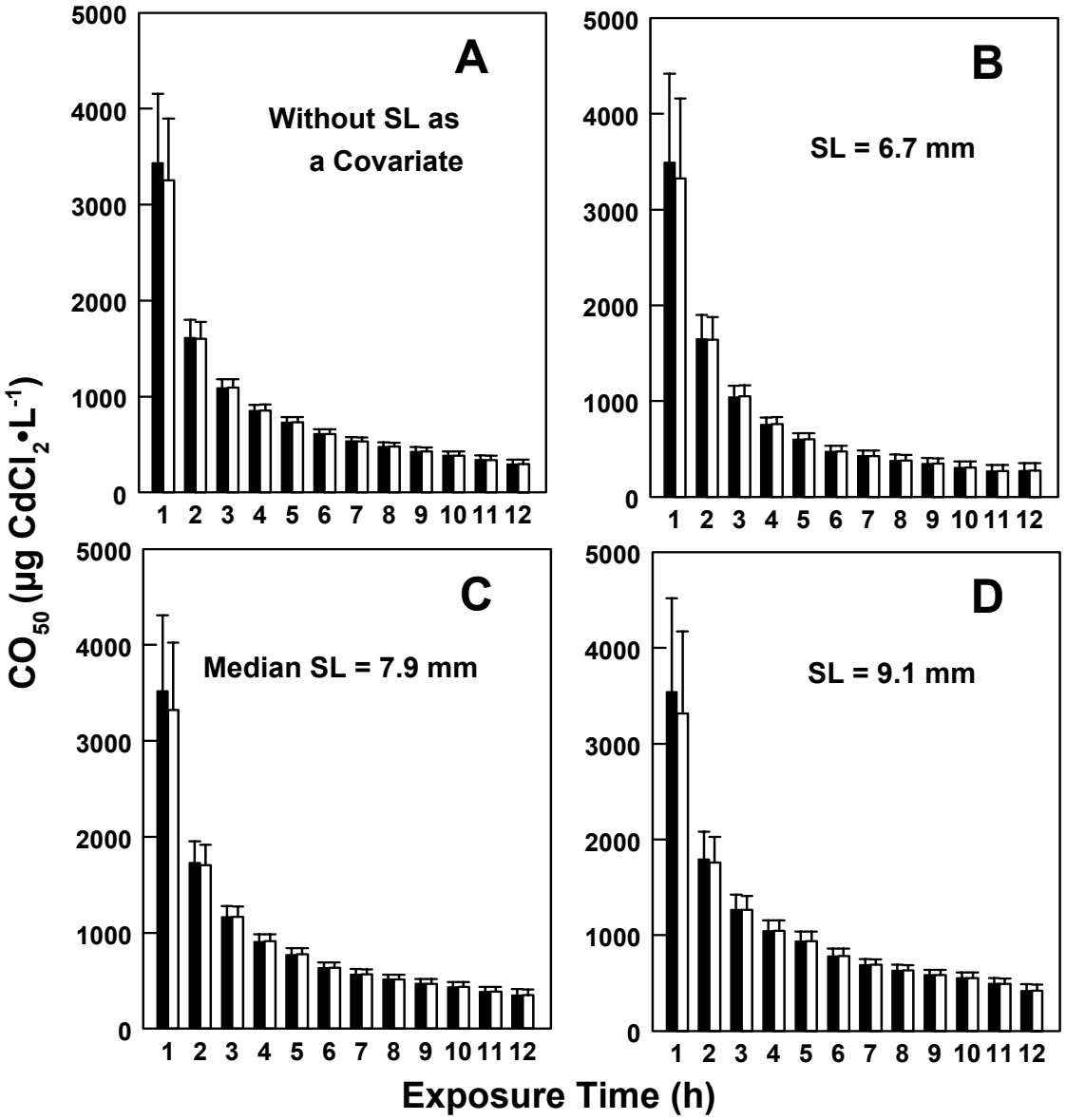


Fig.2.23 Comparison of estimated 50 % sample crawl-out response (CO_{50}) (i.e., crawling out of the medium) computed by maximum likelihood probit (solid histograms) and logistic regression analyses (open histograms) for samples(n = 90) of *Physa acuta* A) without shell length (SL) as a covariate and B-D) with inclusion of SL as a covariate for B) individuals in the 25th (SL = 6.7 mm), C) 50th (median SL = 7.9 mm) and D) 75th of the SL quantile (SL = 9.1) exposed to media for 12 h with different concentrations of cadmium chloride ($CdCl_2$) ranging from 0-2000 µg $CdCl_2 \cdot L^{-1}$. The horizontal axis is exposure time in hours and the vertical axis, 50 % sample crawl-out response (CO_{50}). Vertical bars above histograms represent error bars.

Comparison of Chronic LC_{50} Values Estimated by Probit and Logistic Regression Analyses

Probit and logistic regression analyses both demonstrated significant effects of $CdCl_2$ concentration and individual size measured as SL on mortality in samples of *P. acuta.* Probit regression analyses resulted in a significant effect of SL being recorded for exposure periods ranging from 96-672 h, while logistic regression analyses demonstrated a significant SL effect for exposure of 96-624 h. Probit regression revealed a significant concentration-SL interaction for exposures of 120-600 h, as opposed to that of 96-576 h revealed by logistic regression analysis. In both analyses, the 24 h estimated LC_{50} were omitted from further consideration due to LC_{50} values falling outside the maximum $CdCl_2$ concentration (i.e., 2000 μg $CdCl_2 \cdot L^{-1}$). Similarly, both probit and logistic analyses resulted in LC_{50} values for the 72, 96 and 120 h exposures that also fell outside the maximum $CdCl_2$ concentration tested for the 75th SL quantile (SL = 7.9 mm). Both analyses revealed essentially similar concentration-response effects of increasing mortality with increased $CdCl_2$ concentration for all sample SL quantile groups with an initial reduction in LC_{50} values between being recorded at exposure durations ranging from 48-72-120 h (Fig. 2.17). Both analyses resulted in essentially similar estimates of LC_{50} values and corresponding standard errors across the entire range of tested exposure durations (i.e., 24-672 h) (Fig. 2.17).

The present investigation involved analyzing the effect of cadmium chloride ($CdCl_2$) on survival and behavioral responses in *P. acuta* using different survivorship methodologies including maxmium likelihood probit versus logistic regression analysis with inclusion versus exclusion of shell length as a covariate. One of the criticisms of LC_{50} determinations is the short exposure time periods utilized (i.e., 24-196 h), which require exposure to high concentrations of toxins in order to determine appropriate environmental levels (Kendall *et al*., 2003). It can be

questioned whether very high levels of pollutants actually remain in the aquatic environments over the observation period studied in acute toxicity studies (between 96 and 192 h), and if those results can be extrapolated across species. In order to overcome the difficulties associated with such short-term toxicity testing, this study also included longer observation periods at lower concentration levels allowing estimation of minimum tolerated levels of $CdCl_2$, a chemical species known to have additive effects leading to mortality during extended exposure times (> 192 h) at lower exposure concentrations during exposure. This study demonstrated the similarity in the calculated acute and chronic survival LC_{50}'s over several observation periods between the regression techniques of maximum likelihood probit and logistical analysis, which corresponds to similarly equivalent results from these two approaches reported in previously published literature (Ellersieck and LaPoint, 1995). The statistical techniques utilized in this study were somewhat unusual in that they allowed additional factors (covariates) to be included in the estimation of LC_{50}, LT_{50} and CO_{50} values. The impacts of covariates such as shell length utilized in this study are rarely considered in environmental toxicity studies even though they are known to generally to greatly impact and likely to confound results if not statistically controlled. Results from acute and chronic survival experiments showed that the addition of shell length (SL) as a covariate in analyses consistently demonstrated a profound influence of size on estimated LC_{50}, LT_{50} and CO_{50} values for *P. acuta*, indicating the need to either experimentally control or statistically account for additional factors (e.g., chemical species, temperature, water hardness, individual size) that could impact exposure tolerance in toxicity tests.

Discrete Logistic Failure Time Survival Analysis Techniques

In contrast to LC_{50} determinations, discrete logistic failure time model (DLFTM) was used to estimate survival times of specimens of *P. acuta* exposed to $CdCl_2$ (Hicks *et al.,* 2000).

The DLFTM analysis allowed the lethal time (LT_{50}) needed to kill 50 % of sampled individuals to be calculated for both acute and chronic exposures to various concentrations of $CdCl_2$. This information is useful when applied to industrial situations or hazards, when concentrations are known and the time for exposure needs to be controlled.

The LT_{50} determinations utilized the same experimental equipment and the same number of experimental organisms, but required a greater number of observations to derive the estimated value, which would increase the cost of this determination in terms of time and handling. The total exposure duration was the same for both LC_{50} and LT_{50}, but for the LC_{50}, the only observation necessary was at the desired exposure time. In contrast, observations for LT_{50} determinations had to be made daily over the entire exposure period (i.e., durations of 192 h for acute studies to 672 h for chronic studies).

Comparison of Sub-lethal CO_{50} Values Estimated by Probit and Logistic Regression Analyses

Analysis of crawl-out response on exposure to $CdCl_2$ by maximum likelihood probit or logistic regression analysis produced very similar results. Both analyses demonstrated significant concentration and SL effects on crawl-out response and significant interactions between concentration and SL. The estimates of CO_{50} for the first hour of exposure in both analyses fell outside the highest tested concentration and with inclusion of SL as a covariate, both analyses revealed that smaller individuals of *P. acuta* were less tolerant of all tested concentrations of $CdCl_2$ than larger animals (Fig. 2.23). Overall, logistic regression analyses generated estimates of CO_{50} that were slightly less than those generated by probit regression analysis at all tested exposure times between 1 and 12 h whether SL was excluded (Fig. 2.23 A) or included as a covariate for the 25th SL quantile (Fig. 2.23 B), median 50th SL quantile (Fig. 2.23 C) or 75th SL quantile SL (Fig. 2.23 D). The extensive overlap of error terms for CO_{50}

values computed by probit and logistic analyses across all tested exposure times (i.e., 1-12 h) and among all tested sample SL quantile groups (Figs. 2.23 A-D) suggested these two methodologies generated essentially equivalent results.

The results for the sub-lethal, crawl-out response determinations suggested that this test could be used to determine the toxicity of $CdCl_2$ to *P. acuta* with both reduced exposure time (<12 h) and handling compared to either LC_{50} or LT_{50} determinations. The effect of $CdCl_2$ on crawl-out response was estimated from the logistic regression analysis as measured by probability of the response for each test concentration and for the 50 % crawl-out response (CO_{50}). The probability for each concentration was derived from the estimated logit along with the standard error estimated using the delta method. The probability provided a more familiar one-number summary for determining the toxicity of $CdCl_2$ with the CO_{50} value being calculated and interpreted in a similar manner to that for the LC_{50} value. This allowed an easier method of comparing the endpoints of survival and the crawl-out response.

Species Comparisons of LC_{50} Values

Previous studies of toxicity of $CdCl_2$ in aquatic organisms have included analytical methods based on percent mortality, transformed percent mortality, or methods to determine end-points such as the probit LC_{50}. The 96 h LC_{50} for the freshwater snail *Radix plicatulus* was estimated from percent mortality (Lam, 1996). In contrast, 24 and 48 h LC_{50} values for *Physa acuta* were estimated by probit analysis (Cheung and Lam, 1998). A 96 h LC_{50} value for the guppy, *Poecilia reticulate*, was calculated from a USEPA computer program using probit regression (Yilmaz *et al.*, 2004).

One issue that has been of concern in the toxicity testing literature is variation in LC_{50}'s within species resulting from differences in experimental procedures (Marke and Solbe, 1998).

Experimental procedures vary in water hardness, age and size of tested individuals, chemical species utilized and duration of exposure to test chemicals. An example of such variation within a species was demonstrated in the acute toxicity testing for *Physa acuta* (Cheung and Lam, 1998). Four-day old juveniles were separated from the adults and tested at 25 °C. The acute 24 h LC_{50} for juveniles was 1320 μg $CdCl_2 \cdot L^{-1}$ (1130-1540 μg $CdCl_2 \cdot L^{-1}$) and the 48 h LC_{50} was 1050 μg $CdCl_2 \cdot L^{-1}$ (810-1360 μg $CdCl_2 \cdot L^{-1}$). This study identified a significant decrease in LC_{50} values with decreasing size in *P. acuta* suggesting that limitation of testing to juveniles would underestimate the 24 and 48 LC_{50} values for adults which in this study were 2960.49 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±66.77) and 1785.30 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±33.20), respectively, or more than 1.7-2.2 times greater than the values for juveniles (Cheung and Lam, 1998) (Table 2.22).

In some cases non-standard exposure periods have been utilized such as the 8 h period utilized to test the acute $CdCl_2$ tolerance of freshwater New Zealand mud snail, *Potamopyrgus antipodarum*, which prevented direct comparison of $CdCl_2$ LC_{50} values for this species with those estimated for other species over more generally utilized, standard exposures periods of 24, 48, 72, 96, and 168 h (Table 2.23). In addition (Jensen and Forbes, 2001) demonstrated that different clones of *P. antipodarum* had different tolerances of $CdCl_2$ exposure indicating a genetic influence on tolerance estimates (Jensen and Forbes, 2001). In addition, Jensen and Forbes (2001) used calcium sulfate ($CaSO_4$) instead the more commonly used calcium carbonate ($CaCO_3$) used in this and other studies to maintain water hardness (Table 2.22).

Several studies of $CdCl_2$ toxicity have suggested that degree of water hardness can impact $CdCl_2$ toxicity (Kinkade, 1977). In this study, all determinations of toxicity to $CdCl_2$ were carried out in artificial media with water hardness at 50 mg $Ca^{2+} \cdot L^{-1}$ to control the impact of varying medium Ca^{+2} on results. An interaction between dissolved cadmium nitrate and dietary

Table 2.22 Comparison of toxicity of different freshwater animal species to cadmium chloride ($CdCl_2$) measured as concentration for 50 % of sample mortality (LC_{50}) in static toxicity tests over specific exposure durations.

Species	Conditions	Exposure Duration (h)	Hardness (mg $Ca \cdot L^{-1}$)	LC_{50} ± s.e ($\mu g\ CdCl_2 \cdot L^{-1}$)	Literature Citation
Freshwater Gastropods					
**Physa acuta*	25°C	24	50.0	2960.49 (±66.79)	This study
"	25°C	48	50.0	1785.34 (±33.20)	"
"	25°C	72	50.0	1631.25 (±32.90)	"
"	25°C	96	50.0	1572.86 (±32.55)	"
"	25°C	168	50.0	1318.17 (±30.38)	"
"	25°C	672	50.0	79.3 (±10.58)	"
Aplexa hypnorum		96	N.A.	93 (54-160)	Phipps and Holcombe, 1985
Brotia hainanensis	Upstream adult	96	N.A.	15210 (±230)	Lam, 1996
"	Downstream Adult	96	N.A.	35940 (± .810)	"
"	Upstream Juvenile (<2 d old)	96	N.A.	770 (±30)	"
"	Downstream Juvenile (<2 d old)	96	N.A.	1090 (±40)	"
Lymnea stagnalis	Adult	48	N.A.	2500	Gomot, 1998
"	1 month old	48	N.A.	1250	"
"	Juvenile	48	4.0	1183	Coeurdassier *et al*. 2004
"	Juvenile	96	N.A.	583	"
"	Adult	48	N.A.	583	Slooff, 1986
Physa acuta	Juvenile	24	N.A	1320 (1130-1540)	Cheung and Lam, 1998
Physa acuta	Juvenile	48	N.A	1050 (810-1360)	Cheung and Lam, 1998
Physa integra		168	44-48	114	Spehar *et al*, 1978
"		672	44-48	10.4	"

Table 2.22 Continued

Potamopyrgus antipodarum	Clone A $CaSO_4$	8	120	1920 (1490-2530)	Jensen and Forbes, 2001
Potamopyrgus antipodarum	Clone B $CaSO_4$	8	120	1290 (1040-1600)	"
"	Clone C $CaSO_4$	8	120	560 (420-730)	"
		Freshwater Cladocerans			
Ceriodaphnia dubia		48	N.A.	63.1 (±7.5)	Suedel *et al*., 1997
"		168	N.A.	11.6 (±1.9)	"
Daphnia magna		24	N.A.	1800	Qureshi *et al*., 1980
"		48	N.A.	1000	"
"	*Clone S-1*	48	N.A.	146.7 (±9.98)	Stuhlbacher et al, 1983
"	*Clone F*	48	N.A.	355.3 (±8.8)	"
"		48	N.A.	24	Nebekeretal , 1986
		Freshwater Crusteceans			
Procambarus clarkia	*Adult, intermolt*	96	N.A.	18400	Del Ramo *et al.*, 1987
"	"	"	"	58500	"
"	"	"	"	10000	Diaz-Mayans *et al.*, 1986
		Freshwater Fish			
Pimephales promelas		48	N.A.	8.9 (±1.2)	Suedel *et al*., 1997
"		48	70-90	35.4	Diamond *et al.,* 1997
"		96	44	1500 (1270-1760)	Phipps and Holcombe, 1985
"		168	47.4 (44.2-53.0)	4.4 (±0.8)	Suedel *et al*., 1997
Oncorhynchus mykiss		96	N.A.	6.0	Kumada *et al,.* 1980
"		96	39-48	2.3	Spehar and Carlson, 1984
"	Juveniles	96	41	1.5	Buhl and Hamilton, 1991
"		96		3 (±.1)	Phipps and Holcombe, 1985
Salvelinus fontinalis		48	47.4	6160 (5200-7300)	Holcombe *et al*., 1983

Table 2.22 Continued

"		72	"	5240 (4440-6190)	"
"		96	"	5080 (4320-5970)	"
Ctenopharyngodon idellus	Cd as $CdSO_4$	"	N.A.	9420	Yorulmazlar and Gül, 2003

calcium, as $CaCl_2$, on Cd^{2-} tolerance has been demonstrated in the rainbow trout, *Oncorhynchus mykiss*. As dietary calcium increased, the accumulation of waterborne cadmium decreased over an exposure period of 168 h (7d) (Baldisserotto *et al.*, 2004). To avoid dietary influences on $CdCl_2$ tolerance in *P. acuta*, specimens in this study were fed the same Tetra® flake fish food both in culture and during testing. The effect of cadmium chloride ($CdCl_2$) on survival computed as LC_{50} values by maximum likelihood probit regression analysis without size as a covariate for *P. acuta* in this study was compared to values for other aquatic animal species in the published literature on several species. Estimated LC_{50}'s from maximum likelihood probit regression analysis for *Physa acuta* in this study ranged from 2960.5 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±66.79) at 24 h to 79.3 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±10.6) at 672 h. The LC_{50} values for juvenile *P. acuta* were lower as expected from the strong influence of size detected in this study, ranging from 1320 μg $CdCl_2 \cdot L^{-1}$ (1130-1540 μg $CdCl_2 \cdot L^{-1}$) at 24 h to 1050 μg (810-1360 μg $CdCl_2 \cdot L^{-1}$) at 48 h (Cheung and Lam, 1998). Another physid species, *Physa integra,* had adult LC_{50} values that ranged from 114 μg $CdCl_2 \cdot L^{-1}$ at 168 h to 10.4 μg $CdCl_2 \cdot L^{-1}$ at 672 h to 168 h (Spehar, 1978) that were much lower than the 168 h LC_{50} value of 1318.2 $CdCl_2 \cdot L^{-1}$ and 672 h value of 79.3 $CdCl_2 \cdot L^{-1}$ determined in this study (Table 2.25). Among other freshwater snails, the prosobranch *Brotia hainanensis*, has been reported to have adult 96 h LC_{50} values for ranging from 152101 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ± 230) to 35940 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±810) (Lam, 1996) which is somewhat higher than reported in this study for *P. acuta* (Table 2.22). Estimates of the 48 h LC_{50} value for the freshwater pulmonate snail, *Lymnea stagnalis*, vary from 583 μg $CdCl_2 \cdot L^{-1}$

(Slooff, 1986) to 2500 μg $CdCl_2 \cdot L^{-1}$at 48 h (Gomot, 1998) which bracket the LC_{50} value of 1785.34 μg $CdCl_2 \cdot L^{-1}$ reported for adult *P. acuta* in this study (Table 2.22). The 8 h LC_{50} range reported for three clones of the freshwater prosobranch snail *Potamopyrgus antipodarum* was 560 μg $CdCl_2 \cdot L^{-1}$ to 1920 μg $CdCl_2 \cdot L^{-1}$ (Jensen and Forbes, 2001) which is considerably lower than even the 24 LC_{50} value for adult specimens of *P. acuta* of 2960.49 μg $CdCl_2 \cdot L^{-1}$ in this study (Table 2.25). Estimates of 96 h LC_{50} values for freshwater snails exposed to $CdCl_2$ range from 93 μg $CdCl_2 \cdot L^{-1}$ for the freshwater pulmonate *Aplexa hypnorum* (Phipps and Holcomb, 1985) to 35940 μg $CdCl_2 \cdot L^{-1}$ for *Brotia hainanensis* (Lam, 1996) (Table 2.22). The calculated LC_{50} for *P. acuta* (this study) estimated at 1572.86 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±32.55) fell in the middle of this huge range of values from other freshwater snail species.

It is also of value to compare the $CdCl_2$ LC_{50} values determined for adult *P. acuta* in this study with that of other non-molluscan aquatic species, particularly those commonly utilized in standard aquatic toxicity testing. Thus, the reported LC_{50} values of the freshwater cladoceran, *Ceriodaphnia dubia*, range from 63.1 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±7.5) at an exposure of 48 h to 11.6 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±1.9) at 168 h (Suedel, 1997) (Table 2.25). For the cladoceran, *Daphnia magna*, 48 h LC_{50} values varied from 0.017 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ± 0.0004) (Guilhermino *et al*, 2000) to 24 μg $CdCl_2 \cdot L^{-1}$ (Nebekeretal, 1986) (Table 2.22). Qureshi *et al.* (1980) estimated $CdCl_2$ LC_{50} values for *Daphnia magna* to be 1800 μg $CdCl_2 \cdot L^{-1}$ and 1000 μg $CdCl_2 \cdot L^{-1}$ at exposures of 24 h and 48 h, respectively (Table 2.22). The calculated LC_{50} values for *P. acuta* (this study)

were 2960.49 (s.e. = ±66.79) and 1785.30 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±33.20) at 24 and 48 h exposure, respectively (Table 2.22). These LC_{50} values for adult *P. acuta* and other freshwater snail species (see above) are considerably elevated compared to those reported for the cladocerans, *C. dubia* and *D. magna*, suggesting that freshwater cladocerans may be generally more sensitive to $CdCl_2$ than freshwater snails. In contrast, *P. acuta* and other freshwater snail species appear to be considerably less tolerant of $CdCl_2$ exposure than crayfish as evidenced by the reported 96 h $CdCl_2$ LC_{50} for specimens of adult *Procambarus clarkia* ranging from 10,000 (Diaz-Mayans *et al.,* 1986) to 58500 μg $CdCl_2 \cdot L^{-1}$ at 96 h (Del Ramo *et al.,* 1987) (Table 2.22).

The tolerance of adult *P. acuta* to $CdCl_2$ exposure falls within a wide range of values reported for standard fish species utilized in toxicity testing. The LC_{50} for the freshwater fathead minnow, *Pimephales promelas,* varied from 1500 μg $CdCl_2 \cdot L^{-1}$ at 96 h (Phipps and Holcombe, 1985) to 4.4 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ± 0.8) at 168 h (Suedel *et al.*, 1997) as opposed to 1572.90 μg $CdCl_2 \cdot L^{-1}$ and 1318.17 μg $CdCl_2 \cdot L^{-1}$ for adult *P. acuta* (this study), respectively. Similarly 48 h, $CdCl_2$ LC_{50} values of 8.9 μg $CdCl_2 \cdot L^{-1}$ (Suedel *et al*, 1997) and 35.4 μg $CdCl_2 \cdot L^{-1}$ (Diamond *et al,* 1997) have been estimated for *P. promelus* by (Suedel *et al.*, 1997) which were which were 50-200 times lower than that for *P. acuta* at 1785.34 μg $CdCl_2 \cdot L^{-1}$ (Table 2.22). These results suggest that the fathead minnow may be more sensitive than *P. acuta* to $CdCl_2$, particularly in exposures of longer duration. In contrast, Phipps and Holcombe (1985) estimated the 96 h, $CdCl_2$ LC_{50} for *P. promelus* to be 1500 μg $CdCl_2 \cdot L^{-1}$ which was very similar to that of 1572.86 μg $CdCl_2 \cdot L^{-1}$ estimated for adult *P. acuta* in this study (Table 2.22). Rainbow Trout

appear to be particularly sensitive to $CdCl_2$ exposure relative to P. *acuta*. The 96 h $CdCl_2$ LC_{50} value for rainbow trout, *Oncorhynchus mykiss*, ranged from 1.5 μg $CdCl_2 \cdot L^{-1}$ for juvenile trout (Buhl and Hamilton, 1991) to 6.0 μg $CdCl_2 \cdot L^{-1}$ for adult trout (Kumada *et al*, 1980), values well below the 96 h value of 1572.86 μg $CdCl_2 \cdot L^{-1}$ recorded for *P. acuta* in this study (Table 2.22). In contrast, the tolerance of adult *P. acuta* for exposure to $CdCl_2$ is much less than that of the brook trout, *Salvelinus fontinalis*, whose 48 h and 96 h $CdCl_2$ LC_{50} values of 6160 μg $CdCl_2$ and 5080 μg $CdCl_2 \cdot L^{-1}$, respectively (Holcombe *et al.*, 1983) (Table 2.22). These brook trout values are roughly three times those reported for adult *P. acuta* in this study.

Several factors complicate the comparison of $CdCl_2$ LC_{50} values between different studies for the same or different species including utilization of different methodologies, different observation periods for determination of LC_{50} values, using samples of different age/size distributions and different levels of dissolved calcium ion in testing media. These differences in method of determination have led to multiple, often conflicting, $CdCl_2$ LC_{50} values being published for the same species (Table 2.22). In addition, many of the results in the published literature do not report associated standard errors making it difficult to determine their validity when comparing data across different reports.

Efficacy of Crawl-out Response as a Measure of $CdCl_2$ Sensitivity

The 12 h crawl-out behavior study demonstrated that crawl-out behavior was a far more sensitive endpoint for determination of $CdCl_2$ sensitivity/toxicity with and without SL as a covariate, with a smaller concentration needed to elicit a response for a

50 % population response (CO_{50}) whether estimated by maximum likelihood probit or logistic regression analyses. Probit regression analysis yielded a CO_{50} of 295.43 µg $CdCl_2 \cdot L^{-1}$ (s.e. = ±45.86) versus a LC_{50} of 3717.79 µg $CdCl_2 \cdot L^{-1}$ (s.e. = ±107.20) without SL as a covariate. Probit regression analysis with SL as a covariate exhibited CO_{50} values that ranged from 276.40 µg $CdCl_2 \cdot L^{-1}$ (s.e. = ±78.47) versus the LC_{50} of 3159.57 µg $CdCl_2 \cdot L^{-1}$ (s.e. = ±106.02) for the 25th SL quantile, to 349.97 µg $CdCl_2 \cdot L^{-1}$ (s.e = ±63.07) versus the LC_{50} of 3626.57 µg $CdCl_2 \cdot L^{-1}$ (s.e. = ±100.69) for the median 50th SL quantile, to 421.40 µg $CdCl_2 \cdot L^{-1}$ (s.e = ±66.57) versus the LC_{50} of 4148.10 µg $CdCl_2 \cdot L^{-1}$ (s.e. = ±152.25) for the 75th SL quantile (Table 2.23). Similarly logistic regression analysis without SL as a covariate yielded a CO_{50} value of 294.81 µg $CdCl_2 \cdot L^{-1}$ (s.e. = ±45.22) versus an LC_{50} value of 3733.73 µg $CdCl_2 \cdot L^{-1}$ (s.e. = ±90.26).

With SL as a covariate, logistic regression analysis estimated CO_{50} values ranged from 276.12 µg $CdCl_2 \cdot L^{-1}$ (s.e. = ±77.74) versus an LC_{50} value of 3242.23 µg $CdCl_2 \cdot L^{-1}$ (s.e. = ±102.42) for the 25th of the sample SL quantile, to 349.07 µg $CdCl_2 \cdot L^{-1}$ (s.e. = ±62.49) versus an LC_{50} of 3653.72 µg $CdCl_2 \cdot L^{-1}$ (s.e. = ±89.92) for the median 50th SL quantile, to 419.95 µg $CdCl_2 \cdot L^{-1}$ (s.e. = ±66.57) versus an LC_{50} of 4082.60 µg $CdCl_2 \cdot L^{-1}$ (s.e. = ±125.42) for the 75th SL quantile (Table 2.24). Efficacy of crawl-out response was investigated by analyzing standard survival 96 h LC_{50} values versus crawl-out response CO_{50} values (Table 2.23 and 2.24). A Wald statistic was used to examine differences between the $CdCl_2$ exposure 12 h CO_{50} value and the 96 h LC_{50} values in *P. acuta.* The 96 h LC_{50} value was chosen for this comparison because it is

Table 2.23. Comparison of 50 % sample mortality (LC_{50}) and 50 % crawl-out response (CO_{50}) estimated by maximum likelihood probit regression determinations for samples of *Physa acuta* exposed to various concentrations of cadmium chloride ($CdCl_2$). In LC_{50} determinations, samples (n = 150) were exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 – 5000 µg $CdCl_2.L^{-1}$, while, for CO_{50} determinations, samples (n = 90) were exposed to media with concentrations ranging from 0 - 2000 µg $CdCl_2.L^{-1}$. Values of LC_{50} for 12 h and 96 h exposure periods are provided for analysis of the entire sample without shell length (SL) as a covariate and with SL as a covariate for individuals in the 25th SL quantile (SL = 6.0 mm), 50th SL quantile (median SL = 7.1 mm) and 75th SL quantile (SL = 8.3 mm). Values of CO_{50} for a 12 h exposure period are provided for the entire sample without shell length (SL) as a covariate and with SL as a covariate for individuals in the 25th SL quantile (SL = 6.7 mm), median 50th SL quantile (median SL = 7.9 mm) and 75th SL quantile (SL = 9.1 mm).

	LethalConcentration		**Crawl-out Response**		
SL for 50 % Response	**96 h LC_{50}**	**s.e.**	**SL for 50 % response**	**12 h CO_{50}**	**s.e.**
Without SL as a Covariate	1572.86	±32.55	**Without SL as a Covariate**	295.43	±45.86
SL= 6.0 mm	1504.57	±39.96	**SL= 6.0 mm**	276.40	±78.47
SL= 7.1 mm	1563.96	±32.78	**SL= 7.1 mm**	349.97	±63.07
SL= 8.3 mm	1628.26	±37.65	**SL= 8.3 mm**	421.39	±66.57

Table 2.24. Comparison of 50 % sample mortality (LC_{50}) and 50 % crawl-out response (CO_{50}) estimated by maximum likelihood logistic regression determinations for samples of *Physa acuta* exposed to various concentrations of cadmium chloride ($CdCl_2$). In LC_{50} determinations, samples (n = 150) were exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 – 5000 μg $CdCl_2.L^{-1}$, while, for CO_{50} determinations, samples (n = 90) were exposed to media with concentrations ranging from 0 - 2000 μg $CdCl_2.L^{-1}$. Values of LC_{50} for 12 h and 96 h exposure periods are provided for analysis of the entire sample without shell length (SL) as a covariate and with SL as a covariate for individuals in the 25th SL quantile (SL = 6.0 mm), median 50th SL quantile (median SL = 7.1 mm) and 75th SL quantile (SL = 8.3 mm). Values of CO_{50} for a 12 h exposure period are provided for the entire sample without shell length (SL) as a covariate and with SL as a covariate for individuals in the 25th SL quantile (SL = 6.7 mm), 50th SL quantile (median SL = 7.9 mm) and 75th SL quantile (SL = 9.1 mm).

	Lethal Concentration				**Crawl-out Response**		
SL for 50 % Response	**12 h LC_{50}**	**s.e.**	**96 h LC_{50}**	**s.e.**	**SL for 50 % response**	**12 h CO_{50}**	**s.e.**
Without SL As a Covariate	3733.73	±90.26	1589.26	±27.51	**Without SL as a Covariate**	294.81	±45.22
SL= 6.0 mm	3242.23	±102.42	1499.32	±33.53	**SL= 6.7 mm**	276.12	±77.74
SL= 7.1 mm	3653.72	±89.92	1577.21	±27.42	**SL= 7.9 mm**	349.07	±62.49
SL= 8.3 mm	4082.60	±125.42	1658.42	±32.04	**SL= 9.1 mm**	419.95	±66.21

the LC_{50} value most commonly used to measure aquatic toxicity. Wald values were computed for these comparisons for the entire sample without SL as a covariate and with SL as a covariate for the 25th SL quantile SL quantile, median 50th SL quantile and 75th SL quantile. In all cases, 12 h CO_{50}values proved significantly (p = 0.001) lower than corresponding 96 h LC_{50} values as detailed below. Maximum likelihood probit analyses without SL as a covariate comparing the 96 h LC_{50} to the 12 h CO_{50} response resulted in w = 1059.50, p = 0.001. All Wald comparisons of maximum likelihood probit analyses with SL as a covariate indicated significant differences between estimates of 12 h CO_{50} and 96 h LC_{50} values as follows: for the 25th SL quantile, w = 1596.80, p = 0.001; for the median 50 th SL quantile, W = 1074.53, p = 0.001; and for the 75th of SL quantile, W = 1417.52, p = 0.001. Wald Analysis comparing maximum likelihood logistic estimations of 96 h LC_{50} to the 12 h CO_{50} without SL as a covariate yielded a W of 756.80, p = 001. All Wald analyses comparing logistic regression estimates of 96 h LC_{50} and 12 h CO_{50} estimates with SL as a covariate proved significant as follows: for the 25th SL quantile, = 1124.26, p = 0.0; for the median 50th SL quantile, w = 751.86, p = 0.0; and for the 75th SL quantile, w= 1026.56, p = 0.0.

This study demonstrated a significant effect of $CdCl_2$ on crawl-out behavior in *P. acuta,* which could be used to quantify its sensitivity to this toxin. The crawl-out response observed on exposure to $CdCl_2$ was very similar that reported to occur in this species in the presence of predator or injured snails (McCarthy and Fisher, 2000). Changes in behavior on exposure to cadmium have also been recorded in the grass carp, *Ctenopharyngodon idellus* Val., 1844. Observed changes include swimming upside

down, swimming more slowly, respiratory difficulty and breathing at the surface (Yorulmazlar and Gül, 2003). Sub-lethal concentrations of cadmium also result in a significant decrease in aggressive interactions between exposed fish, which affected social dominance hierarchies. A significant decrease in aggressive interactions between individuals has been reported in rainbow trout, *Oncorhynchus mykiss*, exposed to cadmium. (Sloman *et al.*, 2003)

The results of this study indicated that crawl-out response measured as 12 h CO_{50} values was able to detect the deleterious effects of $CdCl_2$ exposure more quickly (12 h) and at lower concentrations than standard 96 h LC_{50} determinations (Tables 2.23 and 2.24). Crawl-out CO_{50} determinations also required a smaller number of organisms (90 versus 150 snails per test) and the crawl-out response was a more accurate and the endpoint more readily and accurately identifiable than snail death defined as immobility in this study. At 295.43 μg $CdCl_2 \cdot L^{-1}$ (probit) to 294.81 μg $CdCl_2 \cdot L^{-1}$ (logistic), CO_{50} values for *P. acuta* in response to $CdCl_2$ exposure are within the range of 48 h and 96 h LC_{50} values reported for the standard aquatic toxicity test species, fathead minnow, *Pimephales promelis* and water flea, *Ceriodaphnia dubia* (Table 2.22).

Conclusions

This study met the criteria of demonstrating an effect of the toxin ($CdCl_2$) on the model organism (Purchase *et al.,* 1998) in order to introduce a new test species (i.e., *Physa acuta*) that could replace use of vertebrate species in toxicity testing. Use of the invertebrate aquatic freshwater snail *P. acuta* in toxicity testing of $CdCl_2$ allowed information to be produced more rapidly, more accurately and/or less expensively in

terms of direct costs and/or labor time than existing tests utilizing standard species. This study meet a second requirement of providing additional information for prediction of toxicity through the introduction of a new toxicity testing methodology (Purchase *et al.*, 1998) through development of a toxicity testing methodology based on the crawl-out behavioral response of *P. acuta*.

The effect of cadmium chloride was demonstrated as a significant effect on the freshwater snail *P. acuta* using the standard toxicity tests of acute and chronic survival. Significant survival endpoints included the concentration that resulted in 50 % sample mortality, LC_{50}, and the time that resulted in 50 % sample mortality, LT_{50}. A comparison between maximum likelihood probit and logistic regression analysis for estimated of LC_{50} demonstrated essentially similar results, allowing comparison to previous studies. Values of LT_{50} in response to $CdCl_2$ exposure were also estimated by the relatively new discrete logistic failure time model regression (Hicks *et al*., 2000).

In addition, this study demonstrated that a new end-point, the crawl-out behavioral response was highly correlated to $CdCl_2$ concentration when analyzed from the logistic regression. This study allowed analysis of this effect using a smaller sample sizes than standard survival methods (90 versus 150), and a shorter overall study duration (12 h versus the standard acute exposure of 96 h). The crawl-out response end-point was easier to identify than the more common survival endpoint of death identified as immobilization. This experimental procedure also had the advantage of portability, allowing analysis to be conducted in the field.

CHAPTER 3

EFFECT OF CADMIUM CHLORIDE ON REPRODUCTION

Introduction

Purchase *et al*. (1998) examined the requirements for developing new toxicity testing methodologies to replace or supplement existing standard methodologies. They suggested that a basic requirement for development of new environmental toxicity test methodologies is that they must be capable of supplying useful information not produced by existing tests and/or produce the information more rapidly, accurately and/or less expensively in terms of direct costs and/or labor than existing standard methodologies. In addition, any newly proposed test methodology should be compatible with existing toxicity tests since no single test can provide all required information. Information resulting from a new methodology should also be capable of being converted into predictive models that allow decision makers to resolve issues more quickly or accurately or to use less information than required from traditional test results when making decisions. Thus, data generated from any new test format must be capable of being compared to that produced by existing standard tests. In addition, statistical analyses utilized to analyze data produced by any new toxicity testing methodology should minimize error leading to more accurate predictions of environmental toxicity and, thus, better decision-making on the part of regulators and industry (Purchase *et al*., 1998).

Any newly developed testing methodology must also be readily and inexpensively transferable among laboratories and produce accurately repeatable results among different

laboratories (Purchase *et al*., 1998). Finally, Purchase *et al.* (1998) highly recommend that any new methodologies provide replacement of vertebrate test species with invertebrate species whenever possible to avoid both the paperwork and public concerns associated with vertebrate toxicity and mortality testing. This chapter describes of the outcomes of sub-lethal cadmium chloride ($CdCl_2$) toxicity testing on aspects of reproduction in the common, North American, pulmonate, pond snail, *Physa acuta*. This testing was also part of an investigation of the efficacy of *P. acuta* as a new animal model for toxicity testing, thus, achieving the goals of developing new toxicity testing models and organisms set out by Purchase *et al.* (1998).

Several studies have investigated reproductive and developmental responses to cadmium exposure in snails (Cheung and Lam, 1998; Gomot, 1998; Holcombe *et al.,* 1984). An investigation of the effect of exposure to cadmium as $CdCl_2$ on the development of embryos of *P. acuta* was conducted (Cheung and Lam, 1998) at test concentrations of 0, 1000, 2000, 3000, and 4000 $\mu g\, CdCl_2 \cdot L^{-1}$ over an exposure period of 48 h. One egg mass laid by an adult snail in artificial water is placed in an aerated 100 ml glass beaker with 80 ml of test solution. This testing reveals that acute 24 h LC_{50} for the embryonic development in this species was 1270 $\mu g \cdot L^{-1}\, CdCl_2$ (95 % CI = 1130 - 1420) and the 48 h LC_{50} was 850 $\mu g\, CdCl_2 \cdot L^{-1}$ (95 % CI = 710 - 1010 $\mu g\, CdCl_2 \cdot L^{-1}$). A chronic toxicity test on embryo hatching was also conducted at exposure concentrations of 100, 200, 300, 400 and 500 $\mu g\ CdCl_2 \cdot L^{-1}$ in order to compare the number of embryos hatching after $CdCl_2$ exposures of 8 and 28 days. Eggs not hatching after 28 days were considered unhatchable. ANOVA indicated a significant decrease in percent hatching with increasing $CdCl_2$ concentration. Hatching is completely inhibited at concentrations greater than 210 $\mu g\ CdCl_2 \cdot L^{-1}$ (Cheung and Lam, 1998).

The toxic effects of cadmium on the sub-lethal responses of oviposition and embryo development have also been studied in the freshwater snail, *Lymnaea stagnalis* (Gomot, 1998). In this study, egg masses were collected twice per week during water changes and placed in Petri dishes with the same $CdCl_2$ concentration to which the ovipositing adult producing the egg mass had been exposed. Each week, the number of egg masses and number of eggs per mass produced by ovipositing adults were counted and embryo development recorded. Length of incubation and number of hatched juveniles were recorded for each treatment. After seven weeks, the mean number of eggs per mass produced under each tested $CdCl_2$ concentration was determined and the percentage of eggs hatched in each treatment is compared to the control held in media without $CdCl_2$. No significant differences were observed in the number of egg masses produced and number of eggs per egg mass between exposures to control media and Cd concentrations of 25, 50 and 100 $\mu g\, Cd^{+2} \cdot L^{-1}$. At a concentration of 200 $\mu g\, Cd^{+2} \cdot L^{-1}$ there was a significant decrease in the number of eggs per egg mass relative to controls, but not in number of egg masses oviposited. At a concentration of 400 $\mu g\, Cd^{+2} \cdot L^{-1}$ there was a significant decrease in both the number of egg masses oviposited and number of eggs per egg mass relative to controls. This study demonstrated that deleterious impacts on egg mass oviposition and number of eggs oviposited per egg mass increase in *L. stagnalis* with increasing $CdCl_2$ concentration.

Time to egg hatching in *L. stagnalis* was also affected by $CdCl_2$ exposure. In the control group eggs hatched 12-14 day subsequent to oviposition. Treatment groups exposed to 25 and 50 $\mu g\, Cd^{+2}$ (as $CdCl_2 \cdot L^{-1}$) started hatching 4-5 days later and the hatching period extended over a 10-day period. A hatching rate of 8 % was recorded at 100 $\mu g\, Cd^{+2}$ (as $CdCl_2 \cdot L^{-1}$), significantly reduced relative to the 15-21 % hatching rate recorded at 0, 25 or 50 $\mu g\, Cd^{+2}$ (as $CdCl_2 \cdot L^{-1}$).

Time to hatching, number of eggs per mass and hatching rate (0.4%) were all significantly reduced at 200 μg Cd^{+2} (as $CdCl_2 \cdot L^{-1}$). There was also a decrease in the size of embryos hatched at this Cd^{2+} concentration compared to controls (Gomot, 1998).

In a 26 d reproductive study using individuals of the freshwater pulmonate, *Aplexa hypnorum,* no oviposition occurred at cadmium exposure concentrations that significantly impacted survival determined from a 96 h LC_{50} of 152 μg $CdCl_2 \cdot L^{-1}$ (95 % CI = 88-262 μg $CdCl_2 \cdot L^{-1}$) (Holcombe *et al.,* 1984). In a 26 d embryo development study for this species, eggs were added to $CdCl_2$ concentrations ranging from 0 - 21.6 μg $CdCl_2 \cdot L^{-1}$. Eighty four percent of all eggs developed normally after four days of chronic exposure to these cadmium concentrations. Hatching occurred 6-9 days after oviposition only at concentrations of 7.82 μg $CdCl_2 \cdot L^{-1}$ and below. No hatching occurred at higher concentrations (>11.71 μg $CdCl_2 \cdot L^{-1}$). At 26 days, no embryos hatched in the 21.07 μg $CdCl_2 \cdot L^{-1}$ treatment, although one embryo hatched in the 21.56 μg $CdCl_2 \cdot L^{-1}$ treatment. There was a significant difference in the number of embryos surviving to the end of the 26 d test period between the control and treatment levels (i.e., 7.82, 11.71, 12.46 and 21.56 μg $CdCl_2 \cdot L^{-1}$) (Holcombe *et al.,* 1984).

The studies described above suggest that reproductive parameters in freshwater pulmonate snails could be used as effective measures of the sub-lethal impacts of cadmium toxicity in aquatic organisms. This section describes a study of the utility of fecundity and development rate parameters in the common, pulmonate, pond snail, *Physa acuta*, as a new model species for sub-lethal aquatic toxicity testing utilizing cadmium chloride ($CdCl_2$) as the toxicant. The effects of $CdCl_2$ exposure concentration and individual size on these endpoints were estimated from various statistical techniques in this study. Determinations of reproductive

endpoints were estimated over a wide range of $CdCl_2$ concentrations in order to provide the basis for comparison with the acute LC_{50} values determined for exposure of specimens of *P. acuta* to $CdCl_2$ in this study (see Chapter 2). Tested $CdCl_2$ concentrations ranged from 0 - 2000 μg $CdCl_2 \cdot L^{-1}$ for all studies except impact on egg diameter, which involved exposures to concentrations ranging from 0 - 300 μg $CdCl_2 \cdot L^{-1}$.

In their review of the lethal and sub-lethal impacts of heavy metal toxicity, Newman and McIntosh (1991) noted that animal body size impacted tissue metal concentration and level of toxicity. In spite of the widely accepted fact that there is an allometry between body size/age and toxicity tolerance, size of test specimens is rarely considered in aquatic toxicity testing (USEPA 2001). In this chapter, all reproductive endpoints were estimated with and without size measured as shell length (SL) as a covariate on the same reproductive data for samples of *P. acuta* utilizing SAS® (Cary, NC) computer program codes developed in collaboration with Dr. D. L. Hawkins of The University of Texas at Arlington. Analysis of the same reproductive data sets with and without size measured as SL as a covariate allowed comparison of the impacts of individual size distributions in samples of *P. acuta* on EC_{50} analysis and on the degree to which the size of individuals tested could impact decisions regarding environmentally acceptable levels of aquatic toxicants.

The research described in this chapter also examines several statistical methodologies for analyzing the reproduction and development data resulting from these studies. The impacts of $CdCl_2$ concentration on long-term reproductive responses were assessed in *P. acuta*, using effect concentration for 50 % sample egg mass oviposition (EC_{50}) as an indicator of toxicity. The EC_{50} value, the concentration required to induce 50 % reduction in individuals ovipositing one or more egg masses, was estimated by both the more standard maximum likelihood probit

regression analysis and the more recently developed and less commonly used maximum likelihood logistic regression analysis. Use of logistic regression analyses to estimate EC_{50} values has been recommended by statisticians (Finney, 1971), but has not been generally adopted in toxicity studies. One of the main objectives of this study was to analyze the same data sets for reproduction of samples of *P. acuta* on exposure to the toxic effects $CdCl_2$ and compare their outcomes in order to determine if there would be major differences in the EC_{50} predictions generated by probit versus logistic regression analysis, that would justify use of one statistical technique over the other.

Another analysis provided a formal evaluation of the effect of $CdCl_2$ exposure on the number of egg masses oviposited by adult specimens of *P. acuta.* Mean numbers of egg masses·individual^{-1} were estimated at each tested $CdCl_2$ concentration along with multiple pair-wise comparisons between concentrations.

Based on the studies of Cheung and Lam (1998), Gomot (1998) and Holcombe *et al.* (1984), which indicated that the oviposition decreased as a general response in freshwater pulmonate snails to increasing $CdCl_2$ concentrations, this study investigated the possibility of using fecundity measured as number of eggs oviposited·individual^{-1} as a response variable for estimating the sub-lethal toxic effects of $CdCl_2$ exposure. Regression analysis provided a determination of the effect of $CdCl_2$ on the number of eggs oviposited over a 672 h (28 d) exposure period.

In addition to the parameters discussed above, the impact of $CdCl_2$ exposure was also recorded for several developmental parameters in *P. acuta.* Among these was the fraction of eggs developing into embryos determined at weekly intervals over an observation period of 672 h (28 d). Regression analysis provided determinations of the effect of cadmium chloride on the

fraction of eggs developing into embryos based on $CdCl_2$ concentration, shell length and weeks after oviposition. Toxicity testing based on fraction of eggs developing into embryos would also require fewer test individuals than standard survival testing, provide an accurate, readily observable endpoint and be cost-effective.

Based on the studies of Cheung and Lam (1998), Gomot (1998), and Holcombe *et al.* (1984), which indicated that the percent of eggs hatching decreased as a general response in freshwater pulmonate snails to $CdCl_2$ exposure, this study investigated egg hatching as a toxicity testing methodology in *P. acuta* using two different developmental endpoints. These two endpoints included, 1) the concentration of $CdCl_2$ resulting in 50 % of individuals ovipositing egg masses in which at least one egg hatched into a juvenile (EC_{50}) and 2) the number of eggs hatching over a 672 h (28 d) exposure period. Toxicity testing based on the egg hatching response would require fewer test individuals than standard survivorship testing, provide an accurate, readily observable endpoint and be more cost-effective and readily adapted to the field.

Prior studies (Cheung and Lam, 1998, Gomot, 1998, and Holcombe *et al.,* 1984) have recorded a temporal delay in embryonic development to hatching with increasing $CdCl_2$ concentration in several species of freshwater pulmonate snails. This study investigated the possibility of using the time to hatching of eggs oviposited by specimens of *P. acuta* as a potential endpoint variable in determining sub-lethal $CdCl_2$ toxicity in this species. Regression analysis provided a determination of the effect of $CdCl_2$ exposure on the number of days to egg hatching over a 672 h (28 d) exposure period.

This chapter also describes the efficacy of a new toxicity testing methodology involving the variation in the size of eggs oviposited by specimens of *P. acuta* measured as egg diameter when exposed to a range of sub-lethal $CdCl_2$ concentrations. To date, a thorough literature

search as part of this research has revealed no published toxicity studies of aquatic pollutants that have used the diameter of individual eggs as an endpoint parameter for any marine, estuarine or freshwater animal test species. One study revealed a significant decrease in the related variable of juvenile size at hatching on exposure to $CdCl_2$ in the freshwater pulmonate snail, *L. stagnalis* (Gomot, 1998), indicating that egg size was a potentially efficacious end point in toxicity testing with *P. acuta.* Toxicity testing based on the egg diameter response would be more rapid (< 96 h), require fewer test individuals than standard survival testing, provide an accurate, readily observable endpoint and be cost effective.

Methods

Collection and Maintenance of Experimental Specimens

Specimens for testing the sub-lethal effects of $CdCl_2$ exposure on individual fecundity, egg size and hatching success in *P. acuta* were collected from a section of Trader Horse Creek in Arlington, Texas, which ran along the south side of the University of Texas at Arlington campus, Arlington, Texas. Individuals were collected by removing them from the upper surface of rocks on the creek substratum or from rocks lifted from the substratum. After collection, snails were returned immediately to the laboratory and held in 21 x 21 x 12.5 cm plastic aquaria filled with 5 L of artificial pond water (composition described below) at a constant temperature of 25 °C. Tank media was replaced every three days until snails were used in experiments. Snails were fed *ad libitum* with flake aquarium fish food (Total Tropical Gourmet Flake Blend® fish food, Wardley, Secaucus, NJ) after each replacement of holding media. Snails were held in the laboratory for no longer than 10 days prior to use in experiments.

Holding and sub-lethal toxicity testing of fecundity, egg diameter and hatching success in *P. acuta* was conducted in artificial pond water (Thomas Dietz personal communication)

composed of 29 $mg \cdot L^{-1}$sodium chloride (NaCl), 17 $mg \cdot L^{-1}$sodium bicarbonate ($NaHCO_3$), 12 $mg \cdot L^{-1}$ potassium chloride (KCl), 15 $mg \cdot L^{-1}$ magnesium sulfate ($MgSO_4$), and 50 $mg \cdot L^{-1}$ Ca^{+2} as calcium chloride ($CaCl_2$) dissolved in distilled water. Appropriate amounts of $CdCl_2$ were added to this artificial medium to achieve the range of $CdCl_2$ treatments utilized in the experiments described below.

Tests of the Effects of $CdCl_2$ Exposure on Fecundity, Hatching Success and Egg Diameter

The sub-lethal effects of exposure to $CdCl_2$ on fecundity, number of egg masses oviposited, number of eggs per mass, and hatching success in *P. acuta* were investigated in this study. In these tests, 30 individual snails were held in separate 88.7 ml plastic cups measuring 5.5 cm wide by 4.5 cm in height filled with 60 ml of medium at $CdCl_2$ test concentrations of 0, 10, 50, 100, 150, 200, 250, 300, 350, 400, 600, 800, 1000, 1200, 1400, 1600 and 2000 μg $CdCl_2 \cdot L^{-1}$ at 25 °C in a constant temperature incubator. Media in all test cups was changed every 3 days during which Total Tropical Gourmet Flake Blend® fish food (Wardley, Secaucus, NJ) was added to the cup allowing individuals to be fed *ad libitum* through the course of the experiment. For fecundity determinations, containers were observed for egg masses at 72 h intervals throughout the course of the experiment. For each snail oviposited egg masses were removed for each 72 h exposure period from the cup by carefully cutting them from their attachment sites with a scalpel blade. The number of eggs laid per egg mass was counted under a dissecting microscope using the procedure developed for *P. acuta* by McMahon (1975). Total number of egg masses and eggs and number of eggs per mass was determined for each individual at each tested $CdCl_2$ concentration during the 672 h (28 d) exposure period. The size of all ovipositing adult snails was measured as shell length to the nearest 0.1 mm with a dial micrometer

At each tested $CdCl_2$ concentration in which snails oviposited egg masses, at least one egg mass from each ovipositing individual was placed in an 88.7 ml cup measuring 5.5 mm in diameter by 4.5 mm in height filled with 60 ml of medium with a $CdCl_2$ concentration equivalent to that in which the egg mass was produced (range = 0-2000 μg $CdCl_2 \cdot L^{-1}$). The number of eggs in the mass was counted under a dissecting microscope prior to placement in the cup. Thereafter, cups were maintained in a constant temperature incubator at 25 °C ±1 °C. Egg masses were maintained in control or $CdCl_2$ treated media for period of 28 days, longer than required for egg development and hatching at 25 °C (after the method of Cheung and Lam, 1998). Four and eight days after placement in the cups, the number of embryos formed in each mass was determined under a dissecting microscope. During these observation periods, all embryos in an egg mass were inspected for rolling movement induced by the activity of epithelial cilia, heart beat and foot movement. Embryos not displaying these indications of viability were recorded as dead. After the initial eight-day observation period, cups were examined daily for the remaining 20 days and the number of hatched juveniles counted. Cup media was replaced every three days during the 28 d observation period. These observations yielded the fraction of eggs in each mass developing into embryos and the fraction of hatched eggs in each mass relative to $CdCl_2$ concentration.

In a separate test, the diameter of 20 eggs was determined in one or more egg masses initially oviposited by 15 adult individuals of *P. acuta* individually held in 60 ml of test media as described above at $CdCl_2$ concentrations of 0, 10, 100, 200 and 300 μg CdCl2$\cdot$ L^{-1} under a test temperature of 25 °C in a constant temperature incubator. After the first 72 h of exposure, containers were inspected for the presence of egg masses every 24 h. Any egg masses found were removed as described above and placed on a slide with a cover slip and viewed at 10X

power under a dissecting microscope. An ocular micrometer mounted in the dissecting microscope was utilized to measure the diameter of all freshly laid eggs (eggs containing embryos were not utilized) in the egg masses. The diameters of eggs in eggs masses were determined in this manner until a sample size of 20 eggs was achieved for each of 15 ovipositing individuals at each tested $CdCl_2$ concentration. The shell lengths (SL) of ovipositing adult snails were measured to the nearest 0.1 mm with a dial micrometer.

Data Analysis

Mean noncumulative egg masses oviposited and cumulative mean eggs oviposited per individual were determined at each consecutive 72 h observation period at each $CdCl_2$ treatment concentration over the course of the 672 h observation period. In order to investigate the effects of $CdCl_2$ exposure on the number of egg masses·individual^{-1}, number eggs·mass·individual^{-1} and time to egg hatching, a generalized linear regression model based on a poisson distribution was selected because the response was a discrete value, and because shell length (SL) was included as a covariate (Nelder and Wedderburn, 1972). All analyses were carried out without and with adult size measured as SL as a covariate. Treatment means for all endpoint variables were examined for significance with a Bonferroni multiple pair-wise comparison test.

Probit analysis is the conventional methodology for determination of acute or chronic survivorship LC_{50} or sub-lethal EC_{50} values (Finney, 1971). In this research maximum likelihood analyses were used to estimate 50 % effect concentration (EC_{50}) utilizing endpoint values of oviposition, multiple oviposition in a 72 h observation period and egg hatching using the probit function. The likelihood was defined as being proportional to the joint probability of all observations in the study. Logistic regression also uses maximum likelihood to estimate EC_{50}

values. To determine the the probability of an individual demonstrating the response of oviposition, multiple oviposition over a 72 h observation period, or egg hatching in a given interval without SL as a covariate the following generalized linear regression model with either the probit or logistic distribution is utilized: $\Gamma^{1}(p) = b_0 + b_1(Lconcentration+1)$. To determine the the probability of an individual demonstrating the response of oviposition, multiple oviposition over a 72 h observation period or egg hatching, in a given interval with SL as a covariate the following generalized linear regression model with either the probit or logistic distribution was utilized:

$\Gamma^{1}(p) = b_0 + b_1(Lconcentration+1)+b_2\ (SL-SL^{0})+b_3[(Lconcentration+1)*SL-SL^{0}]$.

For this analysis, snails ovipositing an egg mass, having multiple egg mass oviposition or having an egg hatch during each 72 h observation period were coded as 1 and snails not ovipositing or having a juvenile hatch during the test period were coded as 0. The proportion of 0 or 1 was determined using a logit transformation. Logistic regression allowed the use of covariates such as individual size in determination of EC_{50} values (Dytham, 2003; Floyd, 2001).

As described above, maximum likelihood probit and logistic regressions were used to describe the relationship between oviposition, multiple oviposition in a 72 h observation period and egg hatching and the predictors, $CdCl_2$ concentration and adult size as SL. The 50 % effect $concentration_0$ (EC_{50}), the statistically estimated concentration causing the response (i.e. presence of egg mass, multiple egg masses over a 72 observation period or hatching of a juvenile) for 50 % of the sample population over a fixed time was calculated for each of these three endpoint variables from maximum likelihood probit and logistic regression methods at consecutive 24-72 h intervals over the entire 672 h exposure period depending on the test (see above). In all three tests, results were analyzed without and with adult SL as a covariate in order

to demonstrate the effect of size on endpoint variables. The equation utilized to determine the probability of of an individual ovipositing, laying multiple eggmasses during a 72 h observation period and egg hatching in a given interval was:

$\Gamma^{1}(p) = b_0 + b_1(Lconcentration+1)+b_2\ (SL-SL^0)+b_3[(Lconcentration+1)*(SL-SL^0)]$.

Maximum likelihood estimates are interpreted as in the survival analysis (see Chapter 2).

Fraction of total eggs oviposited·individual^{-1} developing into embryos was determined at 7, 14 and 21 days of exposure to each tested $CdCl_2$ concentration. The resulting data was analyzed using a generalized linear model regression analysis allowing repeated measures (Nelder and Wedderburn, 1972). All analyses were carried out without and with adult size measured as SL as a covariate. Treatment means for all endpoint variables were examined for significance with a Bonferroni multiple pair-wise comparison test.

The effect of $CdCl_2$ on egg diameter was analyzed with a mixed regression model that allowed both fixed and random effects, due to its repeated measure design involving measuring of the diameter of 20 eggs from a single adult individual (Sokol and Rolf, 1995). Data were analyzed without and with adult size measured as SL as a covariate. A Bonferroni multiple pair-wise comparison analysis was used to examine significance among treatment means.

Results

Fecundity

Egg Mass Oviposition

There was an evident impact of chronic $CdCl_2$ exposure on the **r**aw percentage of adults in samples of *Physa acuta* ovipositing egg masses at concentrations ranging from 0-2000 μg $CdCl_2 \cdot L^{-1}$ over the 672 h (28 day) exposure period. The percent sample individuals ovipositing at least one egg mass during a 72 h observation period declined from values ranging from

6.06-78.79 % at 0 μg $CdCl_2 \cdot L^{-1}$ to near-zero levels at $CdCl_2$ concentrations ≥ 1000 μg $CdCl_2 \cdot L^{-1}$ over the 672 h (28 d) exposure period (Table 3.1, Fig. 3.1).

Values greater than 50 % of sampled individuals ovipositing an egg mass occurred only at concentrations of 0, 5 and 150 μg $CdCl_2 \cdot L^{-1}$ during the first 72 h of exposure. The highest percent sample egg mass oviposition occurred in concentrations of 0 μg $CdCl_2 \cdot L^{-1}$ (78.79 %) followed by 5 μg $CdCl_2 \cdot L^{-1}$ (73.33 %). No oviposition was recorded at concentrations of 150, 300, 800 and 1000 μg $CdCl_2 \cdot L^{-1}$ in this initial 72 h period (Table 3.1, Fig. 3.1).

Between 72 and 144 h of exposure, the highest percent of sample egg mass oviposition occurred at 400 μg $CdCl_2 \cdot L^{-1}$ (16.67 %) followed by 100 μg $CdCl_2 \cdot L^{-1}$ (10.00 %). No oviposition occurred in concentrations of 150, 200, 300, 450, 600, 800, 1000, 1200, and 2000 μg $CdCl_2 \cdot L^{-1}$ (Table 3.1, Fig. 3.1). Between 144 and 216 h of exposure, the highest percent of egg mass oviposition occurred in concentrations 150 μg $CdCl_2 \cdot L^{-1}$ (60.00 %) followed by 10 μg $CdCl_2 \cdot L^{-1}$ (46.67 %). No oviposition was recorded at concentrations of 800, 1000 and 1200 μg $CdCl_2 \cdot L^{-1}$. Between 216 and 288 h of exposure, the highest percent of egg mass oviposition occurred in 0 μg $CdCl_2 \cdot L^{-1}$ (51.51 %) followed by 450 and 600 μg $CdCl_2 \cdot L^{-1}$ (33.33 %). No oviposition was recorded at concentrations of 10, 50, 200, 1000, 1400, 1600 and 2000 μg $CdCl_2 \cdot L^{-1}$. Between 288 and 360 h of exposure, the highest percent of egg mass oviposition occurred at 5 μg $CdCl_2 \cdot L^{-1}$ (40.0 %) followed by 350 μg $CdCl_2 \cdot L^{-1}$ (23.33 %). No oviposition was recorded at 200, 300, 400, 450, 600, 1000, 1200, 1400 and 2000 μg $CdCl_2 \cdot L^{-1}$. Between 360 and 432 h exposure, the highest percent of egg mass oviposition occurred at 0 μg $CdCl_2 \cdot L^{-1}$ (18.18 %) followed by 250 μg $CdCl_2 \cdot L^{-1}$ (10.00 %). Ovisposition was not recorded at 5, 10, 50,

Table 3.1 Effect of cadmium chloride ($CdCl_2$) concentration and exposure time on the number of adult individuals in samples of *Physa acuta* ovipositing egg masses over a concentration range of 0-2000 µg $CdCl_2 \cdot L^{-1}$ as determined every 72 h during a 672 h (28d) total exposure period [n = number individuals in sample, # Ovipos. (%) = percent of sampled individuals ovipositing and egg mass or masses; Masses·Ind^{-1} = mean number of egg masses oviposited per individual in the sample.

		72 h Exposure		**144 h Exposure**		**216 h Exposure**		**288 h Exposure**		**360 h Exposure**	
µg $CdCl_2 \cdot L^{-1}$	**n**	**# Ovipos. (%)**	**Masses·Ind.$^{-1}$**	**# Ovipos. (%)**	**Masses·Ind.$^{-1}$**	**# Ovipos. (%)**	**Masses·Ind.$^{-1}$**	**# Ovipos. (%)**	**Masses·Ind.$^{-1}$**	**# Ovipos. (%)**	**Masses·Ind.$^{-1}$**
0	33	78.79	1.79	6.06	1.85	18.18	2.09	51.51	2.85	12.12	3.12
5	30	73.33	0.90	3.33	0.93	3.33	0.96	3.33	1.06	40.00	1.50
10	30	16.66	0.27	0.0	0.27	46.67	0.77	0.0	0.77	3.33	0.77
50	30	3.33	0.03	3.33	0.03	33.33	0.43	0.0	0.43	6.67	0.50
100	30	16.67	0.17	10.00	0.27	30.00	0.73	13.33	1.07	10.00	1.20
150	30	0.00	0.00	0.00	0.00	60.00	0.60	3.33	0.63	10.00	0.80
200	30	40.00	0.40	0.00	0.40	36.67	0.87	0.00	0.87	0.00	0.87
250	30	26.67	0.37	3.33	0.43	13.33	0.60	6.67	0.73	10.00	0.83
300	30	0.00	0.00	0.00	0.00	3.33	0.03	13.33	0.27	0.00	0.27
350	30	13.33	0.17	6.67	0.27	33.33	0.67	6.67	0.77	23.33	1.07
400	30	20.00	0.20	16.67	0.43	13.33	0.50	10.00	0.70	0.00	0.70
450	30	3.33	0.10	0.00	0.10	43.33	0.67	33.33	1.07	0.00	1.07
600	30	6.67	0.07	0.00	0.07	20.00	0.30	33.33	0.67	0.00	0.67
800	30	0.00	0.0	0.00	0.0	0.00	0.0	20.00	0.20	3.33	0.23
1000	30	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0
1200	30	3.33	0.03	0.00	0.03	0.00	0.03	0.00	0.03	0.00	0.03
1400	30	16.67	0.13	6.67	0.20	3.33	0.20	0.00	0.20	0.00	0.20
1600	30	10.00	0.10	6.67	0.20	6.67	0.27	0.00	0.27	3.33	0.27
2000	30	6.67	0.07	0.00	0.07	3.33	0.10	0.00	0.10	0.00	0.10

Table 3.1 Continued

		432 h Exposure		504 h Exposure		576 h Exposure		648 h Exposure		672 h Exposure	
µg $CdCl_2 \cdot L^{-1}$	n	# Ovipos. (%)	Masses·Ind.$^{-1}$	Oviposit. (%)	Masses·Ind.$^{-1}$	# Ovipos. (%)	Masses·Ind.$^{-1}$	# Ovipos. (%)	Masses·Ind.$^{-1}$	# Ovipos. (%)	Masses·Ind.$^{-1}$
0	33	18.18	3.33	54.55	4.03	27.27	4.39	9.09	4.52	36.36	4.94
5	30	0.00	1.50	33.33	1.90	3.33	1.93	0.00	1.93	20.00	1.93
10	30	0.00	0.77	0.0	0.77	3.33	0.80	0.00	0.80	20.00	1.03
50	30	0.00	0.50	20.00	0.77	0.00	0.77	0.00	0.77	6.67	0.83
100	30	6.67	1.30	6.67	1.40	3.33	1.50	3.33	1.53	3.33	1.60
150	30	0.00	0.80	6.67	0.87	13.33	1.00	0.00	1.00	26.67	1.33
200	30	0.00	0.87	0.00	0.87	13.33	1.03	0.00	1.03	0.00	1.03
250	30	10.00	0.97	13.33	1.20	10.00	1.40	0.00	1.40	3.33	1.43
300	30	0.00	0.27	0.00	0.27	16.67	0.43	0.00	0.43	46.67	1.00
350	30	0.00	1.07	3.33	1.10	3.33	1.13	0.00	1.13	6.67	1.20
400	30	0.00	0.70	3.33	0.73	0.00	0.73	0.00	0.73	0.00	0.73
450	30	0.00	1.07	10.00	1.23	3.33	1.27	0.00	1.27	23.33	1.53
600	30	0.00	0.67	10.00	0.80	0.00	0.80	0.00	0.80	0.00	0.80
800	30	0.00	0.23	23.33	0.47	0.00	0.47	0.00	0.47	0.00	0.47
1000	30	0.00	0.0	0.00	0.00	0.00	0.0	0.00	0.0	0.00	0.0
1200	30	0.00	0.03	0.00	0.03	0.00	0.03	0.00	0.03	0.00	0.03
1400	30	0.00	0.20	0.00	0.20	0.00	0.20	0.00	0.20	0.00	0.27
1600	30	0.00	0.27	0.00	0.27	0.00	0.27	0.00	0.27	0.00	0.33
2000	30	0.00	0.10	0.00	0.10	0.00	0.10	0.00	0.10	0.00	0.10

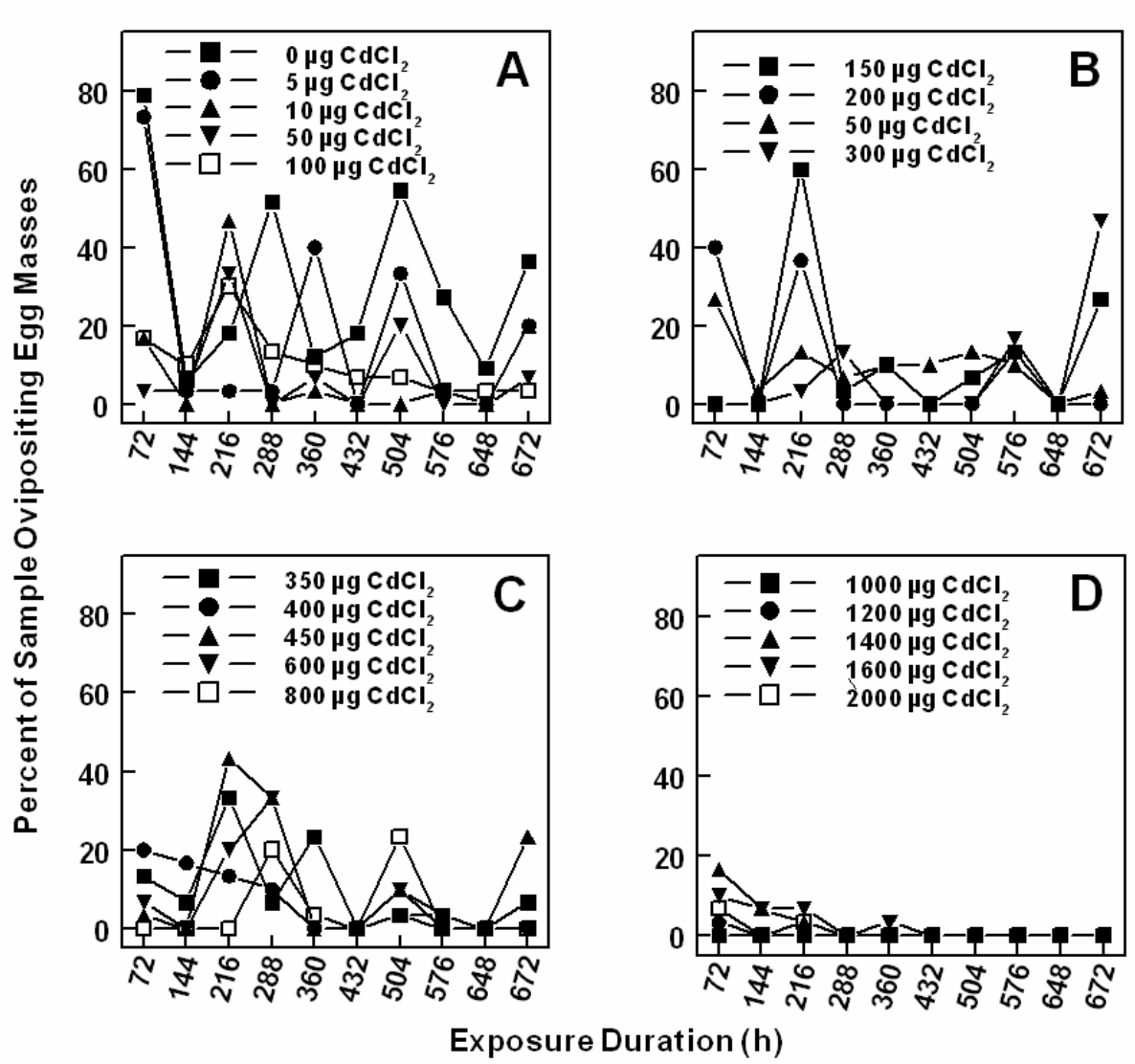


Fig.3.1. The effect of exposure of samples of *Physa acuta* to various concentrations of cadmium chloride ($CdCl_2$) on the percent of individuals ovipositing at least one egg mass (vertical axis) over a total exposure period of 672 h (i.e., 28 d, horizontal axis). The results for each tested exposure concentration are as indicated in the legend displayed in the center of the figure.

150, 200, 300, 350, 400, 450, 600, 800, 1000, 1200, 1400 and 2000 μg $CdCl_2 \cdot L^{-1}$. Between 432 and 504 h exposure, the highest percent of egg mass oviposition occurred at 0 μg $CdCl_2 \cdot L^{-1}$ (54.55 %) followed by 5 μg $CdCl_2 \cdot L^{-1}$ (33.33 %). No oviposition occurred at 10, 200, 300, 1000, 1200, 1400, 1600 and 2000 μg $CdCl_2 \cdot L^{-1}$. Between 504 and 576 of exposure, the highest percent of egg mass oviposition occurred at 0 μg $CdCl_2 \cdot L^{-1}$ (27.27 %) followed by 300 μg $CdCl_2 \cdot L^{-1}$ (16.67 %). No oviposition was recorded at 50, 400, 600, 800, 1000, 1200, 1400, 1600 and 2000 μg $CdCl_2 \cdot L^{-1}$. Between 576 and 648 h of expsosure, the highest percent of egg mass oviposition occurred at 0 μg $CdCl_2 \cdot L^{-1}$ (9.09 %) followed by 100 μg $CdCl_2 \cdot L^{-1}$ (3.33 %). There was no oviposition at 5, 10, 50, 150, 200, 250, 300, 350, 400, 450, 600, 800, 1000, 1200, 1400, 1600 and 2000 μg $CdCl_2 \cdot L^{-1}$ (Fig. 3.1). Between 648 and 672 h of exposure, the highest percent of egg mass oviposition occurred at 300 μg $CdCl_2 \cdot L^{-1}$ (46.67 %) followed by 0 μg $CdCl_2 \cdot L^{-1}$ (36.36 %), with no oviposition recorded at 200, 400, 600, 800, 1000, 1200, 1400,1600 and 2000 μg $CdCl_2 \cdot L^{-}1$ (Table 3.1, Fig. 3.1).

Three concentrations demonstrated values of percent sample egg mass oviposition above 50 % at least once during the 672-h exposure period (Table 3.1, Fig. 3.1). At 0 $CdCl_2 \cdot L^{-1}$, sample oviposition remained above 50 % in three observation periods (i.e., 0-72 h, 216-288 h and 432-504 h. Sample percent oviposition rose above 50 % in only one observation period at 5 μg $CdCl_2 \cdot L^{-1}$ (0-72 h) and 150 μg $CdCl_2 \cdot L^{-1}$ (144-216 h). At least some oviposition in all observation periods throughout the 672 h exposure was recorded only at 0 μg $CdCl_2 \cdot L^{-1}$. In all tested $CdCl_2$ concentrations (i.e., 5-2000 μg $CdCl_2 \cdot L^{-1}$) complete inhibition of oviposition occurred in at least one 72 h observation period during the 672 h exposure (Table 3.1, Fig. 3.1). These data indicate that increasing $CdCl_2$ concentration from 5-2000 $CdCl_2 \cdot L^{-1}$ progressively

inhibit oviposition in *P. acuta* with oviposition essentially ceasing at concentrations ≥ 1000 μg $CdCl_2 \cdot L^{-1}$ (Table 3.1, Fig. 3.1).

Cumulative Mean Number Egg Masses Oviposited Per Individual

Samples of *P. acuta* exposed to 0 μg $CdCl_2 \cdot L^{-1}$ had the highest cumulative egg mass oviposition rate measured as mean cumulative number of egg masses oviposited per individual per 72 h observation period over the entire 672 h duration of exposure to $CdCl_2$. Mean cumulative number of egg masses oviposited per individual over the entire 672 h exposure period were 4.94 egg masses·individual^{-1} at 0 μg $CdCl_2 \cdot L^{-1}$; 1.93 at 5 μg $CdCl_2 \cdot L^{-1}$; 1.03 at 10 μg $CdCl_2 \cdot L^{-1}$; 0.83 at 50 μg $CdCl_2 \cdot L^{-1}$; 1.60 at100 μg $CdCl_2 \cdot L^{-1}$; 1.33 at 150 μg $CdCl_2 \cdot L^{-1}$; 1.03 at 200 μg $CdCl_2 \cdot L^{-1}$; 1.43 at 250 μg $CdCl_2 \cdot L^{-1}$; 1.00 at 300 μg $CdCl_2 \cdot L^{-1}$; 1.20 at 350 μg $CdCl_2 \cdot L^{-1}$; and 1.53 egg masses·individual^{-1} at 450 μg $CdCl_2 \cdot L^{-1}$ (Table 3.1, Fig. 3.2). At concentrations of 400 - 2000 μg $CdCl_2 \cdot L^{-1}$ the range of cumulative egg mass oviposition after 672 h of exposure ranged from 0-0.83 egg masses·individual^{-1} (Table 3.1, Fig. 3.2). Like the data on egg mass oviposition described above, this data indicates that exposure to $CdCl_2$ at concentrations as low as 5 μg $CdCl_2 \cdot L^{-1}$ has major detrimental impacts on fecundity in *P. acuta*, suggesting that this parameter may be a highly efficacious endpoint for toxicity testing with this species. Statistical analyses of various fecundity and developmental endpoints in response to $CdCl_2$ exposure are detailed below to further assess their utility in toxicity testing with this species.

Egg Mass Oviposition EC_{50} Analysis

Maximum likelihood probit analysis was conducted to determine the effect of $CdCl_2$ concentration on egg mass oviposition by specimens of *P. acuta* without SL as a covariate. This analysis revealed a significant relationship between egg mass oviposition and $CdCl_2$

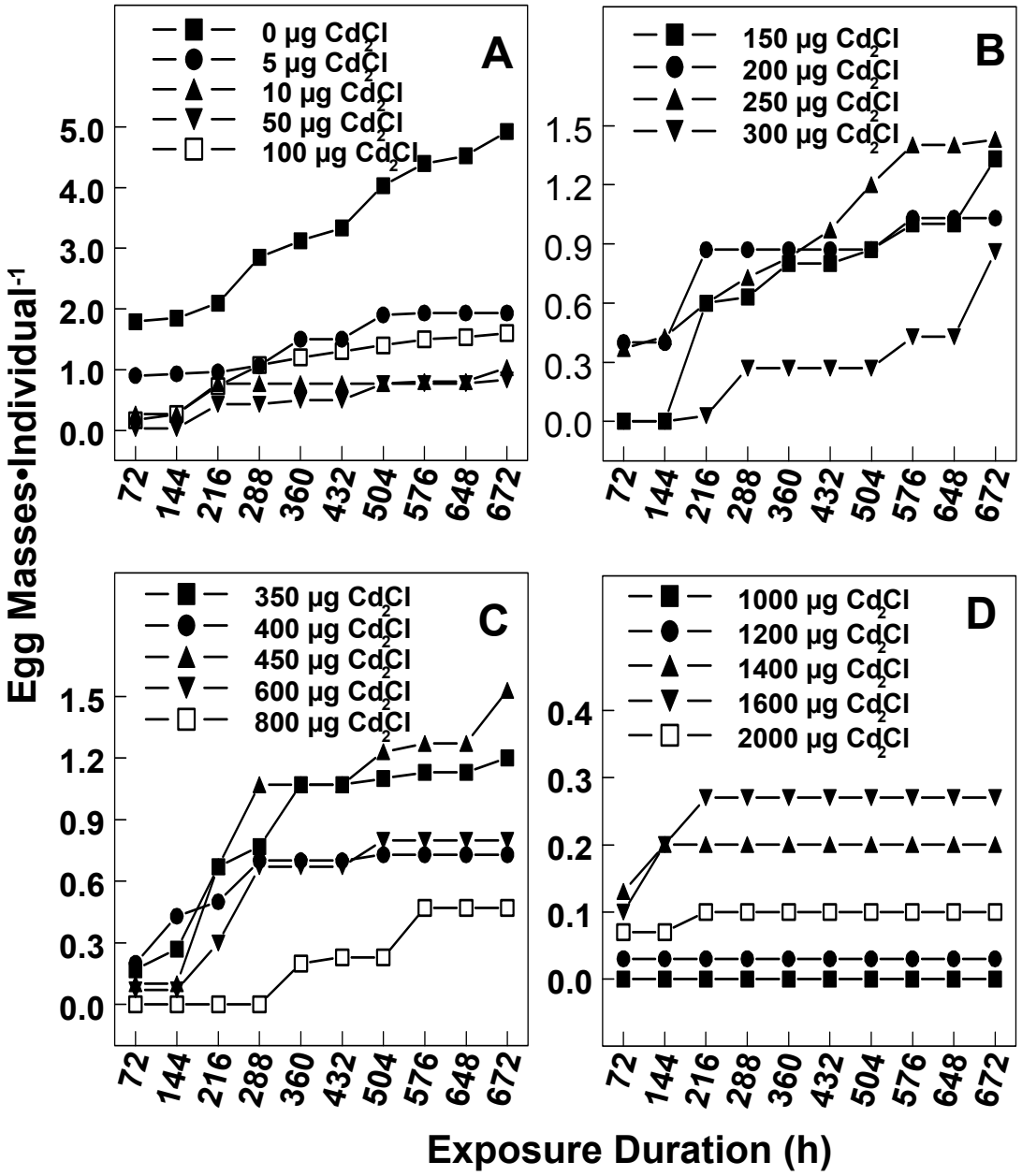


Fig.3.2 Effect of exposure of samples of *Physa acuta* to cadmium chloride ($CdCl_2$) on raw mean number of egg masses oviposited per individual (egg masses·individual^{-1}) per 72 h observation period (vertical axis) during a total exposure period of 672 h (i.e., 28 d, horizontal axis) for A) Exposure to $CdCl_2$ concentrations ranging from 0-100 µg·L^{-1}. B) Exposure to $CdCl_2$ concentrations ranging from 150-300 µg·L^{-1}. C) Exposure to $CdCl_2$ concentrations ranging from 350-800 µg·L^{-1}. D) Exposure to $CdCl_2$ concentrations ranging from 1000-2000 µg·L^{-1}. Note that $CdCl_2$ concentration ranges vary among the different figure panels.

concentration at each tested 72 h exposure duration over a 672 observation period across which chi-square and p values ranged from chi-square = 86.23, p <0.0001 at 432-672 h to chi-square = 87.98, p <0.0001 at 144 h (Table 3.2). Egg mass EC_{50} values decreased from 188.203 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±42.144) at an exposure time of 672 h through 168.737 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±39.715) at an exposure time of 72 h (Table 3.3, Fig. 3.3 A).

Maximum likelihood probit analysis allowed egg mass oviposition EC_{50} values to also be computed with ovipositing adult SL as a covariate. This analysis did not reveal a significant correlation between adult SL and egg mass oviposition over the 672 h exposure period with chi-squares ranging from 0.50, p = 0.477 at 72 h (3 d) to 0.94 at 216 h, p = 0.3335 at 216 h (9 d) (Table 3.4), but did reveal a significant effect of $CdCl_2$ concentration. There was also no significant relationship between egg mass oviposition and the interaction between concentration and SL demonstrated throughout the 672 h exposure period with chi-squares ranging from 1.56, p = 0.2113 at 216 h (9 d) to 2.43 p = 0.1189 at 72 h (3d) (Table 3.4). Because neither SL or the concentration-SL interaction significantly effected egg mass oviposition, egg mass oviposition EC_{50} values were computed only for the median 50th sample SL quantile (Median SL = 8.7 mm) (Table 3.3, Fig. 3.3 B). For an individual with a median SL of 8.7 mm in the 50th sample SL quantile, there was a significant relationship between egg mass and $CdCl_2$ concentration at each tested 72 h exposure duration through the entire 672 h exposure period across which chi-square and p values ranged from chi-square = 75.91, p <0.0001 at 432-672 h to chi-square = 77.50, p <0.0001 at 72 h (Table 3.2). Egg Mass EC_{50} values decreased from 127.154 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±26.787) at 672 h through 107.927 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±23.348)

Table 3.2 Chi-square and probability (p) values for maximum likelihood probit regression analysis determinations of chronic cadmium chloride ($CdCl_2$) effect concentration values (EC_{50} values) for 50 % of individuals of *Physa acuta* ovipositing egg masses when exposed to media with concentrations ranging from 0 - 2000 µg $CdCl_2 \cdot L^{-1}$ over a total exposure period of 672 h (28 days). Chi-square values are provided for analysis carried out without shell length (SL) as a covariate to describe the probability of an individual laying an eggmass in the given interval: $\Gamma^{1}(p) = b_0 + b_1(Lconcentration+1)$.The probability of of an individual laying an eggmass in a given interval: $\Gamma^{1}(p) = b_0 + b_1(Lconcentration+1)+b_2\ (SL-SL^0)+b_3[(Lconcentration+1)*SL-SL^0]$ with shell length as a covariate allowing determination of EC_{50} values for individuals in the 50th SL quantile (median SL = 8.7 mm).

Exposure Duration (hours)	Chi-Square SL not a Covariate	P	Exposure Duration (hours)	Chi-Square Median SL = 8.7 mm	P
72	87.30	<0.0001*	72	77.50	<0.0001*
144	87.98	<0.0001*	144	78.88	<0.0001*
216	86.70	<0.0001*	216	77.62	<0.0001*
288	87.59	<0.0001*	288	77.36	<0.0001*
360	87.59	<0.0001*	360	77.36	<0.0001*
432	86.23	<0.0001*	432	75.91	<0.0001*
504	86.23	<0.0001*	504	75.91	<0.0001*
576	86.23	<0.0001*	576	75.91	<0.0001*
648	86.23	<0.0001*	648	75.91	<0.0001*
672	86.23	<0.0001*	672	75.91	<0.0001*

* indicates a significant difference at $p < 0.005$

Table 3.3 Sub-lethal reproduction sample egg mass effect concentration values (EC_{50}) estimated by maximum likelihood probit regression analysis for 50 % of individuals of *Physa acuta* ovipositing egg masses when exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 - 2000 µg $CdCl_2 \cdot L^{-1}$ at 72 h intervals over a total exposure period of 672 h (28 days). Egg mass EC_{50} values are provided for analyses of the entire sample without shell length (SL) included as a covariate, and with inclusion of SL as a covariate for individuals in the median 50th SL quantile (median SL = 8.7 mm).

Exposure Time (h)	EC_{50} SL not a Covariate	s.e.	EC_{50} Med. SL = 8.7 mm	s.e.
72	168.737	±39.715	107.927	±23.348
144	170.695	±39.766	109.618	±23.276
216	173.060	±40.375	113.233	±24.083
288	185.538	±41.501	124.959	±26.108
360	185.538	±41.501	124.959	±26.108
432	188.203	±42.144	127.154	±26.787
504	188.203	±42.144	127.154	±26.787
576	188.203	±42.144	127.154	±26.787
648	188.203	±42.144	127.154	±26.787
672	188.203	±42.144	127.154	±26.787

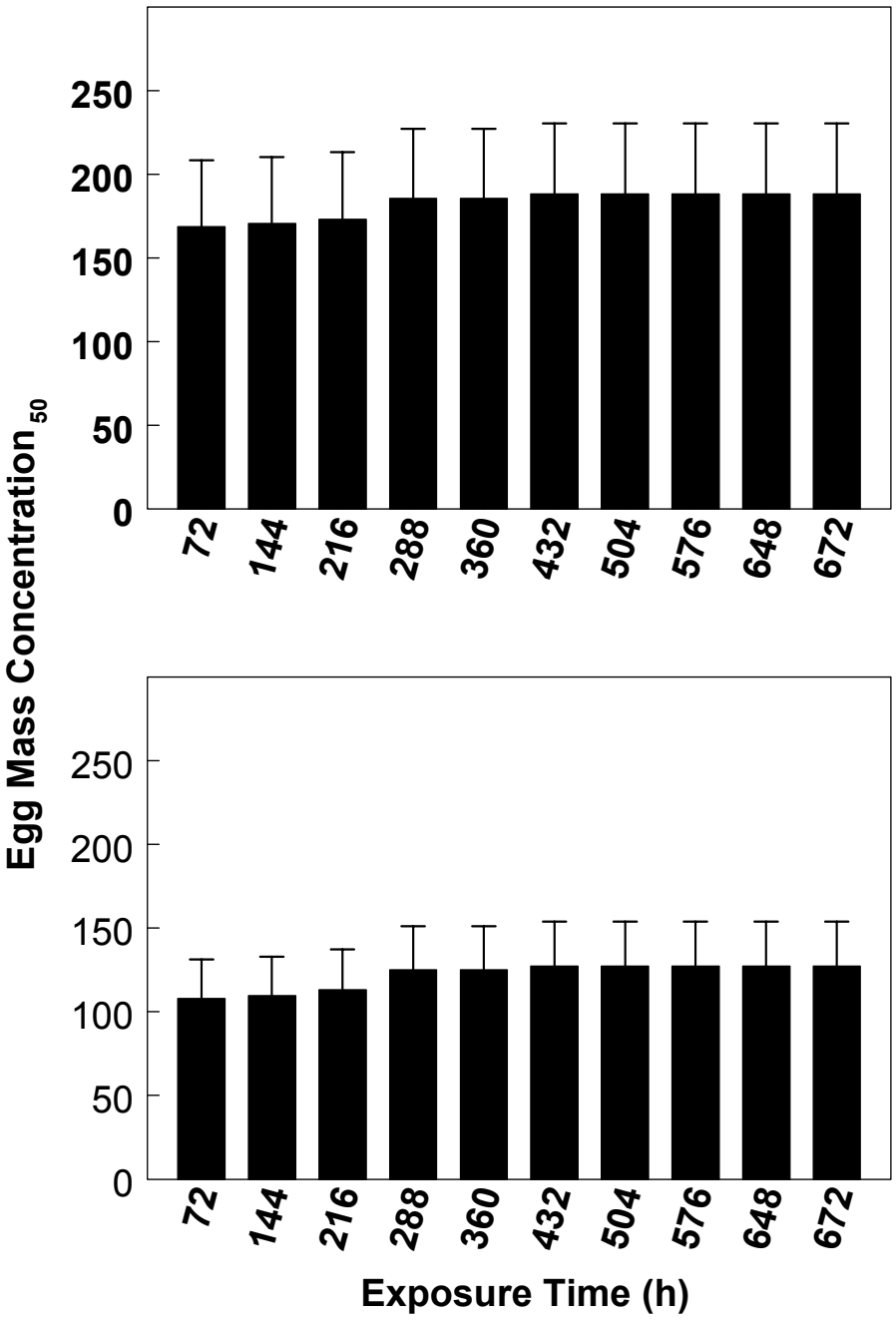


Fig.3.3 Effect concentration (EC_{50}) estimated by maximum likelihood probit regression for 50 % of individuals in a sample of *Physa acuta* (n = 30) ovipositing egg masses when exposed to varying concentrations of cadmium chloride (µg $CdCl_2 \cdot L^{-1}$, horizontal axis) every 72 h over a total exposure time of 672 h (i.e., 28 d) as computed for the entire sample without shell length as a covariate (A) and with inclusion of SL as a covariate for individuals in the 50th SL quanitile (median SL = 8.7 mm) (B). Error bars above histograms represent standard error of the EC_{50} value.

Table 3.4 Shell length and concentration-shell length interaction chi-square and probability (p) values for egg mass effect concentration (EC_{50}) for 50 % of individuals of *Physa acuta* with egg mass response determined by maximum likelihood probit regression analysis when exposed to concentrations of cadmium chloride ($CdCl_2$) ranging from 0-2000 µg $CdCl_2 \cdot L^{-1}$ over a total exposure period of 672 h (28 days). Chi-square values are provided for analysis carried out without shell length (SL) as a covariate to describe the probability of an individual laying an eggmass in the given interval: $\Gamma^{1}(p) = b_0 + b_1(Lconcentration+1)$. The probability of of an individual laying an eggmass in a given interval: $\Gamma^{1}(p) = b_0 + b_1(Lconcentration+1)+b_2\ (SL-SL^0)+b_3[(Lconcentration+1)*SL-SL^0]$with shell length as a covariate allowing determination of EC_{50} values for individuals in the 50th SL quantlie (median SL = 8.7 mm).

Exposure Duration (hours)	**Chi-Square SL as a Covariate**	**P**	**Chi-Square D SL as a Covariate**	**P**
72	0.50	0.4774	2.43	0.1189
144	0.74	0.3891	2.01	0.1565
216	0.94	0.3335	1.56	0.2113
288	0.52	0.4689	1.84	0.1774
360	0.52	0.4689	1.84	0.1774
432	0.52	0.4689	1.84	0.1774
504	0.52	0.4689	1.84	0.1774
576	0.52	0.4689	1.84	0.1774
648	0.52	0.4689	1.84	0.1774
672	0.52	0.4689	1.84	0.1774

at 72 h (Table 3.3, Fig. 3.2 B).

Application of logistic regression analysis without SL as a covariate to egg mass oviposition data for *P. acuta* revealed a significant relationship between egg mass oviposition and $CdCl_2$ concentration at each tested 72 h exposure duration over the 672 h exposure period across which chi-square and p values ranged from chi-square = 73.67, $p < 0.0001$ at exposures ranging from 432-672 h to chi-square = 74.27, $p < 0.0001$ at an exposure of 216 h (Table 3.5). Egg mass oviposition EC_{50} values based on this analysis decreased from 199.302 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±48.285) at an exposure times of 432 to 672 h through 179.780 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±45.652) at an exposure time of 72 h (Table 3.6, Fig. 3.4 A).

As with probit regression analysis described above, maximum likelihood logistic analysis did not reveal a significant relationship between egg mass oviposition and adult size when adult SL was included as a covariate over the 672 h (28 d) exposure period, chi-square = 0.37, p = 0.5443 at h 72 h (3 d) to chi-square = 0.78 p =0.3761 at 216 h (9 d), but did reveal a significant effect of $CdCl_2$ concentration. There was also no significant relationship between egg mass oviposition and the interaction between concentration and SL over the 672 h (28 d) exposure period, chi-square = 1.26, p = 0.2610 at 216 h (9 d) to 2.08, p = 0.1490 at 72 h (3 d) (Table 3.7). Because SL or the concentration-SL interaction significantly effected egg mass oviposition, egg mass oviposition EC_{50} values were computed only for the median 50th SL quantile (median SL = 8.7 mm) (Table 3.6, Fig. 3.4 B). For an individual with a median SL of 8.7 mm, there was a significant relationship between egg mass and $CdCl_2$ concentration at each tested 72 h exposure duration over the 672 h exposure period across which chi-square and p values ranged from chi-square = 65.50, $p < 0.0001$ at exposures ranging from 432-672 h to chi-

Table 3.5 Chi-Square and probability (p) values for maximum likelihood logistic regression analysis determinations of chronic cadmium chloride ($CdCl_2$) effect concentration values (EC_{50}) for 50 % of individuals of *Physa acuta* ovipositing egg masses when exposed to media with concentrations ranging from 0 - 2000 µg $CdCl_2 \cdot L^{-1}$ at 72 h intervals over a total exposure period of 672 h (28 days). Chi-square values are provided for analysis carried out without shell length (SL) as a covariate to describe the probability of an individual laying an eggmass in the given interval: $\Gamma^1(p) = b_0 + b_1(Lconcentration+1)$.The probability of of an individual laying an eggmass in a given interval: $\Gamma^1(p) = b_0 + b_1(Lconcentration+1)+b_2\ (SL-SL^0)+b_3[(Lconcentration+1)*SL-SL^0]$.with shell length as a covariate allowing determination of EC_{50} values for individuals in the median 50 th of the sample SL range (median SL = 8.7 mm).

Exposure Duration (hours)	Chi-Square SL not a Covariate	p	Chi-Square Median SL = 8.7 mm	p
72	74.36	<0.0001*	66.72	<0.0001*
144	75.20	<0.0001*	67.84	<0.0001*
216	74.27	<0.0001*	66.96	<0.0001*
288	74.65	<0.0001*	66.59	<0.0001*
360	74.65	<0.0001*	66.59	<0.0001*
432	73.67	<0.0001*	65.50	<0.0001*
504	73.67	<0.0001*	65.50	<0.0001*
576	73.67	<0.0001*	65.50	<0.0001*
648	73.67	<0.0001*	65.50	<0.0001*
672	73.67	<0.0001*	65.50	<0.0001*

Table 3.6 Sub-lethal reproduction sample egg mass effect concentration values (EC_{50}) estimated by maximum likelihood logistic regression analysis for 50 % of individuals of *Physa acuta* ovipositing egg masses when exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 - 2000 µg $CdCl_2 \cdot L^{-1}$ at 72 h intervals over a total exposure period of 672 h (28 days). Egg mass EC_{50} values are provided for analyses of the entire sample without shell length (SL) included as a covariate, and with inclusion of SL as a covariate for individuals in the 50th SL quanililee (median SL = 8.7 mm).

Exposure Time (h)	EC_{50} SL not a Covariate	s.e.	EC_{50} Med. SL = 8.7 mm	s.e.
72	179.780	±45.652	108.493	±24.312
144	180.727	±45.510	109.277	±24.134
216	182.939	±46.097	112.526	±24.878
288	196.850	±47.680	125.180	±27.082
360	196.850	±47.680	125.180	±27.082
432	199.302	±48.285	127.292	±27.739
504	199.302	±48.285	127.292	±27.739
576	199.302	±48.285	127.292	±27.739
648	199.302	±48.285	127.292	±27.739
672	199.302	±48.285	127.292	±27.739

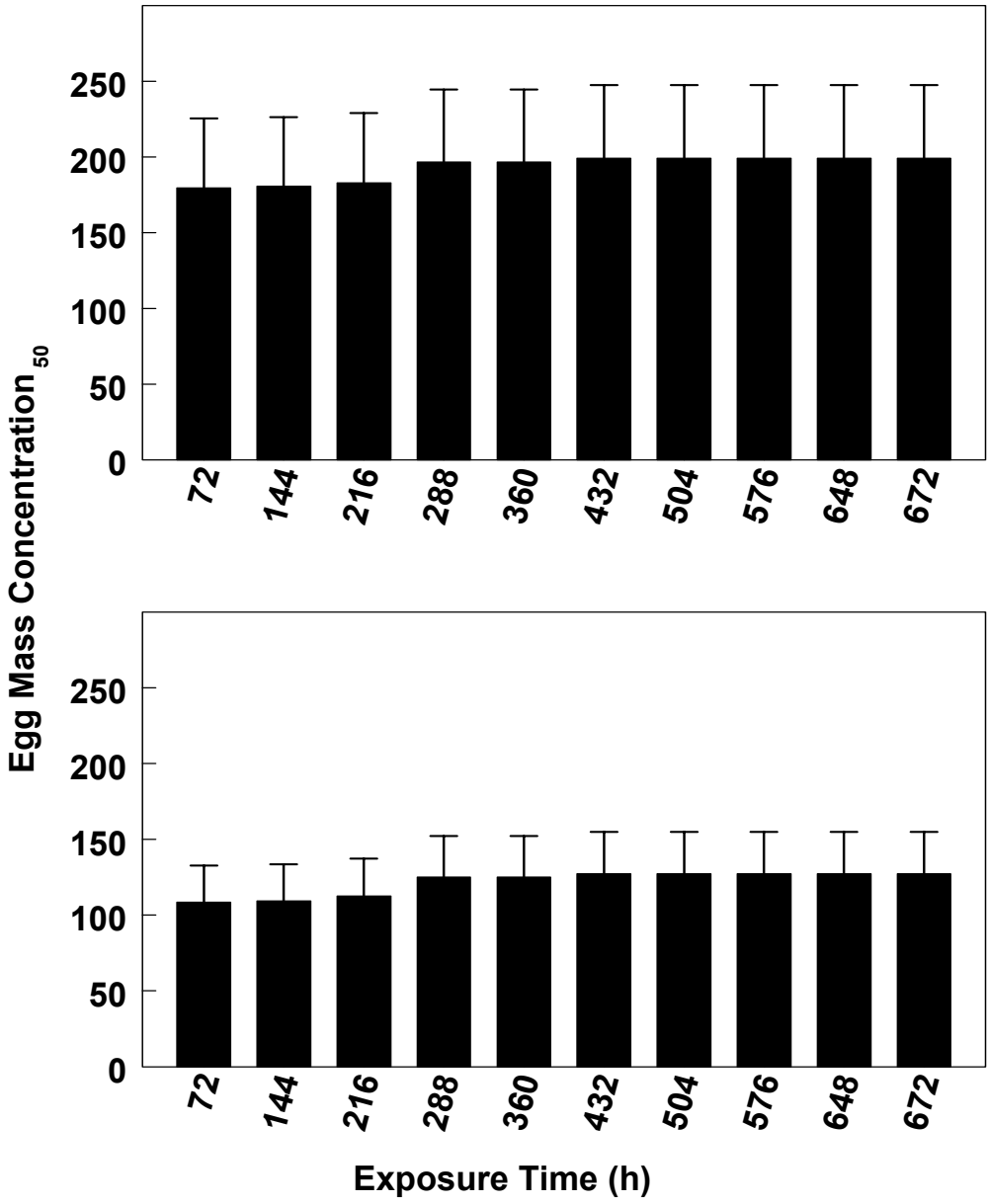


Fig.3.4 Effect concentration (EC_{50}) estimated by maximum likelihood logistic regression for 50 % of individuals in a sample of *Physa acuta* (n = 30) without shell length as a covariate (A) and with inclusion of SL as a covariate for individuals in the 50th SL quanitle (SL = 8.7 mm) (B), respectively ovipositing egg masses when exposed to varying concentrations of cadmium chloride (μg $CdCl_2 \cdot L^{-1}$, horizontal axis) every 72 h over a total exposure time of 672 h (i.e., 28 d). Error bars above histograms represent standard error of the EC_{50} value.

Table 3.7 Shell length and concentration-shell length interaction chi-square and probability (p) values for egg mass effect concentration (EC_{50}) for 50 % of individuals of *Physa acuta* with egg mass response determined by maximum likelihood logistic regression analysis when exposed to concentrations of cadmium chloride ($CdCl_2$) ranging from 0-2000 µg $CdCl_2 \cdot L^{-1}$ over a total exposure period of 672 h (28 days). Chi-square values are provided for analysis carried out without shell length (SL) as a covariate to describe the probability of an individual laying an eggmass in the given interval: $\Gamma^{1}(p) = b_0 + b_1(Lconcentration+1)$. The probability of of an individual laying an eggmass in a given interval: $\Gamma^{1}(p) = b_0 + b_1(Lconcentration+1)+b_2\ (SL-SL^0)+b_3[(Lconcentration+1)*SL-SL^0]$ with shell length as a covariate allowing determination of EC_{50} values for individuals in the median 50 th of the sample SL range (median SL = 8.7 mm).

Exposure Duration (hours)	Chi-Square SL as a Covariate	P	Chi-Square DSL as a Covariate	P
72	0.37	0.5443	2.08	0.1490
144	0.61	0.4330	1.62	0.2034
216	0.78	0.3761	1.26	0.2610
288	0.40	0.5272	1.56	0.2112
360	0.40	0.5272	1.56	0.2112
432	0.40	0.5272	1.56	0.2112
504	0.40	0.5272	1.56	0.2112
576	0.40	0.5272	1.56	0.2112
648	0.40	0.5272	1.56	0.2112
672	0.40	0.5272	1.56	0.2112

square = 67.84, p <0.0001 at 144 h (Table 3.5). Egg mass oviposition EC_{50} values based on this analysis decreased from 127.292 µg $CdCl_2 \cdot L^{-1}$ (s.e. = ±27.739) at exposures ranging from 432-672 h through 108.493 µg $CdCl_2 \cdot L^{-1}$ (s.e. = ±24.312) at 72 h (Table 3.6, Fig. 3.4 B).

Efficacy of egg mass response was investigated by analyzing standard survival 96 h LC_{50} values versus egg mass EC_{50} values. Egg mass oviposition over a 432 h exposure period EC_{50} values were much lower than the 96 h survival LC_{50} values estimated from probit regression across al SL analyses. Egg mass EC_{50} values for an exposure period of 432 h ranged from 127.154 µg $CdCl_2 \cdot L^{-1}$ (s.e. = ±26.787) for median SL = 8.7 mm to 188.203 µg $CdCl_2 \cdot L^{-1}$ (s.e. = ±42.144) for the analysis without SL as a covariate, while 96 h LC_{50} values ranged from 1563.96 (s.e. = ±32.78) for median SL =7.1 mm to 1572.86 µg $CdCl_2 \cdot L^{-1}$ (s.e. =±32.55) for the analysis without SL as a covariate (Table 3.8).

Egg mass oviposition over a 432 h exposure period EC_{50} values were much lower than the 96 h survival LC_{50} values estimated from logistic regression across all SL analyses. Egg mass EC_{50} values at an exposure period of 432 h ranged from 127.292 µg $CdCl_2 \cdot L^{-1}$ (s.e. = ±27.739) for 50^{th} SL quantile = 7.1 mm to 199.302 µg $CdCl_2 \cdot L^{-1}$ (s.e. = ±48.285) without SL as a covariate, while 96 h LC_{50} values ranged from 1563.96 (s.e. = ±32.78) for median SL =7.1 mm to 1572.86 µg $CdCl_2 \cdot L^{-1}$ (s.e. = ±32.55) without SL as a covariate (Table 3.9). A Wald statistic was used to examine differences between the $CdCl_2$ exposure 432 h egg mass EC_{50} value and the 96 h LC_{50} values in *P. acuta.* The 96 h LC_{50} value was chosen for this comparison because it is the LC_{50} value most commonly used to measure aquatic toxicity. Wald values were computed for these comparisons for the entire sample without SL as a covariate and with SL as a covariate for the 50^{th} SL quantile. In all cases, 432 h EC_{50} values for egg masses oviposited proved

Table 3.8 Comparison of 50 % sample mortality (LC_{50}), 50 % reproductive egg mass response (EC_{50}) and 50 % reproductive multiple egg mass oviposition over one observation period response (EC_{50}) estimated by maximum likelihood probit regression determinations for samples of *Physa acuta* exposed to various concentrations of cadmium chloride ($CdCl_2$). In LC_{50} determinations, samples (n = 150) were exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 – 5000 µg $CdCl_2.L^{-1}$ for egg mass EC_{50} determinations, samples (n=30) were exposed to media with concentrations ranging from 0-2000 µg $CdCl_2.L^{-1}$, while for multiple egg mass oviposiion over one observation period EC_{50} determinations, samples (n=30) were exposed to media with concentrations ranging from 0-2000 µg $CdCl_2.L^{-1}$. Values of LC_{50} for 96 h exposure periods are provided for analysis of the entire sample without shell length (SL) as a covariate and with SL as a covariate for individuals in the 25th quantile of sample SL range (SL = 6.0 mm), median 50th quantile of sample SL range (median SL = 7.1 mm) and 75th quantile SL range (SL = 8.3 mm). Values of egg mass EC_{50} for a 432 h exposure period are provided for the entire sample without shell length (SL) as a covariate and with SL as a covariate for individuals in the 25th of sample SL range (SL = 6.7 mm), median 50th quantile of sample SL range (median SL = 8.7 mm) and 75th quantile of sample SL range (SL = 11.1 mm). Values of multiple egg mass oviposition over one observation period EC_{50} for a 432 h exposure period are provided for the entire sample without shell length (SL) as a covariate and with SL as a covariate for individuals in the lower 25th quantile of sample SL range (SL = 6.7 mm), median 50th quantile of sample SL range (median SL = 8.7 mm) and 75th quantile of sample SL range (SL = 11.1 mm).

	Survival LC_{50}			**Reproduction Egg Mass EC_{50}**			**Reproduction Multiple Egg Mass Over One 72 h Observation Period EC_{50}**		
Analysis	**SL (mm)**	**96 h LC_{50}**	**s.e.**	**SL (mm)**	**432 h EC_{50}**	**s.e.**	**SL (mm)**	**432 h EC_{50}**	**s.e.**
Without SL as a Covariate	N.A.	1572.86	±32.55	N.A.	188.203	±42.144	N.A.	17.041	±6.435
25th sample SL quantile	6.0	1504.57	±39.96	N.A.	N.A.	N.A.	6.7	9.809	±3.091
50th sample SL quantile	7.1	1563.96	±32.78	8.7	127.154	±26.787	8.7	11.985	±3.605
75th sample SL qunatile	8.3	1628.26	±37.65	N.A.	N.A.	N.A.	11.1	17.753	±10.411

Table 3.9 Comparison of 50 % sample mortality (LC_{50}), 50 % reproductive egg mass response (EC_{50}) and 50 % reproductive multiple egg mass oviposition over one observation period response (EC_{50}) estimated by maximum likelihood logistic regression determinations for samples of *Physa acuta* exposed to various concentrations of cadmium chloride ($CdCl_2$). In LC_{50} determinations, samples (n = 150) were exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 – 5000 µg $CdCl_2.L^{-1}$, for egg mass EC_{50} determinations, samples (n = 30) were exposed to media with concentrations ranging from 0-2000 µg $CdCl_2.L^{-1}$, while for multiple egg mass oviposition over one observation period EC_{50} determinations, samples (n = 30) were exposed to media with concentrations ranging from 0-2000 µg $CdCl_2.L^{-1}$. Values of LC_{50} for 96 h exposure periods are provided for analysis of the entire sample without shell length (SL) as a covariate and with SL as a covariate for individuals in the 25th SL quantile (SL = 6.0 mm), 50th SL quantile (median SL = 7.1 mm) and 75th SL quantile (SL = 8.3 mm). Values of egg mass EC_{50} for a 432 h exposure period are provided for the entire sample without shell length (SL) as a covariate and with SL as a covariate for individuals in the 25th SL quantile (SL = 6.7 mm), median 50th SL quantile (median SL = 8.7 mm) and 75th quantile SL quantile, of sample SL range (SL = 11.1 mm). Values of multiple egg mass oviposition over one observation period EC_{50} for a 432 h exposure period are provided for the entire sample without shell length (SL) as a covariate and with SL as a covariate for individuals in the 25th SL quantile (SL = 6.7 mm), 50th SL quantile (median SL = 8.7 mm) and 75th SL quantile (SL = 11.1 mm).

	Survival LC50			**Reproduction Egg Mass EC50**			**Reproduction Multiple Egg Mass Over One 72 h Observation Period EC_{50}**		
Analysis	**Median SL (mm)**	**96 h LC_{50}**	**s.e.**	**Median SL (mm)**	**432 h EC_{50}**	**s.e.**	**Median SL (mm)**	**432 h EC_{50}**	**s.e.**
Without SL as a Covariate	N.A.	1589.26	±27.51	N.A.	199.302	±48.285	N.A.	16.599	±6.27
25th SL quantile	6.0	1499.32	±33.53	N.A.	N.A.	N.A.	6.7	10.016	±3.195
50th SL quantile	7.1	1577.21	±27.42	8.7	127.292	±27.739	8.7	11.472	±3.347
75th SL quantile	8.3	1658.42	±32.04	N.A.	N.A.	N.A.	11.1	16.844	±9.705

significantly (p = 0.001) lower than corresponding 96 h LC_{50} values as detailed below. Maximum likelihood probit analyses without SL as a covariate comparing the 96 h LC_{50} to the 432 h EC_{50} for oviposition of egg masses resulted in w = 2354.90, p = 0.0. The Wald comparison of maximum likelihood probit analysis with the 50th SL quantile included as a covariate resulted in W = 2298.85, p = 0.001.

A Wald statistic was used to examine differences between the $CdCl_2$ exposure for the 432 h egg mass EC_{50} value and the 96 h LC_{50} values estimated with logistic regression in *P. acuta.* Wald values were computed for these comparisons for the entire sample without SL as a covariate and with SL as a covariate for the 50^{th} SL quantile. In all cases, 432 h EC_{50} values for egg masses oviposited proved significantly (p = 0.001) lower than corresponding 96 hLC_{50} values as detailed below. Maximum likelihood logistic analyses without SL as a covariate comparing the 96 h LC_{50} to the 432 h EC_{50} for oviposition of egg masses resulted in W = 3354.44, p = 0.0. The Wald comparison of maximum likelihood logistic analysis with median SL included as a covariate resulted in W = 3351.93, p = 0.001.

Values of egg mass oviposition EC_{50} computed by either maximum likelihood probit or logistic regression analyses proved essentially similar at all tested $CdCl_2$ concentrations at all 72 h exposure intervals throughout the 672 h exposure period whether estimated without (Tables 3.3 and 3.6, Fig. 3.5A) or with SL included as a covariate (Tables 3.3 and 3.6 and 3.5, Fig. 3.5 B). In all cases, EC_{50} values fell well within the standard error of the means of paired values computed by either method suggesting that they were not statistically different from each other.

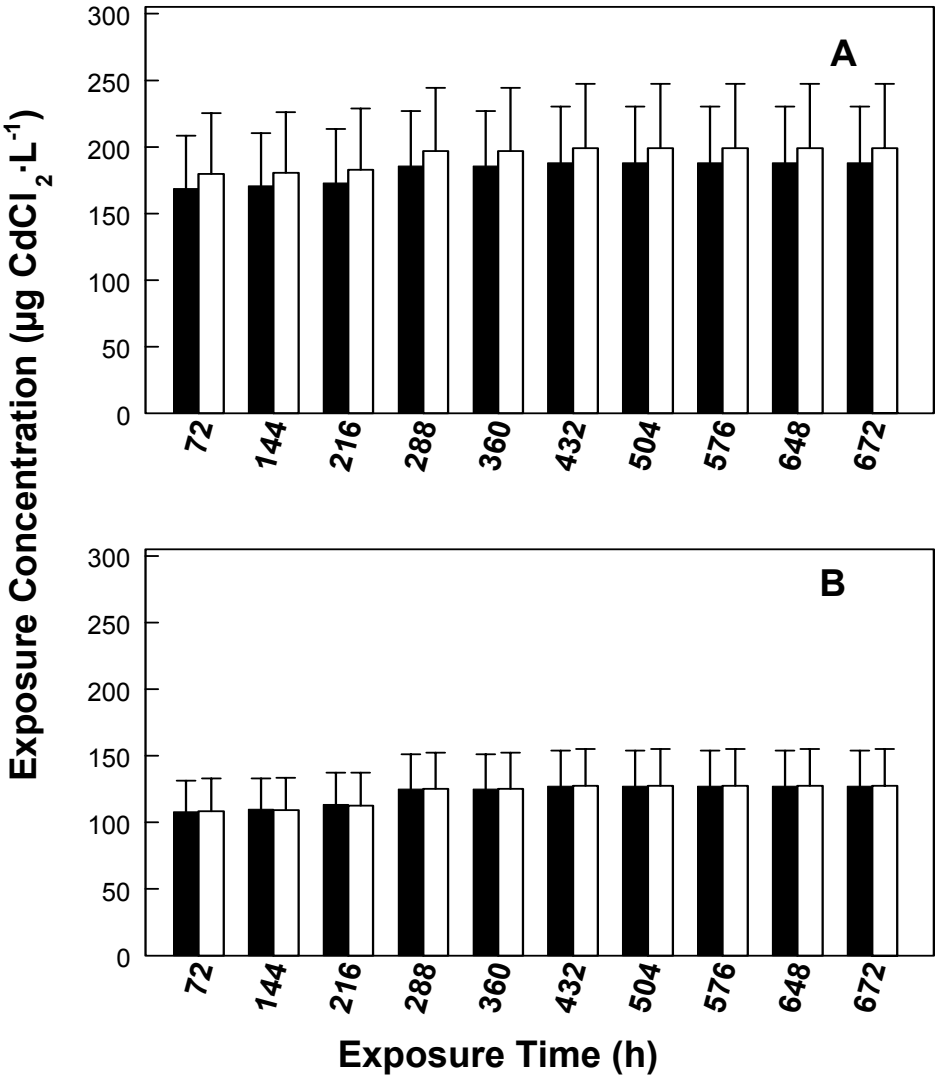


Fig.3.5 A-B Comparison of EC_{50} values computed by maximum likelihood probit analysis (solid histograms) to those computed by maximum likelihood logistic regression analysis (open histograms) of individuals in a sample of *Physa acuta* (n = 30) without shell length as a covariate (A) and with inclusion of SL as a covariate for the 50th SL quantile (median SL = 8.7 mm) (B), respectively ovipositing egg masses when exposed to varying concentrations of cadmium chloride (µg $CdCl_2 \cdot L^{-1}$,horizontal axis) every 72 h over a total exposure time of 672 h (i.e., 28 d). The horizontal axis is exposure time in hours and the vertical axis, the estimated egg mass effect concentration in µg $CdCl_2 \cdot L^{-1}$. Vertical bars above histograms represent standard errors.

Multiple Egg Mass Oviposition over a Single 72 h Observation Period EC_{50}

Maximum likelihood probit analysis without inclusion of ovipositioning adult size as SL as a covariate revealed a significant relationship between ovipostion of multiple egg masses (i.e., two or more egg masses) and $CdCl_2$ concentration at each tested 72 h exposure duration across which chi-square and p values ranged from chi-square = 96.03, $p < 0.0001$ at 72 h to chi-square = 101.58, $p < 0.0001$ at 360-672 h (Table 3.10). Values of EC_{50} for oviposition of multiple egg masses at a single observation period computed by maximum likelihood probit regression without adult SL as a covariate decreased from 17.041 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±6.435) at exposure times ranging from 432 to 672 h through 14.093 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±5.611) at an exposure time of 72 h (Table 3.11, Fig. 3.6 A).

Maximum likelihood probit analysis of the effect of $CdCl_2$ concentration with ovipositioning adult size as SL as a covariate allowed EC_{50} values to be calculated for 25th, median 50 % and 75th SL quantile groups. This analysis again revealed significant effects of $CdCl_2$ concentration on the number of individuals ovipositing multiple egg masses at 72 h observation periods across the 672 h exposure duration. It also revealed a lack of correlation between multiple egg mass oviposition and adult SL (chi-square = 0.78, p = 0.3773), but did reveal a significant interaction between $CdCl_2$ concentration and shell length (chi-square = 8.07, p = 0.0045) (Table 3.12).

For the 25th SL quantile group (SL 6.7 mm), maximum likelihood probit analysis revealed a significant relationship between oviposition of multiple egg masses during a single 72 h observation period and $CdCl_2$ concentration at each tested exposure duration (72-672 hours) across which chi-square and p values ranged from chi-square = 87.71 ($p < 0.0001$) at 72 h to chi-

Table 3.10 Chi-square and probability (p) values for maximum likelihood probit regression analysis determinations of chronic cadmium chloride ($CdCl_2$) mass effect concentration values (EC_{50}) for 50 % of individuals of *Physa acuta* ovipositing multiple egg masses over a single 72 h observation period when exposed to media with concentrations of cadmium chloride ranging from 0 - 2000 µg $CdCl_2 \cdot L^{-1}$ over a total exposure period of 672 h (28 days). Chi-square values are provided for analysis carried out without shell length (SL) as a covariate to describe the probability of an individual laying an eggmass in the given interval: $\Gamma^{1}(p) = b_0 + b_1(Lconcentration+1)$. The probability of of an individual laying an eggmass in a given interval: $\Gamma^{1}(p) = b_0 + b_1(Lconcentration+1)+b_2 (SL-SL^0)+b_3[(Lconcentration+1)*SL-SL^0]$ with shell length as a covariate allowing determination of EC_{50} values for individuals in the 25th SL quantile (SL = 6.7 mm), median 50th SL quantile (median SL = 8.7 mm) and the 75th SL quantile (SL = 11.1 mm).

Exposure Duration (hours)	Chi-Square SL not a Covariate	P	Chi-Square SL = 6.7 mm	p	Chi-Square SL = 8.7 mm	p	Chi-Square SL = 11.1 mm	P
72	96.03	<0.0001*	87.71	<0.0001*	91.38	<0.0001*	20.42	<0.0001*
144	98.26	<0.0001*	88.26	<0.0001*	91.91	<0.0001*	20.51	<0.0001*
216	98.49	<0.0001*	88.03	<0.0001*	94.00	<0.0001*	22.23	<0.0001*
288	98.07	<0.0001*	87.56	<0.0001*	93.31	<0.0001*	22.00	<0.0001*
360	101.06	<0.0001*	89.54	<0.0001*	96.07	<0.0001*	22.81	<0.0001*
432	100.76	<0.0001*	89.54	<0.0001*	95.62	<0.0001*	22.81	<0.0001*
504	101.58	<0.0001*	89.54	<0.0001*	95.62	<0.0001*	22.81	<0.0001*
576	101.58	<0.0001*	89.54	<0.0001*	95.62	<0.0001*	22.81	<0.0001*
648	101.58	<0.0001*	90.18	<0.0001*	95.62	<0.0001*	22.81	<0.0001*
672	101.58	<0.0001*	90.18	<0.0001*	95.62	<0.0001*	22.81	<0.0001*

* indicates a significant difference at $p < 0.005$

Table 3.11 Sub-lethal reproduction effect concentration values (EC_{50}) estimated by maximum likelihood probit regression analysis for 50 % of individuals of *Physa acuta* ovipositing multiple egg masses over a 72 h observation period when exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 - 2000 µg $CdCl_2 \cdot L^{-1}$ over a total exposure period of 672 h (28 days). Multiple egg mass EC_{50} values are provided for analyses of the entire sample without shell length (SL) included as a covariate, and with inclusion of SL as a covariate for individuals in the 25th SL quantile (SL = 6.7 mm), 50th SL quantile (SL = 8.7 mm) and 75th SL quantile (SL = 11.1 mm).

Exposure Time (h)	EC_{50} SL not a Covariate	s.e.	EC_{50} SL = 6.7 mm	s.e.	EC_{50} SL = 8.7 mm	s.e.	EC_{50} SL = 11.1 mm	s.e.
72	14.093	±5.611	8.267	±2.797	9.535	±3.075	13.081	±8.875
144	15.115	±5.885	9.450	±3.133	10.405	±3.301	12.878	±8.762
216	16.139	±6.221	9.031	±3.014	10.957	±3.390	16.535	±10.037
288	16.383	±6.319	9.228	±3.091	11.162	±3.563	16.709	±10.170
360	17.457	±6.559	9.809	±3.195	11.855	±3.563	17.753	±10.411
432	17.041	±6.435	9.809	±3.195	11.985	±3.605	17.753	±10.411
504	17.041	±6.435	9.809	±3.195	11.985	±3.605	17.753	±10.411
576	17.041	±6.435	9.809	±3.195	11.985	±3.605	17.753	±10.411
648	17.041	±6.435	9.809	±3.195	11.985	±3.605	17.753	±10.411
672	17.041	±6.435	9.809	±3.195	11.985	±3.605	17.753	±10.411

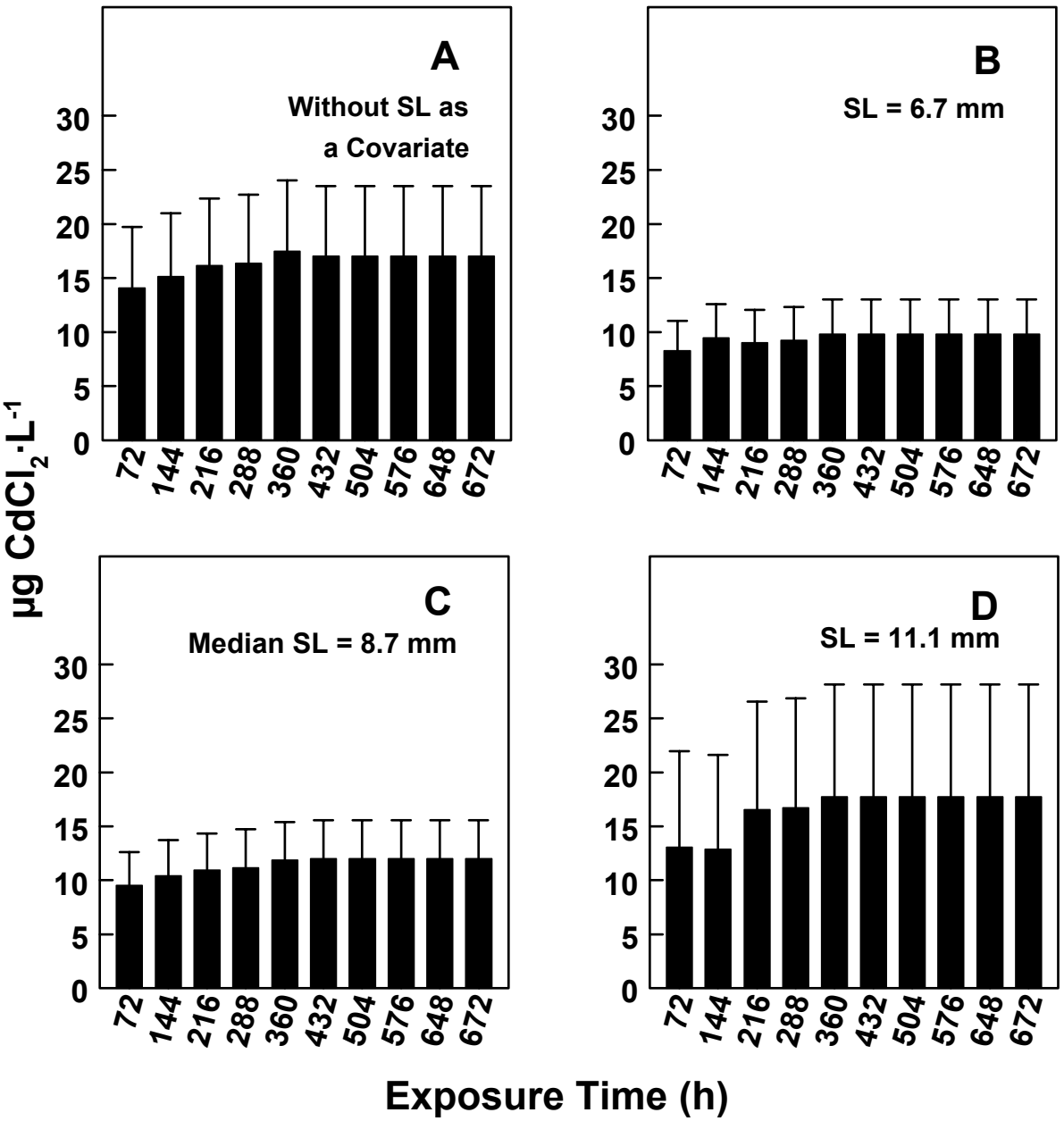


Fig.3.6 Effect concentration (EC_{50}) estimated by maximum likelihood probit regression for 50 % of individuals in a sample of *Physa acuta* (n = 30) without shell length (SL) as a covariate (A) and with inclusion of SL as a covariate for individuals in the 25th (SL = 6.7 mm) (B), 50th (median SL = 8.7 mm) (C) and 75th (SL-11.1 mm) SL quantiles (D), respectively, ovipositing multiple egg masses (> 1 egg mass) during any one 72 h observation period when exposed to varying concentrations of cadmium chloride (µg $CdCl_2 \cdot L^{-1}$, horizontal axis) over a total exposure time of 672 h (28 d). The horizontal axis is exposure time in hours and the vertical axis, the estimated egg mass effect concentration in µg $CdCl_2 \cdot L^{-1}$. Vertical bars above histograms represent standard error of the EC_{50} value.

Table 3.12 Effect of shell length (SL) and the interaction between concentration and SL on sub-lethal 50 % sample of *P. acuta* ovipositing multiple egg masses over a 72 h exposure period (EC_{50}) exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 - 2000 µg $CdCl_2 \cdot L^{-1}$. Response chi-square and probability (p) values were estimated by maximum likelihood probit regression analysis determinations for samples of *Physa acuta* over a total exposure period of 672 h. The probability of of an individual laying an eggmass in a given interval: $\Gamma^1(p) = b_0 + b_1(Lconcentration+1)+b_2\ (SL-SL^0)+b_3[(Lconcentration+1)*SL-SL^0]$ with shell length as a covariate allowing determination of EC_{50} values. Probability values are consistent across all SL calculations for individuals in the 25th SL quantile (SL = 6.7 mm), individuals in the 50th SL quantile (SL = 8.7 mm) and individuals in the 25th SL quantile (SL = 11.1 mm) based on analysis with SL as a covariate.

Exposure Duration (h)	**SL Effect on EC_{50} Chi-square**	**p**	**Concentration-SL Interaction Effect on EC_{50} Chi-square**	**P**
72	1.02	0.3120	11.15	0.0008*
144	1.41	0.2356	10.94	0.0009*
216	0.81	0.3681	10.16	0.0014*
288	0.81	0.3669	9.89	0.0017*
360	0.97	0.3251	10.39	0.0013*
432	0.93	0.3336	8.84	0.0030*
504	0.93	0.3336	8.84	0.0030*
576	0.93	0.3336	8.84	0.0030*
648	0.93	0.3336	8.84	0.0030*
672	0.93	0.3336	8.84	0.0030*

*indicates a significant difference at $p<0.05$

square = 90.18 ($p < 0.0001$) at exposure periods ranging from 648-672 h (Table 3.10). Corresponding EC_{50} values for this group decreased from 9.809 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±3.195) at exposure times ranging from 360-674 h through 8.267 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ± 2.797) at 72 h (Table 3.11, Fig. 3.6 B). For the 50th SL quantile group (SL = 8.7 mm), there was a significant relationship between multiple egg mass oviposition at a single 72 h observation period and $CdCl_2$ concentration at each tested exposure duration (72-672 hours) across which chi-square and p values ranged from chi-square = 91.38 ($p < 0.0001$) at 72 h to chi-square = 95.62 ($p < 0.0001$) at observation periods ranging from 360-762 h (Table 3.10). Values of EC_{50} for the median SL quantile group decreased from 11.985 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±3.605) at an exposure times ranging from 360-672 h through 9.535 μg $CdCl_2 \cdot L^{-1}$ (s.e = ±3.075) at 72 h (Table 3.11, Fig. 3.6 C).

For the 75th SL quantile (SL = 11.1 mm), there was a significant relationship between multiple egg mass oviposition during each 72 h observation period and $CdCl_2$ concentration throughout the 672 h exposure duration across which chi-square and p values ranged from chi-square = 22.81 ($p < 0.0001$) at exposure periods ranging from 360-672 h to chi-square = 20.42 ($p < 0.0001$) at 72 h (Table 3.10). Values of EC_{50} determined by maximum likelihood probit analysis decreased from 17.753 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±10.411) at an exposure durations ranging from 360-672 h through 12.878 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±8.762) at 144 h (Table 3.11, Fig. 3.6 D).

As described above for maximum likelihood probit analysis, maximum likelihood logistic regression analysis was applied to the same data set for multiple egg mass oviposition within sequential 72 h exposure periods across a total $CdCl_2$ exposure of 672 h both with and

without ovipositing adult SL as a covariate. Application of this analysis without ovipositing adult SL as a covariate revealed a significant effect of $CdCl_2$ concentration on multiple egg mass oviposition at each sequential 72 h exposure duration across the 672 h exposure period across which chi-square and p values ranged from chi-square = 89.56 ($p < 0.0001$) at an exposure of 72 h to chi-square = 93.25 ($p < 0.0001$) at exposures ranging from 360-672 h (Table 3.13). Values of EC_{50} values derived from this analysis decreased from 16.559 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±6.271) at exposures ranging from 360-672 h through 13.425 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±5.334) at the initial 72 h exposure (Table 3.14, Fig. 3.7 A).

For the 50th SL quantile (SL = 8.7 mm) this again analysis revealed a significant relationship between sample multiple egg Maximum likelihood logistic regression analysis of the effect of $CdCl_2$ on multiple egg mass oviposition within sequential 72 h observation periods with ovipositing adult SL as a covariate revealed a significant impact of SL on multiple egg mass oviposition. It also revealed a lack of significant correlation between multiple egg mass oviposition and adult SL (chi-square = 1.06, p = 0.3030), but a significant interaction between $CdCl_2$ concentration and shell length (chi-square = 9.90, p= 0.0017) (Table 3.15). For the 25th SL quantile group (SL 6.7 mm), maximum likelihood logistic regression analysis revealed a significant relationship between oviposition of multiple egg masses during a single 72 h observation period and $CdCl_2$ concentration at each tested exposure duration (72-672 hours) which chi-square and p values ranged from chi-square = 77.45 ($p < 0.0001$) at an exposure of 288 h to chi-square = 78.16 ($p < 0.0001$) at exposures ranging from 360-672 h (Table 3.13). Values of EC_{50} for multiple egg mass oviposition based on this analysis decreased from 10.016 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±3.195) at exposures ranging from 432-672 h through 9.155 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±2.815) at an exposure of 72 h (Table 3.14, Fig. 3.7 B).

Table 3.13 Chi-square and probability (p) values for maximum likelihood logistic regression analysis determinations of chronic cadmium chloride ($CdCl_2$) effect concentration values (EC_{50}) for 50 % of individuals of *Physa acuta* ovipositing multiple egg masses during a 72 h observation period when exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 - 2000 µg $CdCl_2 \cdot L^{-1}$ over a total exposure period of 672 h (28 days). Chi-square values are provided for analysis carried out without shell length (SL) as a covariate to describe the probability of an individual laying multiple eggmasses during a 72 h observation period in the given interval: $\Gamma^1(p) = b_0 + b_1(Lconcentration+1)$. The probability of of an individual laying multiple eggmasses during a 72 h observation period in a given interval: $\Gamma^1(p) = b_0 + b_1(Lconcentration+1)+b_2\ (SL-SL^0)+b_3[(Lconcentration+1)*SL-SL^0]$ with shell length as a covariate allowing determination of EC_{50} values for individuals in the 25th SL quantile (SL = 6.7 mm), 50th SL quantile (median SL = 8.7 mm) and the 75th SL quantile (SL = 11.1 mm).

Exposure Duration (hours)	Chi-Square SL not a Covariate	p	Chi-Square SL =6.7 mm	P	Chi-Square SL =8.7 mm	P	Chi-Square SL =11.1 mm	P
72	89.56	<0.0001*	77.97	<0.0001*	86.11	<0.0001*	21.77	<0.0001*
144	91.62	<0.0001*	77.55	<0.0001*	86.28	<0.0001*	21.68	<0.0001*
216	91.43	<0.0001*	77.89	<0.0001*	88.15	<0.0001*	23.33	<0.0001*
288	91.01	<0.0001*	77.45	<0.0001*	87.56	<0.0001*	23.01	<0.0001*
360	93.25	<0.0001*	78.16	<0.0001*	89.28	<0.0001*	23.73	<0.0001*
432	93.25	<0.0001*	78.16	<0.0001*	89.28	<0.0001*	23.73	<0.0001*
504	93.25	<0.0001*	78.16	<0.0001*	88.64	<0.0001*	23.73	<0.0001*
576	93.25	<0.0001*	78.16	<0.0001*	88.64	<0.0001*	23.73	<0.0001*
648	93.25	<0.0001*	78.16	<0.0001*	88.64	<0.0001*	23.73	<0.0001*
672	93.25	<0.0001*	78.16	<0.0001*	88.64	<0.0001*	23.73	<0.0001*

* indicates a significant difference at $p < 0.005$

Table 3.14 Sub-lethal reproduction effect concentration values (EC_{50}) estimated by logistic regression analysis for 50 % of individuals of *Physa acuta* ovipositing multiple egg masses during a 72 h observation period when exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 - 2000 µg $CdCl_2 \cdot L^{-1}$ over a total exposure period of 672 h (28 days). Multiple egg mass EC_{50} values are provided for analyses of the entire sample without shell length (SL) included as a covariate, and with inclusion of SL as a covariate for individuals in the 25th SL quantile (SL = 6.7 mm), median 50th SL quantile (median SL = 8.7 mm) and 75th SL quantile (SL = 11.1 mm).

Exposure Time (h)	EC_{50} SL not a Covariate	s.e.	EC_{50} Med. SL = 6.7 mm	s.e.	EC_{50} Med. SL = 8.7 mm	s.e.	EC_{50} Med. SL = 11.1 mm	s.e.
72	13.425	±5.334	7.972	±2.646	9.155	±2.815	12.694	±8.271
144	14.310	±5.562	9.131	±2.999	9.990	±3.042	12.348	±8.136
216	15.277	±5.912	8.681	±2.861	10.419	±3.103	15.774	±9.341
288	15.503	±6.011	8.860	±2.936	10.603	±3.176	15.920	±9.478
360	16.559	±6.271	9.493	±3.075	11.304	±3.291	16.844	±9.705
432	16.559	±6.271	10.016	±3.195	11.304	±3.291	16.844	±9.705
504	16.559	±6.271	10.016	±3.195	11.472	±3.347	16.844	±9.705
576	16.559	±6.271	10.016	±3.195	11.472	±3.347	16.844	±9.705
648	16.559	±6.271	10.016	±3.195	11.472	±3.347	16.844	±9.705

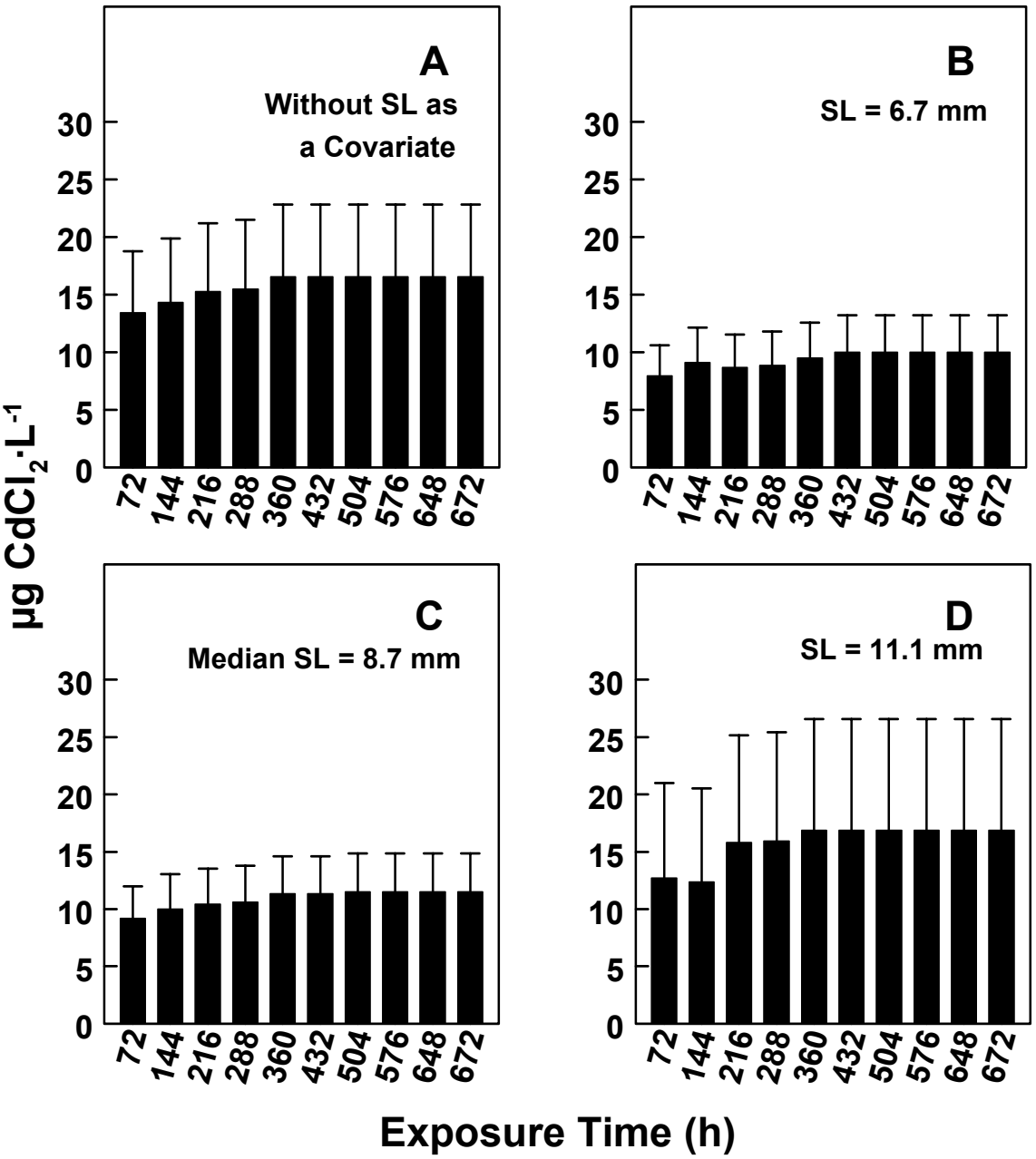


Fig.3.7 Effect concentration (EC_{50}) estimated by logistic regression for 50 % of individuals in a sample of *Physa acuta* (n = 30) without SL as a covariate (A) and with inclusion of SL as a covariate for individuals in the 25th (SL = 6.7 mm), (B), 50th (SL = 8.7 mm) (Panel C) and 75th (SL = 11.1) SL quantiles (D), respectively. ovipositing multiple egg masses (> 1 egg mass) during any one 72 h observation period when exposed to varying concentrations of cadmium chloride (µg $CdCl_2 \cdot L^{-1}$, horizontal axis) over a total exposure time of 672 h (28 d). The horizontal axis is exposure time in hours and the vertical axis, the estimated egg mass effect concentration in µg $CdCl_2 \cdot L^{-1}$. Vertical bars above histograms represent standard error of the EC_{50} value.

Table 3.15 Effect of shell length (SL) and the interaction between concentration and SL on sub-lethal 50 % sample of *P. acuta* ovipositing multiple egg masses over a 72 h exposure period (EC_{50}) exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 - 2000 μg $CdCl_2 \cdot L^{-1}$. Response chi-square and probability (p) values were estimated by maximum likelihood logistic regression analysis determinations for samples of *Physa acuta* over a total exposure period of 672 h. The probability of of an individual laying multiple eggmasses during a 72 h observation period in a given interval: $I^{-1}(p) = b_0 + b_1(Lconcentration+1)+b_2\ (SL-SL^0)+b_3[(Lconcentration+1)*(SL-SL^0)]$ with shell length as a covariate allowing determination of EC_{50} values for individuals in the 25th SL quantile (SL = 6.7 mm), median 50th SL quantile (median SL = 8.7 mm) and the 75th SL quantile (SL = 11.1 mm). Probability values are consistent across all SL calculations for individuals in the 25th SL quantile (SL = 6.7 mm), individuals in the 50th SL quantile (median SL = 8.7 mm) and individuals in the 75th SL quantile (SL = 11.1 mm) based analysis with SL as a covariate.

Exposure Duration (h)	SL Effect for EC_{50} Chi-square	p	Concentration-SL Interaction for EC_{50} Chi-square	p
72	1.02	0.3120	11.15	0.0008*
144	1.41	0.2356	10.94	0.0009*
216	0.81	0.3681	10.16	0.0014*
288	0.81	0.3669	9.89	0.0017*
360	0.97	0.3251	10.39	0.0013*
432	0.93	0.3336	8.84	0.0030*
504	0.93	0.3336	8.84	0.0030*
576	0.93	0.3336	8.84	0.0030*
648	0.93	0.3336	8.84	0.0030*
672	0.93	0.3336	8.84	0.0030*

*indicates a significant difference at $p<0.05$

mass oviposition and $CdCl_2$ concentration at each tested 72 h exposure duration (72-672 hours) across which chi-square and p values ranged from chi-square = 86.11 ($p < 0.0001$) at 72 h to chi-square = 89.28 ($p < 0.0001$) at exposures of 360 and 432 h (Table 3.13). Values of EC_{50} based on this analysis decreased from 11.472 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±3.347) at exposures ranging from 360-672 h to 9.155 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±2.815) at 72 h (Table 3.14, Fig. 3.7 C). Similarly, a significant effect of size on multiple egg mass oviposition occurred in the 75thSL quantile (SL = 11.1 mm) at each 72 h observation period throughout the 672 h exposure duration (72-672 hours) across which chi-square and p values ranged from chi-square = 21.77 ($p < 0.0001$) at 72 h to chi-square = 23.73 ($p < 0.0001$) at exposures ranging from 360-672 h (Table 3.13). Values of EC_{50} for multiple egg mass oviposition based on maximum likelihood regression analysis of the data decreased from 16.844 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±9.705) across exposure times ranging from 360-672 h to 12.348 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±8.136) at 144 h (Table 3.14 Fig. 3.7 D).

Efficacy of egg mass response was investigated by analyzing standard survival 96 h probit LC_{50} values versus multiple egg mass EC_{50} values. Multiple egg mass oviposition over a 72 h exposure period EC_{50} values were much lower than the 96 h survival LC_{50} values estimated from probit regression across all SL analyses. Egg mass EC_{50} values ranged from 9.809 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±3.091) for median SL = 6.7 mm to 17.753 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±10.411) for median SL = 11.1 mm, while 96 h LC_{50} values ranged from 1504.57 (s.e. = ±39.96) for median SL =6.00 mm to 1628.26 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±37.65) for median SL = 8.3 mm (Table 3.8).

A Wald statistic was used to examine differences between the 432 h EC_{50} values for multiple egg mass oviposition and the 96 h LC_{50} values in *P. acuta.* Wald values were computed for these comparisons for the entire sample without SL as a covariate and with SL as a covariate for the 25th SL quantile) of the sample SL quantile, SL quantle and 75th SL quantile. In all

cases, 432 h EC_{50} values for multiple egg masses oviposited over a single 72 h observation period proved significantly ($p = 0.001$) lower than corresponding 96 h LC_{50} values as detailed below. Maximum likelihood probit analyses without SL as a covariate comparing the 96 h LC_{50} to the 432 h EC_{50} for multiple oviposition of egg masses over a single 72 h observation response resulted in $W = 2341.97$, $p = 0.001$. All Wald comparisons of maximum likelihood probit analyses with SL as a covariate indicated significant differences between estimates of 432 h EC_{50} for multiple egg mass oviposition over a single 72 h observation period and 96 h LC_{50} values as follows: for the 25th SL quantile, $W = 1427.74$, $p = 0.0$; for the 50th SL quantile, $W = 2287.37$, $p = 0.001$; and for the 75th SL quantile, $W = 1873.23$, $p = 0.001$.

Multiple egg mass oviposition over a 72 h exposure period EC_{50} values were much lower than the 96 h survival LC_{50} values estimated from logistic regression across all SL analyses. Multiple egg mass EC_{50} values at an exposure period of 432 h ranged from 10.016 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±3195) for median SL = 6.7 mm to 16.844 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±9.705) for median SL = 11.1 mm, while 96 h LC_{50} values ranged from 1499.32 (s.e. = ±33.53) for SL =6.00 mm to 1658.42 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±32.04) for SL = 8.3 mm (Table 3.9).

Wald analysis comparing maximum likelihood logistic estimations of 96 h LC_{50} to the 432 h EC_{50} for multiple egg mass oviposition over a single 72 h observation period without SL as a covariate yielded a $W = 3344.41$, $p = 0$. All Wald analyses comparing logistic regression estimates of 96 h LC_{50} and 432 h EC_{50} estimates for multiple egg mass oviposition over a single 72 h observation period with SL as a covariate proved significant as follows: for the 25 th SL quantile SL, $W = 2009.33$, $p = 0.001$; for the 50 th SL quantile of sample SL, $W = 3320.35$, $p = 0.001$; and for the 75 th SL quantile sample SL, $W = 2682.21$, $p = 0.001$.

A generalized linear regression analysis for poisson data distributions without and with SL as a covariate was used to analyze data on the cumulative number of egg masses oviposited·individual^{-1} by specimens of *P. acuta* exposed to concentrations of $CdCl_2$ ranging from 0-2000 μg $CdCl_2 \cdot L^{-1}$ for 672 h. This analysis revealed that exposure to increasing $CdCl_2$ concentrations (0-2000 μg $CdCl_2 \cdot L^{-1}$) significantly reduced the number of egg masses oviposted during the observation period (chi-square = 6.63, p = 0.0100). Resulting adjusted means for number egg masses oviposited·individual^{-1} during the 672 hour observation period ranged from 0.9942 (s.e. = ±0.00467) at a concentration of 0 μg $CdCl_2 \cdot L^{-1}$ to 0.9033 (s.e. = ±0.0004334) at a

Multiple egg mass oviposition EC_{50} values computed by either maximum likelihood probit or logistic regression analyses proved essentially similar at all tested $CdCl_2$ concentrations at all 72 h exposure intervals throughout the 672 h exposure period whether estimated without (Tables 3.10 and 3.14, Fig. 3.8 A) or with SL included as a covariate (Tables 3.10 and 3.14, Figs. 3.8 B, C and D). In all cases, EC_{50} values fell well within the standard error of the means of paired values computed by either method suggesting that they were not statistically different from each other.

Number of Egg Masses Oviposited Per Individual

A gerneralized linear regression analysis for poisson duistributions without and with SL as a covariate was used to analyze data on the cumulative number of egg masses oviposited·individual^{-1} by specimens of *P. acuta* exposed to concentrations of $CdCl_2$ ranging from 0-2000 μg $CdCl_2 \cdot L^{-1}$ for 672 h. This analysis revealed that exposure to increasing $CdCl_2$ concentrations significantly reduced the number of egg masses oviposited during the observation period (chi-square = 6.63, p = 0.1000). Resulting adjusted means for number egg masses

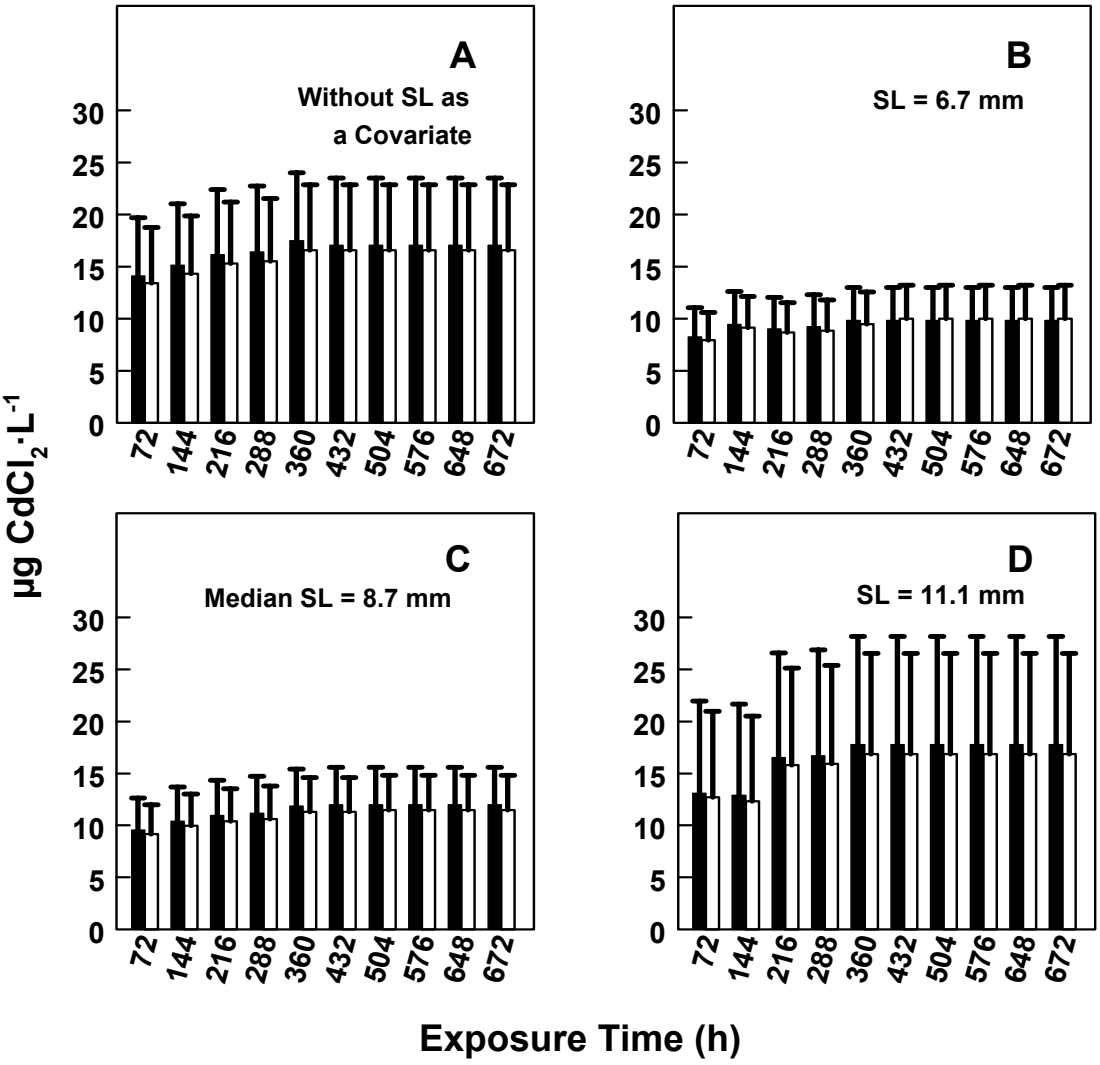


Fig.3.8 Comparison of EC_{50} values computed by maximum likelihood probit analysis (solid histograms) to those computed by maximum likelihood logistic regression (open histograms) of individuals in a sample of *Physa acuta* (n = 30) without shell length (SL) as a covariate (A) and with inclusion of SL as a covariate for individuals in the 25th (SL = 6.7 mm) (B), 50th (median SL = 8.7 mm) (C) and 75th (SL=11.1mm) SL quantiles (D), respectively, ovipositing multiple egg masses during any one 72 h observation period when exposed to varying concentrations of cadmium chloride (µg $CdCl_2 \cdot L^{-1}$, horizontal axis) over a total exposure time of 672 h (i.e., 28 d). The horizontal axis is exposure time in hours and the vertical axis, the estimated egg mass effect concentration in µg $CdCl_2 \cdot L^{-1}$. Vertical bars above histograms represent standard errors.

oviposited·individual^{-1} during the 672 h observation period ranged from 0.9942 (s.e. = ±0.00467) at a concentration of 0 μg $CdCl_2 \cdot L^{-1}$ to 0.9033 (s.e. = ±0.0004334) at a concentration of 2000 μg $CdCl_2 \cdot L^{-1}$ (Table 3.16). The relatively low mean oviposition rates resulting from this analysis resulted from large numbers of sampled individuals that did not oviposite an egg mass during the entire 672 h observation period over the entire range of tested $CdCl_2$ concentrations. A Bonferroni pair-wise comparison analysis indicated that mean number of egg masses oviposited·individual^{-1} during the 672 hour observation period were significantly different from each other at all tested concentrations.

Application of a generalized linear regression analysis for poisson data distributions to egg masses oviposited·individual^{-1} data on exposure to $CdCl_2$ concentrations ranging from 0-2000 μg $CdCl_2 \cdot L^{-1}$ during a 672 h exposure period with inclusion of ovipositing adult size as SL as a covariate allowed adjusted mean egg mass oviposition values to be calculated for the 25th, median and 75th SL quantiles. As did a similar analysis without ovipositing adult SL as a covariate (see above), this analysis revealed a significant decrease in the number of egg masses oviposited·individual^{-1} with exposure to increasing $CdCl_2$ concentration (chi-square = 12.87, p = 0.0003) and a significant correlation in egg masses oviposited·individual^{-1} with SL (chi-square = 6.99, p = 0.0082). The number of egg masses oviposited·individual^{-1} increased with increasing SL of ovipositing adults. Adjusted mean values for egg masses oviposited ·individual^{-1} in the 25th SL quantile (SL = 6.7 mm) decreased from 0.9821 (s.e. = ±0.0004749) at 0 μg $CdCl_2 \cdot L^{-1}$ to 0.8380 (s.e. = ±0.0009504) at 2000 μg $CdCl_2 \cdot L^{-1}$ (Table 3.16, Fig. 3.9 B). Adjusted mean values for egg masses oviposited ·individual^{-1} for the median SL quantile (median SL = 8.7 mm) decreased from 1.0104 (s.e. = ±0.0005182) at 0 μg $CdCl_2 \cdot L^{-1}$ to 0.8621 (s.e. = ±0.000633) at

Table 3.16 Sub-lethal reproduction mean number of egg masses concentration^{-1} estimated by a generalized linear model analysis for individuals of *Physa acuta* ovipositing eggsover a 672 h (28 d) observation period at 72 h intervals when exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 - 2000 µg $CdCl_2 \cdot L^{-1}$. Mean number egg mass values are provided for analyses of the entire sample without shell length (SL) included as a covariate, and with inclusion of SL as a covariate for individuals in the 25th SL quantile (SL = 6.7 mm), median 50th SL quantile (median SL = 8.7 mm) and 75th SL quantile (SL = 11.1 mm).

Exposure Concentration (µg $CdCl_2 \cdot L^{-1}$)	**SL not a Covariate**	**s.e.**	**SL = 6.7 mm**	**s.e.**	**SL = 8.7 mm**	**s.e.**	**SL = 11.1 mm**	**s.e.**
0	69.894	±49.2354	68.851	±50.9405	72.332	±46.244	76.742	±157.0568
5	43.647	±10.3976	30.407	±6.2142	37.849	±6.795	49.220	±32.9631
10	35.671	±5.4258	21.422	±2.9375	28.675	±3.274	40.689	±15.9468
50	22.275	±1.6442	9.461	±0.7996	15.004	±1.019	26.096	±2.8244
100	18.152	±1.2197	6.631	±0.5042	11.322	±0.740	21.514	±1.5814
150	16.148	±1.0800	5.413	±0.3897	9.639	±0.628	19.266	±1.2803
200	14.835	±1.003	4.672	±0.3233	8.578	±0.560	17.785	±1.1632
250	13.910	±0.9544	4.178	±0.2803	7.851	±0.512	16.737	±1.1109
300	13.197	±0.9183	3.813	±0.2492	7.303	±0.477	15.927	±1.0844
350	12.594	±0.8886	3.516	±0.2243	6.848	±0.447	15.239	±1.0695
400	12.124	±0.8656	3.291	±0.2058	6.499	±0.424	14.702	±1.0619
450	11.706	±0.8453	3.097	±0.1900	6.192	±0.403	14.223	±1.0574
600	10.754	±0.7983	2.673	±0.1564	5.511	±0.357	13.130	±1.0529
800	9.908	±0.7550	2.319	±0.1292	4.924	±0.317	12.154	±1.0520
1000	9.264	±0.7205	2.063	±0.1102	4.488	±0.286	11.406	±1.0506
1200	8.789	±0.6940	1.883	±0.0972	4.175	±0.264	10.854	±1.0480
1400	8.411	±0.6721	1.745	±0.0875	3.930	±0.246	10.414	±1.0445
1600	8.074	±0.6519	1.625	±0.0792	3.715	±0.231	10.019	±1.0399
2000	7.571	±0.6206	1.454	±0.0677	3.401	±0.208	9.429	±1.0299

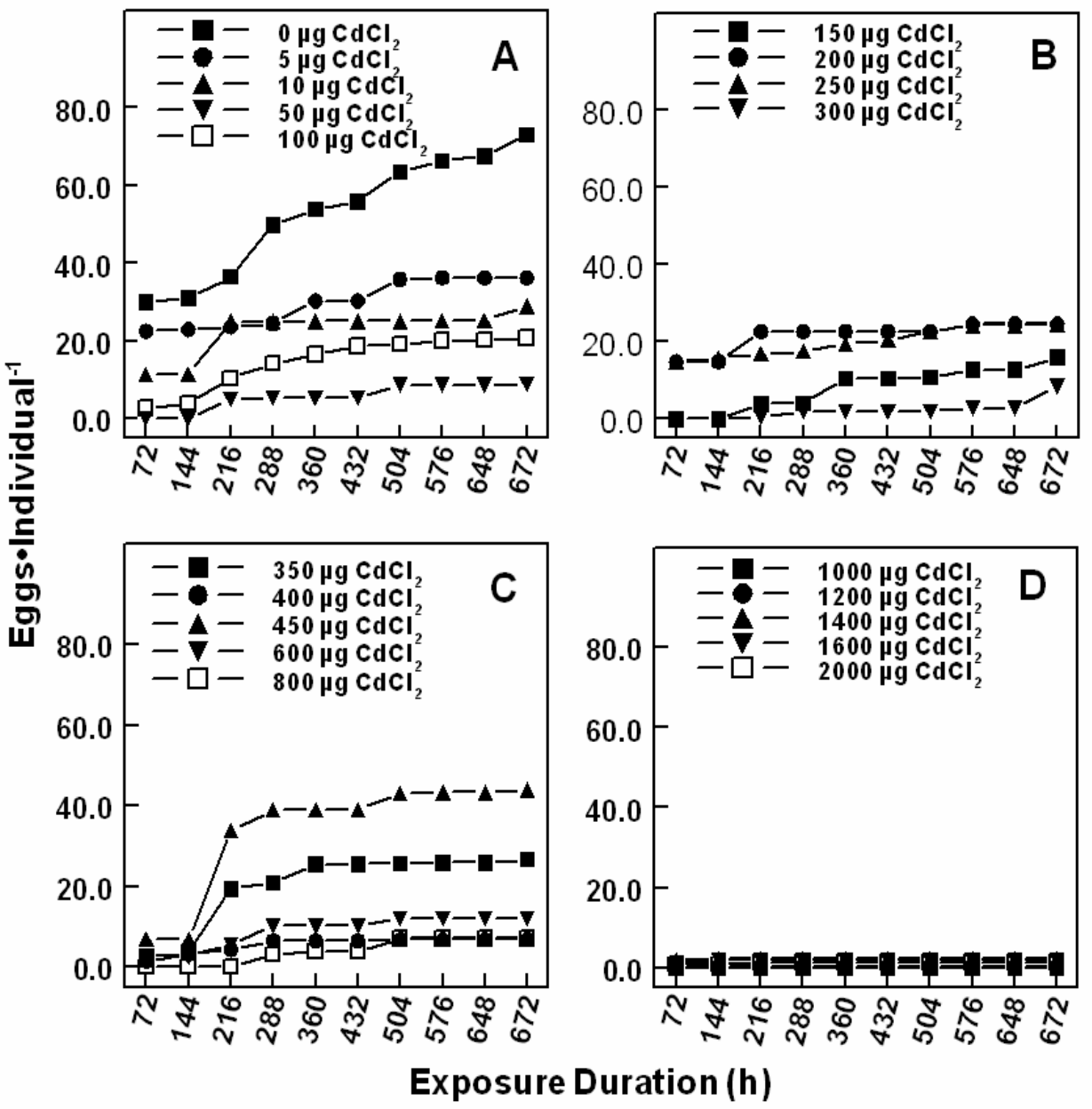


Fig.3.9 Effect of exposure of samples of *Physa acuta* to various concentrations of cadmium chloride ($CdCl_2$) ranging from A) 0-100 µg·L^{-1}, B) 150-300 µg·L^{-1}, C) 350-800 µg·L^{-1} and D) 1000-2000 µg·L^{-1} for raw data on mean cumulative number of eggs oviposited per individual (egg·$individual^{-1}$) (n =20) per 72 h observation period (vertical axis) during a total exposure period of 672 h (i.e., 28 d, horizontal axis).

2000 μg $CdCl_2 \cdot L^{-1}$ (Table 3.16, Fig. 3.9 C). Adjusted mean values for number of egg masses oviposited ·individual^{-1} for the 75th SL quantile (SL = 11.1 mm) decreased from 1.0278 (s.e. = ±0.0006634) at an exposure concentration of 0 μg $CdCl_2 \cdot L^{-1}$ through 0.8770 (s.e. = ±0.0005089) at an exposure concentration of 2000 μg $CdCl_2 \cdot L^{-1}$ (Table 3.16, Fig. 3.9 D). For all three sample SL quantile groups, Bonferroni pair-wise comparisons indicated that adjusted mean values for egg masses oviposited ·individual^{-1} were significantly different from each other at all tested $CdCl_2$ concentrations at $p < 0.0001$.

Number of Eggs Oviposited Per Individual

Both $CdCl_2$ concentration (0-2000 μg $CdCl_2 \cdot L^{-1}$) and exposure time appeared to impact the fecundity of adult specimens of *P. acuta* when determined at consecutive 72 h intervals over a 672 h (28 day) exposure period (Table 3.17, Fig. 3.10). The greatest cumulative fecundity in specimens of *P. acuta* as eggs oviposited·individual^{-1} were recorded at 0 μg $CdCl_2 \cdot L^{-1}$ (Table 3.17, Fig. 3.10) with fecundity decreasing progressively with increasing $CdCl_2$ concentration until it reached near-zero levels at concentrations ≥1000 μg $CdCl_2 \cdot L^{-1}$ over the entire 672 h exposure period (Table 3.17, Fig 3.10 D). At $CdCl_2$ concentrations of 5-1000 μg $CdCl_2 \cdot L^{-1}$ fecundity rate declined with increasing exposure time, with the majority of egg oviposition occurring within the first 360 h of exposure (Figs. 3.10 A, B and C), following which oviposition was greatly inhibited for the remainder of the 672 h exposure period. Only individuals not exposed to any $CdCl_2$ (i.e., 0 μg $CdCl_2 \cdot L^{-1}$) appeared to produce eggs at a relatively constant rate throughout out the entire exposure period (Fig. 3.10), suggesting that exposure concentrations of $CdCl_2$ even at levels as low as 5 μg $CdCl_2 \cdot L^{-1}$ can have major impacts on fecundity in *P.*

Table 3.17 Effect of cadmium chloride ($CdCl_2$) concentration and exposure time on the number of adult individuals in samples of *Physa acuta* ovipositing eggs over a concentration range of 0-2000 µg $CdCl_2 \cdot L^{-1}$ as determined every 72 h during a 672 h (28d) total exposure period [# Ovipos. = non-cumulative number of individuals sample ovipositing; $Eggs \cdot Ind^{-1}$ = Cumulative eggs oviposited per individual in the sample.

		72 h Exposure		144 h Exposure		216 h Exposure		288 h Exposure		360 h Exposure	
µg $CdCl_2$ · L^{-1}	**n**	**# Individuals With Eggs**	**$Eggs \cdot Ind.^{-1}$**	**# Individuals With Eggs**	**$Eggs \cdot Ind.^{-1}$**	**# Individuals With Eggs**	**$Eggs \cdot Ind.^{-1}$**	**# Individuals With Eggs**	**$Eggs \cdot Ind.^{-1}$**	**# Individuals With Eggs**	**$Eggs \cdot Ind.^{-1}$**
0	33	26	29.91	2	31.03	3	36.52	13	49.91	4	53.91
5	30	16	22.50	1	22.93	1	23.67	1	24.50	12	30.27
10	30	5	11.30	0	11.30	14	24.97	0	24.97	1	25.10
50	30	0	0.00	0	0.0	8	5.03	0	5.03	1	5.37
100	30	6	2.60	3	3.87	9	10.37	4	14.17	3	16.60
150	30	0	0.00	0	0.00	18	3.93	1	4.07	3	10.47
200	30	12	14.83	0	14.83	11	22.63	0	22.63	0	22.63
250	30	8	14.97	1	15.83	2	16.93	1	17.53	3	19.33
300	30	0	0.00	0	0.00	1	0.77	1	1.93	0	1.93
350	30	4	2.43	2	3.57	10	19.43	2	20.90	7	25.50
400	30	5	1.50	5	3.23	2	4.37	3	6.53	0	6.53
450	30	1	6.87	0	6.87	13	33.97	8	38.97	0	38.97
600	30	2	2.70	0	2.70	6	5.50	10	10.20	0	10.20
800	30	0	0.00	0	0.00	0	0.00	6	3.07	1	3.93
1000	30	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
1200	30	1	0.20	0	0.20	0	0.20	0	0.20	0	0.20
1400	30	4	1.77	2	2.33	0	2.33	0	2.33	0	2.33
1600	30	3	0.97	2	1.77	2	2.00	0	2.00	0	2.00
2000	30	2	1.00	0	1.00	1	1.40	0	1.40	0	1.40

Table 3.17 Continued

µg $CdCl_2$ · L^{-1}	n	432 h Exposure		504 h Exposure		576 h Exposure		648 h Exposure		672 h Exposure	
		# Individuals With Eggs	Eggs·Ind.$^{-1}$	# Individuals With Eggs #	Eggs·Ind.$^{-1}$	# Indivduals With Eggs	Eggs·Ind.$^{-1}$	# Individuals With Eggs	Eggs·Ind.$^{-1}$	# Individuals With Eggs	Eggs·Ind.$^{-1}$
0	33	4	55.82	18	63.6	8	66.42	3	67.64	10	73.09
5	30	0	30.27	9	35.87	1	36.23	0	36.23	0	36.23
10	30	0	25.10	0	25.10	1	25.27	0	25.27	5	28.93
50	30	0	5.37	6	8.67	0	8.67	0	8.67	1	8.83
100	30	2	18.70	2	19.13	1	19.97	1	20.23	1	20.87
150	30	0	10.47	2	10.87	3	12.73	0	12.73	8	16.0
200	30	0	22.63	0	22.63	4	24.60	0	24.60	0	24.60
250	30	3	20.40	4	22.67	3	24.27	0	24.27	1	24.40
300	30	0	1.93	0	1.93	4	2.83	0	2.83	13	8.60
350	30	0	25.50	1	25.77	1	25.93	0	25.93	2	26.67
400	30	0	6.53	1	6.83	0	6.83	0	6.83	0	6.83
450	30	0	38.97	3	43.07	1	43.33	0	43.33	3	44.00
600	30	0	10.20	3	12.07	0	12.07	0	12.07	0	12.07
800	30	0	3.93	6	7.07	0	7.07	0	7.07	0	7.07
1000	30	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
1200	30	0	0.20	0	0.20	0	0.20	0	0.20	0	0.20
1400	30	0	2.33	0	2.33	0	2.33	0	2.33	0	2.33
1600	30	0	2.00	0	2.00	0	2.00	0	2.00	0	2.00
2000	30	0	1.40	0	1.40	0	1.40	0	1.40	0	1.40

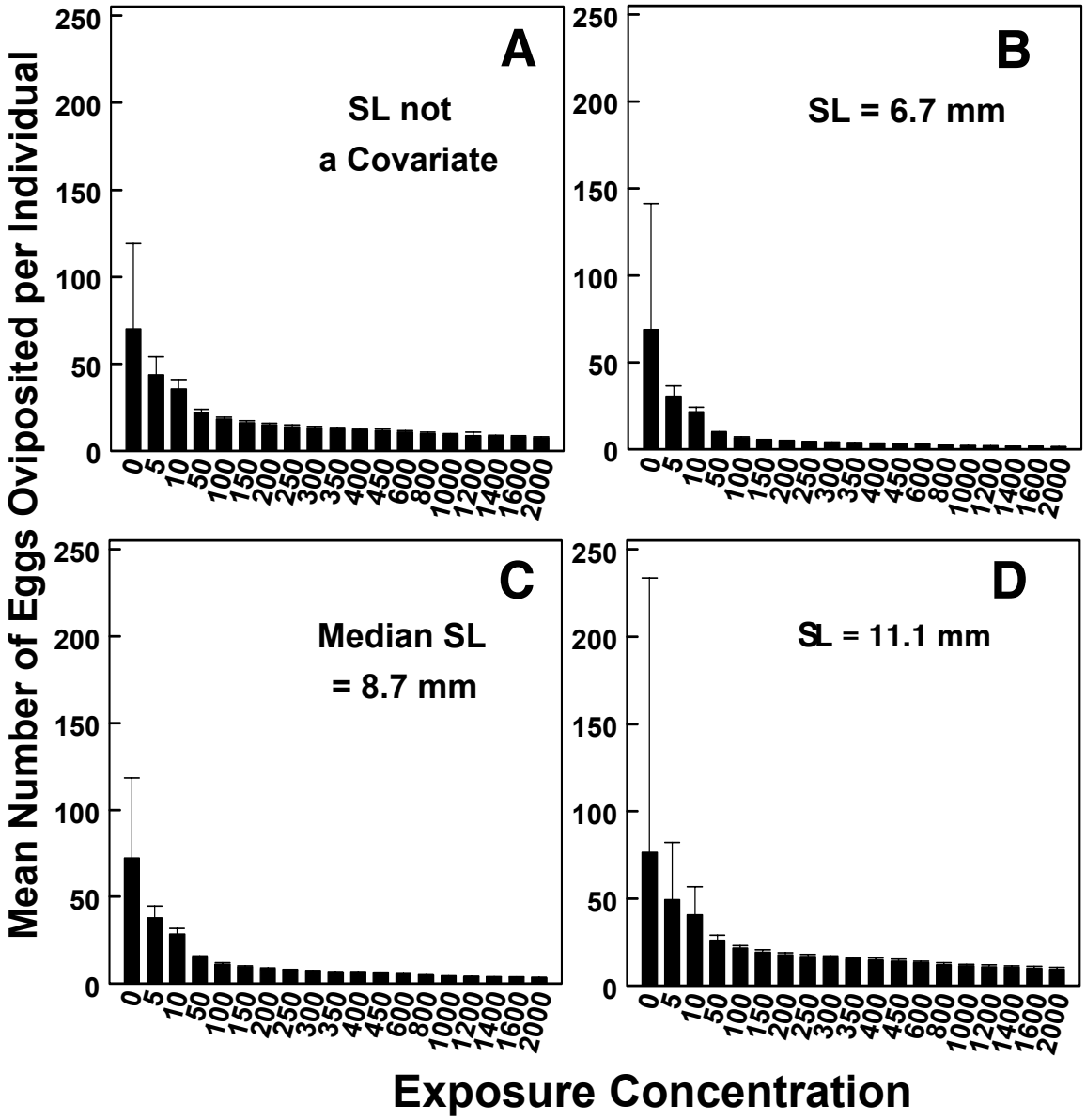


Fig.3.10 Size adjusted mean number of eggs oviposited per individual (vertical axis) in samples of *Physa acuta* (n = 30) without shell length (SL) as a covariate (A) and with inclusion of SL as a covariate for individuals in the 25th (SL = 6.7 mm) (B), 50th (median SL = 8.7 mm) (C) and 75th (SL=11.1mm) SL quantiles (D), respectively, exposed to varying concentrations of cadmium chloride (µg $CdCl_2 \cdot L^{-1}$, Horizontal axis) for 672 h (i.e., 28 d). Bonferroni pair-wise comparison testing indicated that all concentration treatment means in each panel were significantly different from each other at $P < 0.5$. Error bars above histograms represent standard error of the EC_{50} value. Where error bars are not visible standard error was too small to be differentiated from the mean.

acuta. That fecundity declines with increasing time of exposure also suggested that the impacts of sub-lethal concentrations of $CdCl_2$ on fecundity in this species might be cumulative, requiring a critical exposure time (i.e., 360 h) before becoming fully expressed (Table 3.17).

Generalized linear model analysis without inclusion of SL as a covariate revealed a significant relationship between eggs oviposited·individual^{-1} over a 672 period and $CdCL_2$ concentration at each tested exposure concentration (0-2000 μg $CdCl_2 \cdot L^{-1}$) (chi-square = 172.60 $p < 0.0001$). Adjusted mean values of eggs oviposited·individual^{-1} resulting from this analysis ranged from 68.894 eggs oviposited·individual^{-1} (s.e. = ±49.2354) at a concentration of 0 μg $CdCl_2 \cdot L^{-1}$ to 7.571 eggs oviposited·individual^{-1} (s.e. = ±0.6206) at a concentration of 2000 μg $CdCl_2 \cdot L^{-1}$ (Table 3.l8, Fig. 3.10 A). A Bonferroni pair-wise comparison test indicated that adjusted means of eggs oviposited·individual^{-1}were significantly different from each other at all tested $CdCl_2$ concentrations, $p < 0.00001$.

A generalized linear model analysis of the effect of a 672 h exposure to $CdCl_2$ concentrations ranging from 0-2000 μg $CdCl_2 \cdot L^{-1}$ on eggs oviposited·individual^{-1} of *P. acuta* with SL included as a covariate revealed that ovipositing adult size measured as SL was not significantly related to fecundity (chi-square = 0.32, p =0.5721). The analysis also revealed a significant interaction between $CdCl_2$ concentration and SL (chi-square = 41.39, $p < 0.0001$). The analysis allowed estimation of mean eggs oviposited·individual^{-1} for the 25th SL quantile, 50th SL and 75th SL quantiles. In all three quantiles, exposure to increasing $CdCl_2$ concentrations significantly reduced the number of eggs oviposited·individual^{-1} (25th SL quantile chi-square = 291.05, $p < 0.0001$, 50th SL quantile chi-square value = 258.43, $p < 0.0001$ and 75th SL quantile chi-square = 74.39, $p < 0.0001$). In the 25th SL quantile (SL = 6.7 mm), mean

Table 3.18 Sub-lethal reproduction total egg mean values estimated by a generalized linear model analysis for individuals of *Physa acuta* ovipositing eggs over a 672 h (28 d) observation period at 72 h intervals when exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 - 2000 µg $CdCl_2 \cdot L^{-1}$. Mean total egg values are provided for analyses of the entire sample without shell length (SL) included as a covariate, and with inclusion of SL as a covariate for individuals in the 25th SL quantile (SL = 6.7 mm), median 50th SL quantile (median SL = 8.7 mm) and 25th SL quantile (SL = 11.1 mm).

Exposure Concentration (µg $CdCl_2 \cdot L^{-1}$)	**Mean SL not a Covariate**	**s.e.**	**Mean SL = 6.7 mm**	**s.e.**	**SL = 8.7 mm**	**s.e.**	**Med. SL = 11.1 mm**	**s.e.**
0	96.274	±23.9679	93.0857	±23.3299	101.959	±30.2462	113.731	±144.8998
5	68.953	±7.0312	50.5651	±5.0780	62.025	±6.2540	79.254	±35.6602
10	59.762	±4.5010	38.9284	±3.3156	50.125	±3.6847	67.883	±18.5255
50	42.802	±2.4679	21.1463	±1.7151	30.492	±1.8672	47.308	±4.0475
100	37.021	±2.2552	16.2182	±1.3328	24.566	±1.5947	40.433	±2.5678
150	34.074	±2.1908	13.9366	±1.1480	21.712	±1.4677	36.963	±2.2293
200	32.086	±2.1561	12.4859	±1.0266	19.853	±1.3811	34.635	±2.1178
250	30.656	±2.1329	11.4869	±0.9412	18.549	±1.3171	32.967	±2.0836
300	29.533	±2.1147	10.7293	±0.8754	17.547	±1.2657	31.662	±2.0786
350	28.569	±2.0987	10.09795	±0.8198	16.701	±1.2205	30.546	±2.0870
400	27.810	±2.0854	9.6124	±0.7767	16.044	±1.1842	29.668	±2.1004
450	27.126	±2.0727	9.1850	±0.7385	15.460	±1.1509	28.880	±2.1166
600	25.543	±2.0403	8.2289	±0.6522	14.136	±1.0717	27.061	±2.1654
800	24.103	±2.0056	7.4003	±0.5767	12.966	±0.9972	25.413	±2.2173
1000	22.981	±1.9742	6.7824	±0.5201	12.077	±0.9374	24.135	±2.2573
1200	22.139	±1.9477	6.3351	±0.4790	11.424	±0.8919	23.180	±2.2874
1400	21.461	±1.9245	5.9850	±0.4469	10.907	±0.8547	22.413	±2.3089
1600	20.847	±1.9017	5.6757	±0.4186	10.446	±0.8207	21.720	±2.3261
2000	19.918	±1.8640	5.2216	±0.3771	9.760	±0.7689	20.674	±2.3474

fecundity decreased from 68.851 eggs oviposited·individual^{-1} (s.e. = ±50.941 at an exposure concentration of 0 μg $CdCl_2 \cdot L^{-1}$ to 1.454 eggs oviposited·individual^{-1} (s.e. = ±0.0677) at 2000 μg $CdCl_2 \cdot L^{-1}$ (Table 3.17, Fig. 3.10 B). In the median SL quantile (median SL = 8.7 mm), mean fecundity decreased from 72.332 eggs oviposited·individual^{-1} (s.e. = ±46.244) at 0 μg $CdCl_2 \cdot L^{-1}$ to 3.401 eggs oviposited·individual^{-1} (s.e. = ±0.208 at 2,000 μg $CdCl_2 \cdot L^{-1}$ (Table 3.17, Fig. 3.10 C). In the 75thSL quantile (SL = 11.1 mm) mean fecundity values decreased from 76.742 eggs oviposited·individual^{-1} (s.e. = ±157.0568) at 0 μg $CdCl_2 \cdot L^{-1}$ to 9.429 eggs oviposited·individual^{-1} (s.e. = ±1.0299) at 2000 μg $CdCl_2 \cdot L^{-1}$ (Table 3.17, Fig 3.10 D). For all SL analyses, Bonferroni pair-wise comparison testing indicated that estimates of eggs oviposited·individual^{-1}were significantly different from each other at all tested $CdCl_2$ concentrations ($p < 0.00001$). This result suggests that the number of eggs oviposited per individual is a highly sensitive measure of sub-lethal $CdCl_2$ toxicity in *P. acuta*, with a significant depression in individual fecundity being recorded at the lowest tested $CdCl_2$ concentration of 5 μg $CdCl_2 \cdot L^{-1}$ regardless of the size of the ovipositing adults utilized.

Egg Development and Hatching Success

Fraction of Eggs in an Egg Mass Developing into Embryos

Logistic regression analysis with SL as a covariate revealed no significant relationship between fraction of eggs developing into embryos (n = 2245) and $CdCL_2$ concentrations ranging from 0–800 μg $CdCl_2 \cdot L^{-1}$ (chi-square = 1.38, p = 0.2400) at weekly intervals over a total exposure period of 672 hours. This analysis also revealed no significant effect of SL on the fraction of eggs in an egg mass developing into embryos (chi-square = 0.31, p = 0.5750), nor was there a significant interaction between $CdCl_2$ concentration and shell length (chi-square = 3.64, p = 0.0564). The analysis demonstrated a significant increase in the fraction of eggs developing

into embryos with increasing weeks of exposure (chi-square = 21.54, p = <0.0001) as well as a significant interaction between $CdCl_2$ concentration and week of exposure (chi-square = 6.26, p = .0124). A Bonferroni multiple pair-wise comparison test indicated that in weeks 1-3 of exposure to $CdCl_2$, the fraction of eggs developing to an embryo in the control treatment of 0 μg $CdCl_2 \cdot L^{-1}$ was almost always significantly greater than that recorded at any tested $CdCl_2$ concentration (i.e., 5-800 μg $CdCl_2 \cdot L^{-1}$) (p range = 0.0201- <0.0001) with the exception of exposure to 10 μg $CdCl_2 \cdot L^{-1}$ in week one (Table 3.19, Fig. 3.11). Analysis was not undertaken for fraction of eggs hatching data for week four as the data was not different from that recorded for week three. This result suggested that the fraction of eggs hatching per egg mass is another highly sensitive measure of sub-lethal $CdCl_2$ toxicity in *P. acuta*, with a significant depression in individual fecundity generally being recorded at the lowest tested $CdCl_2$ concentration of 5 μg $CdCl_2 \cdot L^{-1}$ in weeks 1-3 over the entire 672 h observation period regardless of the size of the ovipositing adults utilized.

Percent of Eggs Hatching into a Functional Juvenile

Exposure to increasing $CdCl_2$ concentrations ranging from 0-2000 μg $CdCl_2 \cdot L^{-1}$ over a 672 h exposure period resulted in a significant reduction in the percent of eggs hatching in specimens of *P. acuta* (Fig. 3.12). Even after the first 72 h of exposure, egg hatching was almost entirely inhibited at $CdCl_2$ concentrations ≥ 300 μg $CdCl_2 \cdot L^{-1}$. Egg hatching ceased at all tested $CdCl_2$ concentrations after 336 h of exposure based on observation of egg hatching at successive 24 h observation periods after the initial eight days of exposure, indicating that 21 days was the maximum period of embryonic development to the hatched juvenile stage in *P. acuta* (Fig. 3.12).

Table 3.19 Sub-lethal reproduction mean values for fraction eggs per egg mass developing into embryos estimated by a logistic regression analysis for individuals of *Physa acuta* ovipositing eggs over a 672 h (28 d) observation period at one week intervals when exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 - 800 μg $CdCl_2 \cdot L^{-1}$. Mean fraction egg values are provided for analyses of the entire sample over 672 h (4 weeks), and for individuals over 168 h (1 week), 336 h (2 weeks) and 504 h (3 weeks).

Difference in Mean for Exposure Concentrations (μg $CdCl_2 \cdot L^{-1}$)	**Week 1 Adjusted Bonferroni *p***	**Week 2 Adjusted Bonferroni *p***	**Week 3 Adjusted Bonferroni *p***
0 – 5	0.0201*	< 0.001*	< 0.001*
0-10	0.2125	0.008*	< 0.001*
0-50	< 0.001*	< 0.001*	< 0.001*
0-100	< 0.001*	< 0.001*	< 0.001*
0-150	< 0.001*	< 0.001*	< 0.001*
0-200	< 0.001*	< 0.001*	< 0.001*
0-250	< 0.001*	< 0.001*	< 0.001*
0-300	< 0.001*	< 0.001*	< 0.001*
0-350	< 0.001*	< 0.001*	< 0.001*
0-400	< 0.001*	< 0.001*	< 0.001*
0-450	< 0.001*	< 0.001*	< 0.001*
0-600	< 0.001*	< 0.001*	< 0.001*
0-800	< 0.001*	< 0.001*	< 0.001*

* indicates significant

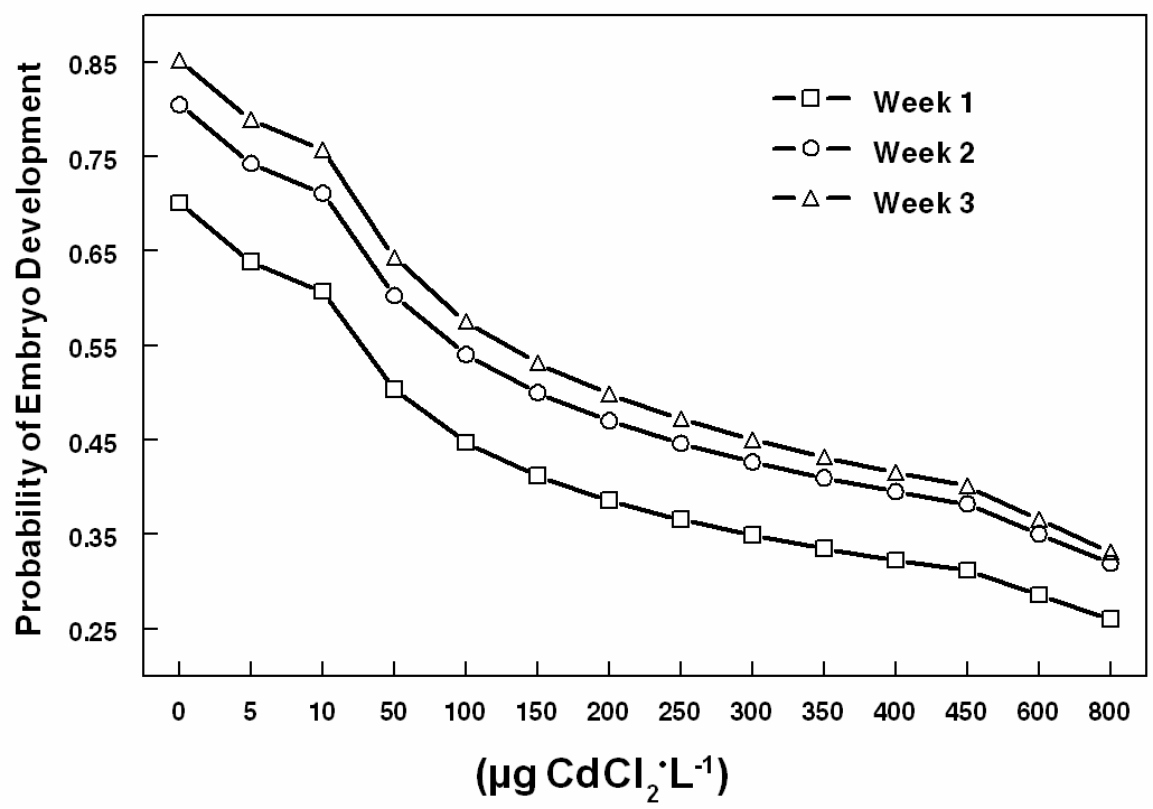


Fig.3.11 Effect of exposure to various concentrations of cadmium chloride ranging from 0-800 µg $CdCl_2 \cdot L^{-1}$ (horizontal axis) on the probability of an egg oviposited by individuals of adult *Physa acuta* developing into a recognizable embryo after one (open squares), two (open circles), and three (open triangles) weeks of development as estimated by maximum likelihood logistic regression analysis.

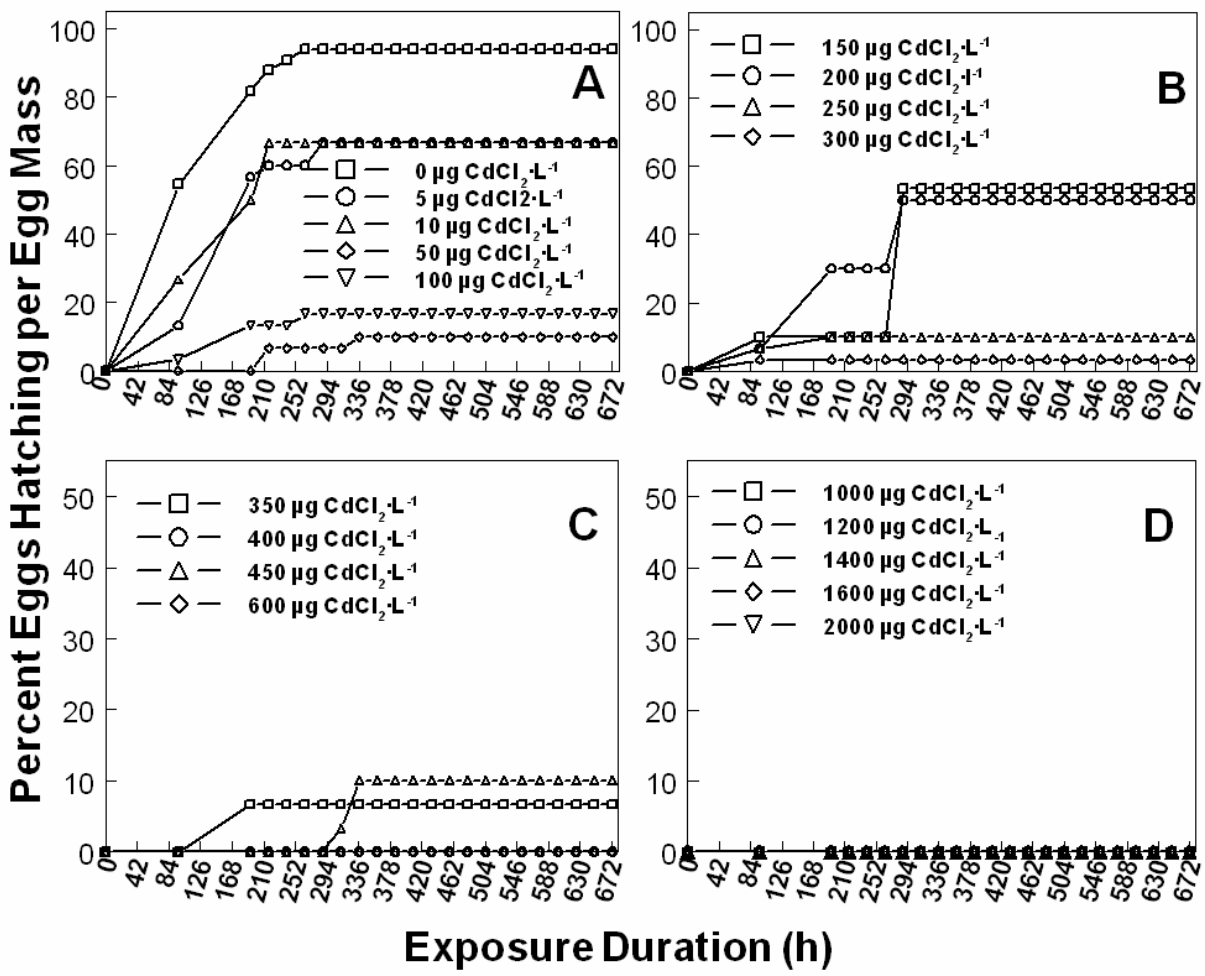


Fig.3.12 Mean cumulative percent of eggs hatching per total eggs oviposited in individual egg masses (vertical axis) oviposited by isolated adult individuals of *Physa acuta* (n = 30-33) exposed to concentrations of cadmium chloride ranging from 0-2000 µg $CdCl_2 \cdot L^{-1}$ (as indicated in figure legends) at 96 h and then consecutive 24 h periods starting 192 h after oviposition over a total exposure time of 672 h (i.e., 28 d) (horizontal axis). Percent egg hatching values are shown for $CdCl_2$ concentrations ranging from (A) 0-100 µg $CdCl_2 \cdot L^{-1}$, (B) 150-300 µg $CdCl_2 \cdot L^{-1}$, (C) 350-800 µg $CdCl_2 \cdot L^{-1}$ and (D) 1000-2000 µg $CdCl_2 \cdot L^{-1}$. Note that in all figures maximum cumulative percentage eggs hatching occurred at 336 h (2 weeks) after egg mass oviposition regardless of $CdCl_2$ concentration (A-D) and that egg hatching was totally inhibited at concentrations > 450 µg $CdCl_2 \cdot L^{-1}$ (C and D).

There was an evident impact of exposure time on hatch. At the first observation period of 96 h (4 days), the highest percent hatch occurred in concentrations of 0 μg $CdCl_2 \cdot L^{-1}$ (54.55 %) followed by 5 μg $CdCl_2 \cdot L^{-1}$ (40.00 %). At all higher $CdCl_2$ concentrations, percent of eggs hatching fell below 33.3 % with no hatching recorded at 150, 300, 800 and 1000 μg $CdCl_2 \cdot L^{-1}$ (Fig. 3.12). This general trend of decreasing hatching success with increasing $CdCl_2$ continued through-out the 672 h observation period at the end of which cumulative percent egg hatching values were 93.94 % at 0 μg $CdCl_2 \cdot L^{-1}$, 66.67 % at 5 μg $CdCl_2 \cdot L^{-1}$, 66.67 % at 10 μg $CdCl_2 \cdot L^{-1}$, 10 % at 50 μg $CdCl_2 \cdot L^{-1}$, 16.67 % at 100 μg $CdCl_2 \cdot L^{-1}$, 53.33 % at 150 μg $CdCl_2 \cdot L^{-1}$, 50 % at 200 μg $CdCl_2 \cdot L^{-1}$, 10 % at 250 μg $CdCl_2 \cdot L^{-1}$, 0 % at 300 μg $CdCl_2 \cdot L^{-1}$, 6.67 % at 350 μg $CdCl_2 \cdot L^{-1}$ and 0 % at concentrations ranging from 400-2000 at μg $CdCl_2 \cdot L^{-1}$ (Fig. 3.12).

Egg Hatch EC_{50} Analysis

Maximum likelihood probit and logistic regression analyses were utilized to determine the effective $CdCl_2$ concentration value (EC_{50}) for the observation of egg hatching in individual egg masses (i.e., one or more eggs hatching to juveniles in an egg mass was counted as successful hatching for that mass while no eggs hatching was counted as unsuccessful hatching in a mass). The egg hatching EC_{50} value was estimated by these two analyses as the $CdCl_2$ concentration at which 50 % of sampled egg masses contained at least one egg that hatched to functional juvenile. Values of EC_{50} were estimated with and without shell length as a covariate.

Maximum likelihood probit regression analysis without inclusion of SL as a covariate revealed a significant relationship between hatch response and $CdCL_2$ concentration at each tested 24 h exposure duration from 96-672 h across which chi-square and p-values ranged from

chi-square = 40.57, p <0.0001 at an exposure period of 96 h to chi-square = 329.79, p <0.0001 at an exposure period of 264 h (Table 3.20). Hatch EC_{50} values increased with increasing duration of exposure reaching a maximum value of 11.9753 µg $CdCl_2 \cdot L^{-1}$ (s.e. = ±2.1306) between 384 and 672 h (Table 3.21, Fig. 3.13 A). The lack of change in the EC_{50} value at exposures of 336 to 672 µg $CdCl_2 \cdot L^{-1}$ were due to lack of further egg hatching at exposures greater than 336 h (Table 3.21, Fig. 3.13 A).

Maximum likelihood probit analysis with inclusion of SL as a covariate revealed a significant relationship between SL and egg hatching at all tested $CdCl_2$ concentrations at all tested exposure times between 96 and 672 h. Chi-square values for the effect of SL on egg hatching ranged from 6.48 (p = 0.0109) at 96 h of exposure to 14.10 (p <0.0002) at 216 h of exposure (Table 3.22). Probit analysis revealed no significant interaction between $CdCl_2$ concentration and SL at any exposure duration (chi-square range = 0.35 - 1.43, range of p = 0.2314 -0.05516) (Table 3.22).

Because maximum likelihood probit analysis revealed a significant impact of shell size on hatch response on exposure to $CdCl_2$, probit analysis was carried out for three SL quantile groups including the 25th, median 50th and 75th sample SL quantile groups. For the 25th SL quantile (median SL = 7.1 mm) there was a significant relationship between hatch response and $CdCL_2$ concentration at each tested exposure duration (96-672 hours) across which chi-square and p values ranged from chi-square = 1.21 (p <0.0001) at 96 h of exposure to chi-square = 225.61, p <0.0001 at the 384 h exposure (Table 3.20). Egg hatching EC_{50} values decreased from 6.8032 µg $CdCl_2 \cdot L^{-1}$ (s.e. = ±1.1220) at an exposure periods 384- 672 h to 0.00 µg $CdCl_2 \cdot L^{-1}$ (s.e. = ±0.0000) at an exposure time of 96 h (Table 3.21, Fig. 3.13 B). Similarly egg hatching in

Table 3.20 Chi-square and probability (p) values for maximum likelihood probit regression analysis determinations of chronic cadmium chloride ($CdCl_2$) hatch effect concentration values (EC_{50}) for 50 % of individuals of *Physa acuta* with egg hatch response when exposed to media with concentrations ranging from 0 - 2000 µg $CdCl_2 \cdot L^{-1}$ over a total exposure period of 672 h (28 days). Chi-square values are provided for analysis carried out without shell length (SL) as a covariate to describe the probability of an individual egg hatching in the given interval: $I^{-1}(p) = b_0 + b_1(Lconcentration+1)$. The probability of of an individual egg hatching in a given interval: $I^{-1}(p) = b_0 + b_1(Lconcentration+1)+b_2\ (SL-SL^0)+b_3[(Lconcentration+1)*(SL-SL^0)]$ with shell length as a covariate allowing determination of EC_{50} values for individuals in the 25th SL quantile (SL = 7.1 mm), 50th SL quantile (median SL = 8.7 mm) and the 75th SL quantile (SL = 11.1 mm).

Exposure Duration (hours)	Chi-Square SL not a Covariate	p	Chi-Square SL = 7.1 mm	P	Chi-Square Median SL = 8.7 mm	p	Chi-Square SL = 11.1 mm	p
96	40.57	<0.0001*	1.21	<0.0001*	40.63	<0.0001*	29.08	<0.0001*
192	216.75	<0.0001*	123.68	<0.0001*	167.80	<0.0001*	109.14	<0.0001*
216	312.35	<0.0001*	174.24	<0.0001*	233.85	<0.0001*	158.24	<0.0001*
240	321.32	<0.0001*	183.54	<0.0001*	241.51	<0.0001*	158.82	<0.0001*
264	329.79	<0.0001*	192.56	<0.0001*	252.76	<0.0001*	161.00	<0.0001*
288	328.83	<0.0001*	216.91	<0.0001*	277.26	<0.0001*	154.79	<0.0001*
312	328.34	<0.0001*	220.63	<0.0001*	278.27	<0.0001*	150.34	<0.0001*
336	320.41	<0.0001*	223.54	<0.0001*	273.53	<0.0001*	140.60	<0.0001*
360	320.41	<0.0001*	223.54	<0.0001*	273.53	<0.0001*	140.60	<0.0001*
384	321.71	<0.0001*	225.61	<0.0001*	276.06	<0.0001*	141.57	<0.0001*
408	321.71	<0.0001*	223.69	<0.0001*	273.12	<0.0001*	139.83	<0.0001*
432	321.71	<0.0001*	223.69	<0.0001*	272.95	<0.0001*	139.75	<0.0001*
456	321.71	<0.0001*	223.69	<0.0001*	272.95	<0.0001*	139.75	<0.0001*
480	321.71	<0.0001*	223.69	<0.0001*	272.95	<0.0001*	139.75	<0.0001*
504	321.71	<0.0001*	223.69	<0.0001*	272.95	<0.0001*	139.75	<0.0001*
528	321.71	<0.0001*	223.69	<0.0001*	272.95	<0.0001*	139.75	<0.0001*
552	321.71	<0.0001*	223.69	<0.0001*	272.95	<0.0001*	139.75	<0.0001*
576	321.71	<0.0001*	223.69	<0.0001*	272.95	<0.0001*	139.75	<0.0001*
600	321.71	<0.0001*	223.69	<0.0001*	272.95	<0.0001*	139.75	<0.0001*
624	321.71	<0.0001*	223.69	<0.0001*	272.95	<0.0001*	139.75	<0.0001*
648	321.71	<0.0001*	223.69	<0.0001*	272.95	<0.0001*	139.75	<0.0001*
672	321.71	<0.0001*	223.69	<0.0001*	272.95	<0.0001*	139.75	<0.0001*

* indicates a significant difference at $p < 0.005$

Table 3.21 Reproduction sample hatch effect concentration values (EC_{50}) estimated by maximum likelihood probit regression analysis for 50 % of individuals of *Physa acuta* (n = 927) with egg hatch response when exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 - 2000 µg $CdCl_2 \cdot L^{-1}$ over a total exposure period of 672 h (28 days). Hatch EC_{50} values are provided for analyses of the entire sample without shell length (SL) included as a covariate, and with inclusion of SL as a covariate for individuals in the 25th SL quantile (SL = 7.1 mm), median 50th SL quantile (median SL = 8.7 mm) and 75th SL quantile (SL = 11.1 mm).

Exposure Time (h)	EC_{50} SL not a Covariate	s.e.	EC_{50} SL = 7.1 mm	s.e.	EC_{50} Med. SL = 8.7 mm	s.e.	EC_{50} SL = 11.1 mm	s.e.
96	0.0043	±0.0036	0.0000	±0.0000	0.0380	±0.0354	0.2105	±0.2353
192	1.9586	±0.4537	1.4790	±0.2980	2.6961	±0.5251	6.1765	±1.7982
216	5.8650	±0.9854	3.7480	±0.5722	6.4706	±0.9140	13.7030	±2.6299
240	6.5777	±1.0961	4.1278	±0.6256	6.9639	±0.9747	14.5207	±2.7727
264	7.2556	±1.1804	4.6572	±0.7051	7.5977	±1.0518	15.0776	±2.8411
288	10.2449	±1.7719	6.1423	±0.9943	10.5763	±1.5099	22.0492	±4.0258
312	10.8632	±1.8874	6.5355	±1.0636	10.9901	±1.5799	22.4365	±4.1730
336	11.6051	±2.0736	6.7813	±1.1166	11.2770	±1.6586	23.2470	±4.5051
360	11.6051	±2.0736	6.7813	±1.1166	11.2770	±1.6586	23.2470	±4.5051
384	11.9753	±2.1306	6.8032	±1.1220	11.3352	±1.6703	23.4612	±4.5525
408	11.9753	±2.1306	6.8032	±1.1220	11.3352	±1.6703	23.4612	±4.5525
432	11.9753	±2.1306	6.8032	±1.1220	11.3352	±1.6703	23.4612	±4.5525
456	11.9753	±2.1306	6.8032	±1.1220	11.3352	±1.6703	23.4612	±4.5525
480	11.9753	±2.1306	6.8032	±1.1220	11.3352	±1.6703	23.4612	±4.5525
504	11.9753	±2.1306	6.8032	±1.1220	11.3352	±1.6703	23.4612	±4.5525
528	11.9753	±2.1306	6.8032	±1.1220	11.3352	±1.6703	23.4612	±4.5525
552	11.9753	±2.1306	6.8032	±1.1220	11.3352	±1.6703	23.4612	±4.5525
576	11.9753	±2.1306	6.8032	±1.1220	11.3352	±1.6703	23.4612	±4.5525
600	11.9753	±2.1306	6.8032	±1.1220	11.3352	±1.6703	23.4612	±4.5525
624	11.9753	±2.1306	6.8032	±1.1220	11.3352	±1.6703	23.4612	±4.5525
648	11.9753	±2.1306	6.8032	±1.1220	11.3352	±1.6703	23.4612	±4.5525
672	11.9753	±2.1306	6.8032	±1.1220	11.3352	±1.6703	23.4612	±4.5525

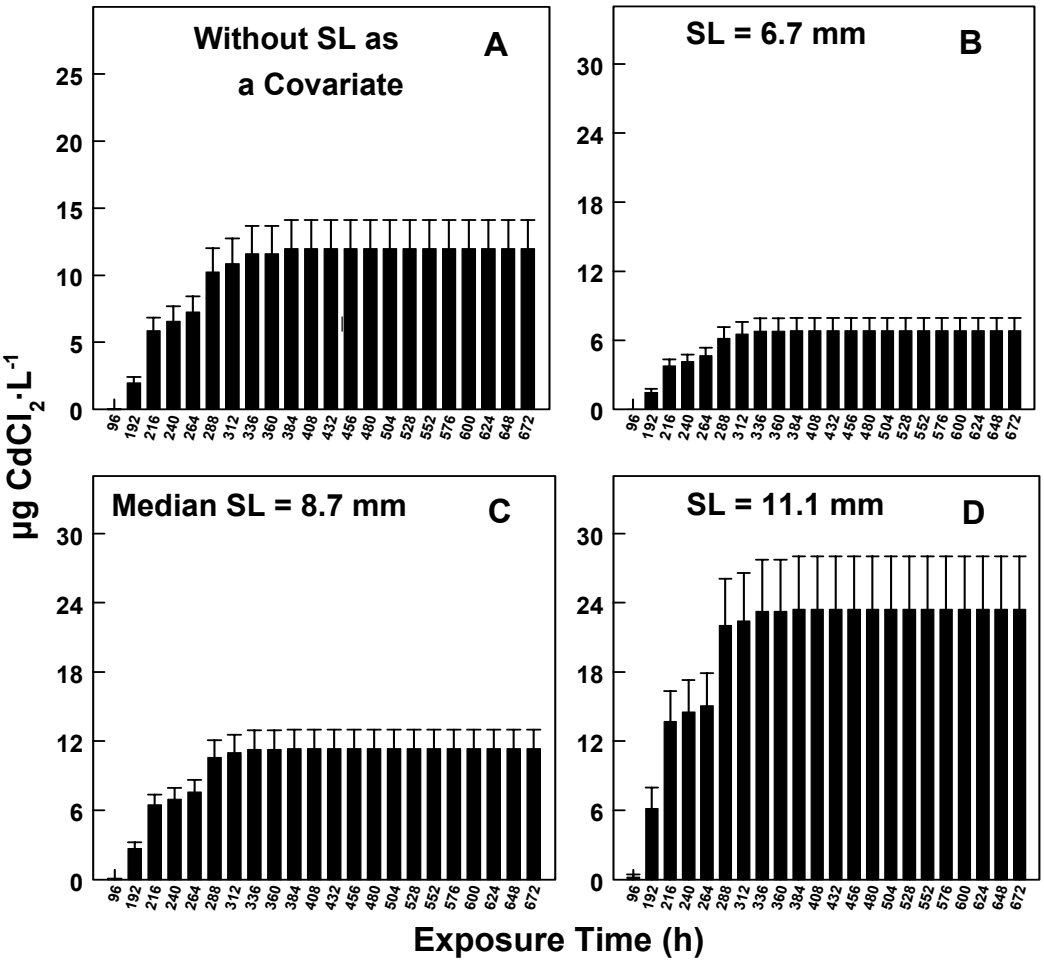


Fig.3.13 Effect concentration (EC_{50}) estimated by maximum likelihood probit regression analysis for 50 % of eggs hatching in individual egg masses oviposited by isolated adult individuals *Physa acuta* (n = 30-33) and exposed to varying concentrations of cadmium chloride ranging from 0-2000 µg $CdCl_2 \cdot L^{-1}$. Numbers of eggs hatching from egg masses were recorded at 96 h and every 24 h from 192 h after oviposition through a total exposure time of 672 h (i.e., 28 d). Egg hatching EC_{50} values were computed without shell length (SL) as a covariate (A) and with inclusion of SL as a covariate for individuals in the 25th (SL = 6.7 mm) (B), 50th (median SL= 8.7 mm) (C) and 75th (SL= 11.1 mm) SL quantiles (D),respectively. Error bars above histograms represent standard error of the EC_{50} values. Note that egg hatching EC_{50} values stabilize after the first 336 h (2-weeks) of exposure. This 336 h period is the development interval for eggs of *P.acuta* after which no eggs hatched (Fig. 3.17). Thus, EC_{50} values estimated beyond 336 h represent the true effect concentration for $CdCl_2$ on this species.

Table 3.22 Shell length and concentration-shell length interaction chi-square and probability (p) values for hatch effect concentration (EC_{50}) for 50 % of individuals of *Physa acuta* with egg hatch response determined by maximum likelihood probit regression analysis when exposed to concentrations of cadmium chloride ($CdCl_2$) ranging from 0-2000 µg $CdCl_2 \cdot L^{-1}$ over a total exposure period of 672 h (28 days). The probability of of an individual egg hatching in a given interval: $\Gamma^{1}(p) = b_0 + b_1(Lconcentration+1)+b_2\ (SL-SL^0)+b_3[(Lconcentration+1)*SL-SL^0]$ with shell length as a covariate allowing determination of EC_{50} values for individuals in the 25th SL quantile (SL = 6.7 mm), median 50th SL quantile (median SL = 8.7 mm) and the 75th SL quantile (SL = 11.1 mm). Chi-square values for SL and the concentration-SL interaction remained constant regardless of SL for individuals in the 25th SL quantile (SL = 7.1 mm), the 50th SL quantile (median SL = 8.7 mm) and the 75th SL quantile (SL = 11.1 mm).

Exposure Duration (hours)	Chi-Square SL as a Covariate	p	Chi-Square Concentration-SL as a Covariate	P
96	6.48	0.0109	0.86	0.3530
192	10.28	0.0013	0.65	0.4218
216	14.10	0.0002	1.09	0.2971
240	12.66	0.0004	0.62	0.4303
264	11.39	0.0007	0.69	0.4076
288	12.67	0.0004	1.43	0.2314
312	11.01	0.0009	1.01	0.3142
336	9.12	0.0025	0.35	0.5516
360	9.12	0.0025	0.35	0.5516
384	9.12	0.0025	0.35	0.5516
408	9.12	0.0025	0.35	0.5516
432	9.12	0.0025	0.35	0.5516
456	9.12	0.0025	0.35	0.5516
480	9.12	0.0025	0.35	0.5516
504	9.12	0.0025	0.35	0.5516
528	9.12	0.0025	0.35	0.5516
552	9.12	0.0025	0.35	0.5516
576	9.12	0.0025	0.35	0.5516
600	9.12	0.0025	0.35	0.5516
624	9.12	0.0025	0.35	0.5516
648	9.12	0.0025	0.35	0.5516
672	9.12	0.0025	0.35	0.5516

* indicates a significant difference at $p < 0.005$

the 50th sample SL quantile (median SL = 8.7 mm) was significantly depressed by exposure to increasing $CdCL_2$ concentrations at each tested exposure duration (96-672 hours) across which chi-square and p values ranged from chi-square = 40.63 ($p < 0.0001$) at 96 h of exposure to chi-square = 277.26 ($p < 0.0001$) at the 288 h exposure (Table 3.20). Egg hatching EC_{50} values in the median SL quantile decreased from 11.3352 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±1.6703) at an exposure periods 384- 672 h to 0.0380 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±0.0354) at 96 h (Table 3.21, Fig. 3.13 C). Like the 25th and median 50th sample SL quantiles, egg hatching response was also significantly depressed with exposure to increasing $CdCl_2$ concentrations in the 75th sample SL quantile (median SL = 11.1 mm) at each tested exposure duration between 96-672 hours across which chi-square and p values ranged from chi-square = 29.08 ($p < 0.0001$) after an exposure of 96 h to chi-square = 139.75 ($p < 0.0001$) at exposure periods ranging from 432-672 h (Table 3.20). In the 75th sample SL quantile egg hatching EC_{50} values decreased from 23.2470 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±4.5051) at an exposure time of 336 h through 0.2105 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±0.2353) at an exposure of 96 h (Table 3.21, Fig. 3.13 D).

Egg hatching oviposition over a 72 h exposure period EC_{50} values were much lower than the 96 h survival LC_{50} values estimated from probit regression across al SL analyses. Egg hatching EC_{50} values ranged from 6.8032 μg $CdCl_2 \cdot L^{-1}$ (s.e = ±1.1220) for median SL = 6.7 mm to 23.4612 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±4.5525) for median SL = 11.1 mm, while 96 h LC_{50} values ranged from 1504.57 (s.e. = ±39.96) for median SL =6.00 mm to 1628.26 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±37.65) for median SL = 8.3 mm (Table 3.23).

Maximum likelihood logistic regression analysis without including SL as a covariate revealed that increasing $CdCl_2$ concentration significantly reduced the egg hatching response in

Table 3.23 Comparison of 50 % sample mortality (LC_{50}), 50 % reproductive egg mass response (EC_{50}) and 50 % reproductive hatch response (EC_{50}) estimated by maximum likelihood probit regression determinations for samples of *Physa acuta* exposed to various concentrations of cadmium chloride ($CdCl_2$). In LC_{50} determinations, samples (n = 150) were exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 – 5000 μg $CdCl_2.L^{-1}$, for egg mass EC_{50} determinations, samples (n=30) were exposed to media with concentrations ranging from 0-2000 μg $CdCl_2.L^{-1}$, while for hatch EC_{50} determinations, samples (n=927) were exposed to media with concentrations ranging from 0-2000 μg $CdCl_2.L^{-1}$. Values of LC_{50} for 96 h exposure periods are provided for analysis of the entire sample without shell length (SL) as a covariate and with SL as a covariate for individuals in the 25th SL quantile (SL = 6.0 mm), median 50th SL quantile (median SL = 7.1 mm) and 75th SL quantile (SL = 8.3 mm). Values of egg mass EC_{50} for a 432 h exposure period are provided for the entire sample without shell length (SL) as a covariate and with SL as a covariate for individuals in the 25th SL quantile (SL = 6.7 mm), median 50th SL quantile (median SL = 8.7 mm) and 75th SL quantile (SL = 11.1 mm). Values of EC_{50} for a 432 h exposure period are provided for the entire sample without shell length (SL) as a covariate and with SL as a covariate for individuals in the 25th SL quantile (SL = 7.1 mm), median 50th SL quanitle (median SL = 8.7 mm) and 75th SL quantile (SL = 11.1 mm).

	Survival LC_{50}			**Reproduction Egg Mass EC_{50}**			**Reproduction Hatch EC50**		
Analysis	**SL (mm)**	**96 h LC_{50}**	**s.e.**	**SL (mm)**	**432 h EC_{50}**	**s.e.**	**SL (mm)**	**432 h EC_{50}**	**s.e.**
Without SL as a Covariate	N.A.	1572.86	±32.55	N.A.	188.203	±42.144	N.A.	11.9753	±2.136
25th SL quantile	6.0	1504.57	±39.96	N.A.	N.A.	N.A.	6.7	6.8032	±1.1220
Median 50th SL quantile	7.1	1563.96	±32.78	N.A.	127.154	±26.787	8.7	11.3352	±1.6703
75th SL quantile	8.3	1628.26	±37.65	N.A.	N.A.	N.A.	11.1	23.4612	±4.5525

P. acuta at each tested 24 h exposure duration from 96-672 h across which chi-square and p-values ranged from chi-square = 37.81 ($p < 0.0001$) at an exposure period of 96 h to chi-square = 274.33 ($p < 0.0001$) at an exposure period of 312 h (Table 3.24). Egg hatching EC_{50} values generated by this analysis increased with increasing duration of exposure reaching a maximum value of 11.4797 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±2.1029) between 384 and 672 h of exposure (Table 3.25, Fig. 3.14A).

Like probit analysis (see above), maximum likelihood logistic analysis with inclusion of SL as a covariate revealed a significant relationship between SL and egg hatching response at all tested $CdCl_2$ concentrations across all tested exposure times of 96-672 h. Chi-square values for the effect of SL on egg hatching ranged from 6.58 ($p = 0.0103$) at 96 h of exposure to 16.28 ($p < 0.0001$) at 216 h of exposure (Table 3.26). Logistic analysis revealed no significant interaction between $CdCl_2$ concentration and SL at any exposure duration (chi-square range = 0.16 – 0.83, range of $p = 0.3618$-0.6848) (Table 3.26).

Because maximum likelihood logistic analysis revealed a significant impact of shell size on hatch response on exposure to $CdCl_2$, logistic analysis was carried out for three SL quantile groups including the 25th, median 50th and 75th sample SL quantiles. For the 25th sample SL quantile (SL = 7.1 mm) there was a significant relationship between hatch response and $CdCl_2$ concentration at each tested exposure duration (96-672 hours) across which chi-square and p values ranged from chi-square = 24.38 ($p < 0.0001$) at 96 h of exposure to chi-square = 196.71 ($p < 0.0001$) at 384 h of exposure (Table 3.24). Egg hatching EC_{50} values decreased from 6.3180 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±1.0676) at an exposure periods ranging from 384-672 h to 0.0251 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±0.0257) at 96 h (Table 3.25, Fig. 3.14 B). For the 50th sample SL quantile (median SL = 8.7 mm) there was a similar significant relationship between egg hatching

Table 3.24 Chi-square and probability (p) values for maximum likelihood logistic regression analysis determinations of chronic cadmium chloride ($CdCl_2$) hatch effect concentration values (EC_{50}) for 50 % of individuals of *Physa acuta* with egg hatch response when exposed to media with concentrations of cadmium chloride ranging from 0 - 2000 µg $CdCl_2 \cdot L^{-1}$ over a total exposure period of 672 h (28 days). Chi-square values are provided for analysis carried out without shell length (SL) as a covariate to describe the probability of an individual egg hatching in the given interval: $\Gamma^1(p) = b_0 + b_1(Lconcentration+1)$. The probability of of an individual egg hatching in a given interval: $\Gamma^1(p) = b_0 + b_1(Lconcentration+1)+b_2\ (SL-SL^0)+b_3[(Lconcentration+1)*SL-SL^0]$ with shell length as a covariate allowing determination of EC_{50} values for individuals in the 25th SL quantile (SL = 6.7 mm), 50th SL quantile (median SL = 8.7 mm) and the 75th SL quantile (SL = 11.1 mm).

Exposure Duration (hours)	Chi-Square SL not a Covariate	p	Chi-Square SL = 7.1 mm	P	Chi-Square Median SL = 8.7 mm	p	Chi-Square SL = 11.1 mm	p
96	37.81	<0.0001*	24.38	<0.0001*	36.54	<0.0001*	29.25	<0.0001*
192	188.13	<0.0001*	106.29	<0.0001*	141.01	<0.0001*	105.81	<0.0001*
216	255.20	<0.0001*	143.91	<0.0001*	187.15	<0.0001*	145.79	<0.0001*
240	261.57	<0.0001*	152.54	<0.0001*	194.31	<0.0001*	146.41	<0.0001*
264	266.43	<0.0001*	157.21	<0.0001*	201.09	<0.0001*	147.99	<0.0001*
288	274.26	<0.0001*	186.83	<0.0001*	233.68	<0.0001*	142.79	<0.0001*
312	274.33	<0.0001*	190.71	<0.0001*	235.90	<0.0001*	138.99	<0.0001*
336	269.97	<0.0001*	195.15	<0.0001*	235.13	<0.0001*	130.73	<0.0001*
360	269.97	<0.0001*	195.15	<0.0001*	235.13	<0.0001*	130.73	<0.0001*
384	271.86	<0.0001*	196.71	<0.0001*	237.09	<0.0001*	131.34	<0.0001*
408	270.83	<0.0001*	195.55	<0.0001*	235.08	<0.0001*	130.02	<0.0001*
432	270.83	<0.0001*	195.35	<0.0001*	235.08	<0.0001*	130.02	<0.0001*
456	270.83	<0.0001*	195.35	<0.0001*	235.08	<0.0001*	130.02	<0.0001*
480	270.83	<0.0001*	195.35	<0.0001*	235.08	<0.0001*	130.02	<0.0001*
504	270.83	<0.0001*	195.35	<0.0001*	235.08	<0.0001*	130.02	<0.0001*
528	270.83	<0.0001*	195.35	<0.0001*	235.08	<0.0001*	130.02	<0.0001*
552	270.83	<0.0001*	195.35	<0.0001*	235.08	<0.0001*	130.02	<0.0001*
576	270.83	<0.0001*	195.35	<0.0001*	235.08	<0.0001*	130.02	<0.0001*
600	270.83	<0.0001*	195.35	<0.0001*	235.08	<0.0001*	130.02	<0.0001*
624	270.83	<0.0001*	195.35	<0.0001*	235.08	<0.0001*	130.02	<0.0001*
648	270.83	<0.0001*	195.35	<0.0001*	235.08	<0.0001*	130.02	<0.0001*
672	270.83	<0.0001*	195.35	<0.0001*	235.08	<0.0001*	130.02	<0.0001*

* indicates a significant difference at $p < 0.005$

Table 3.25 Reproduction hatch effect concentration values (EC_{50}) estimated by maximum likelihood logistic regression analysis for 50 % of individuals of *Physa acuta* (n = 927) with hatching eggs when exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 - 2000 µg $CdCl_2 \cdot L^{-1}$ over a total exposure period of 672 h (28 days). Hatch EC_{50} values are provided for analyses of the entire sample without shell length (SL) included as a covariate, and with inclusion of SL as a covariate for individuals in the 25th SL quantile (SL = 7.1 mm), median 50th SL quantile (median SL = 8.7 mm) and 75th SL quantile (SL = 11.1 mm).

Exposure Time (h)	EC_{50} SL not a Covariate	s.e.	EC_{50} Med. SL = 7.1 mm	s.e.	EC_{50} Med. SL = 8.7 mm	s.e.	EC_{50} Med. SL = 11.1 mm	s.e.
96	0.0112	±0.0081	0.0251	±0.0257	0.0809	±0.06224	0.3687	±0.3516
192	1.9258	±0.4109	1.4697	±0.2589	2.6509	±0.4688	6.1234	±1.6233
216	5.5666	±0.9191	3.4096	±0.5011	5.9457	±0.8151	12.9964	±2.3667
240	6.2280	±1.0249	3.7394	±0.5516	6.384	±0.8735	13.7471	±2.5093
264	6.8560	±1.1080	4.1643	±0.6219	6.9130	±0.9464	14.2707	±2.5809
288	9.7789	±1.7304	5.6230	±0.9220	9.7160	±1.4260	20.7457	±3.8773
312	10.3876	±1.8523	6.0058	±0.9961	10.1479	±1.5030	21.1985	±4.0356
336	11.1248	±2.0434	6.2902	±1.0609	10.5047	±1.5921	22.0614	±4.3689
360	11.1248	±2.0434	6.2902	±1.0609	10.5047	±1.5921	22.0614	±4.3689
384	11.5987	±2.1110	6.3180	±1.0676	10.5665	±1.6052	22.2657	±4.4189
408	11.4797	±2.1029	6.3180	±1.0676	10.5665	±1.6052	22.2657	±4.4189
432	11.4797	±2.1029	6.3180	±1.0676	10.5665	±1.6052	22.2657	±4.4189
456	11.4797	±2.103	6.3180	±1.0676	10.5665	±1.6052	22.2657	±4.4189
480	11.4797	±2.103	6.3180	±1.0676	10.5665	±1.6052	22.2657	±4.4189
504	11.4797	±2.103	6.3180	±1.0676	10.5665	±1.6052	22.2657	±4.4189
528	11.4797	±2.103	6.3180	±1.0676	10.5665	±1.6052	22.2657	±4.4189
552	11.4797	±2.103	6.3180	±1.0676	10.5665	±1.6052	22.2657	±4.4189
576	11.4797	±2.103	6.3180	±1.0676	10.5665	±1.6052	22.2657	±4.4189
600	11.4797	±2.103	6.3180	±1.0676	10.5665	±1.6052	22.2657	±4.4189
624	11.4797	±2.103	6.3180	±1.0676	10.5665	±1.6052	22.2657	±4.4189
648	11.4797	±2.103	6.3180	±1.0676	10.5665	±1.6052	22.2657	±4.4189
672	11.4797	±2.103	6.3180	±1.0676	10.5665	±1.6052	22.2657	±4.4189

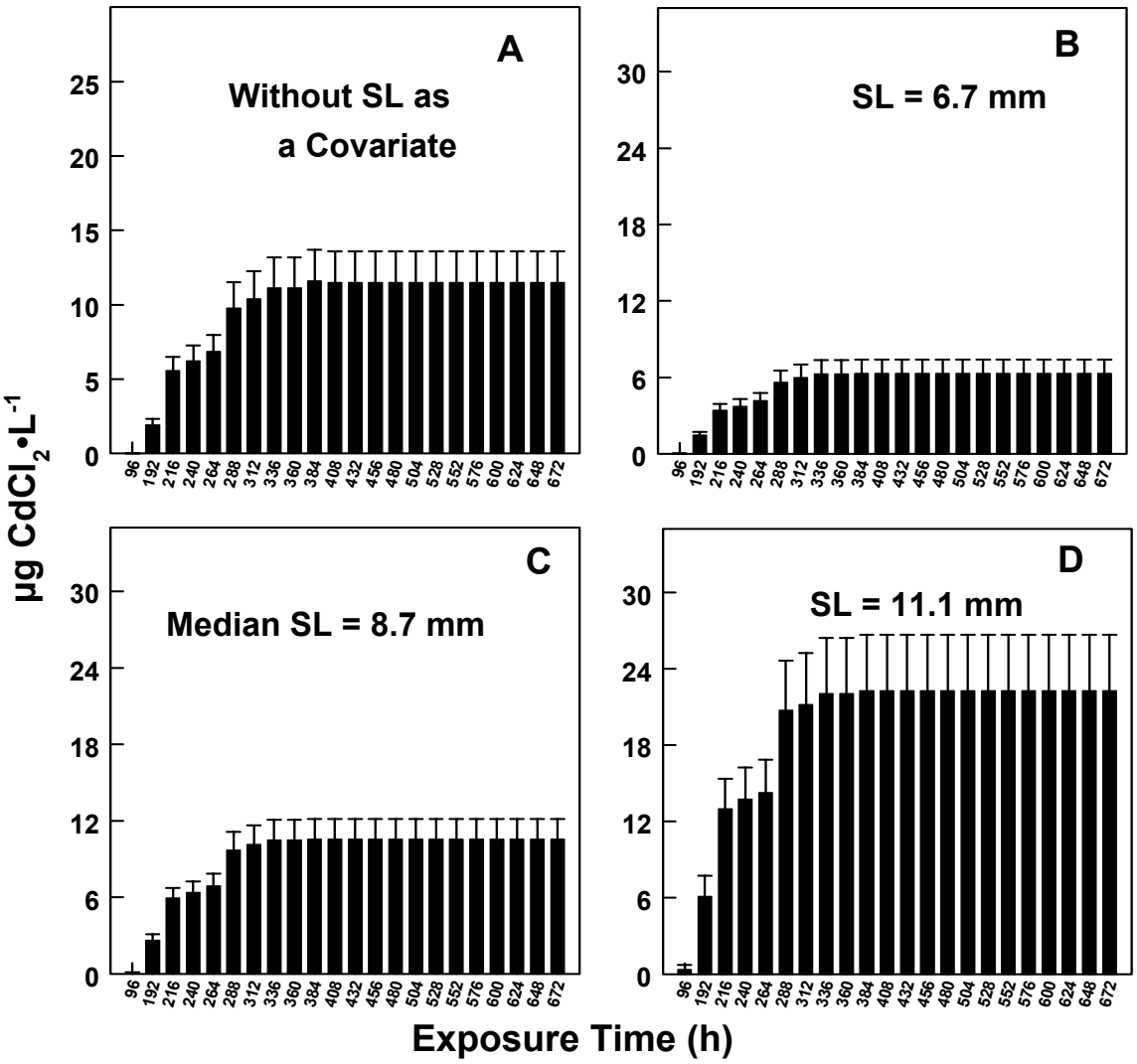


Fig.3.14 A-D Effect concentration (EC_{50}) estimated by maximum likelihood logistic regression analysis for 50 % of eggs hatching in individual egg masses oviposited by isolated adult individuals *Physa acuta* (n = 30-33) and exposed to varying concentrations of cadmium chloride ranging from 0-2000 µg $CdCl_2 \cdot L^{-1}$. Percent of eggs in egg hatching in egg masses were recorded at 96 h and then for every 24 h from 192 h after oviposition through a total exposure time of 672 h (i.e., 28 d). Egg hatching EC_{50} values were computed for the entire sample without shell length (SL) as a covariate (A) and with inclusion of SL as a covariate for individuals in the 25th (SL = 6.7 mm) (B), 50th (median SL = 8.7 mm) (C) and 75th (SL=11.1 mm) SL quantiles (D), respectively. Error bars above histograms represent standard error of the EC_{50} values. Note that egg hatching EC_{50} values stabilize after the first 336 h (2-weeks) of exposure. This 336 h period this is the maximum development interval for eggs of *P. acuta* (Fig. 3.17). Thus, EC_{50} values estimated beyond 336 h represent the true effect concentration for $CdCl_2$ on this species.

Table 3.26 Shell length and concentration-shell length interaction chi-square and probability (p) values for hatch effect concentration (EC_{50}) for 50 % of individuals of *Physa acuta* with egg hatch response determined by maximum likelihood logistic regression analysis when exposed to concentrations of cadmium chloride ($CdCl_2$) ranging from 0-2000 µg $CdCl_2 \cdot L^{-1}$ over a total exposure period of 672 h (28 days). The probability of an individual egg hatching in a given interval: $I^{-1}(p) = b_0 + b_1(Lconcentration+1)+b_2\ (SL-SL^0)+b_3[(Lconcentration+1)*(SL-SL^0)]$ with shell length as a covariate allowing determination of EC_{50} values for individuals in the 25th SL quantile (SL = 6.7 mm), 50th SL quantile (median SL = 8.7 mm) and the 75th SL quantile (SL = 11.1 mm). Chi-quare values for SL and the concentration-SL interaction remained constant regardless of SL.

Exposure Duration (hours)	Chi-Square SL as a Covariate	P	Chi-Square Concentration-SL as a Covariate	p
96	6.58	0.0103	0.49	0.4831
192	12.05	0.0005	0.32	0.5724
216	16.28	< 0.0001	0.65	0.4216
240	14.52	0.0001	0.32	0.5695
264	13.11	0.0003	0.36	0.5493
288	12.42	0.0004	0.83	0.3618
312	10.80	0.0010	0.57	0.4494
336	8.96	0.0028	0.16	0.6848
360	8.96	0.0028	0.16	0.6848
384	8.96	0.0028	0.16	0.6848
408	8.96	0.0028	0.16	0.6848
432	8.96	0.0028	0.16	0.6848
456	8.96	0.0028	0.16	0.6848
480	8.96	0.0028	0.16	0.6848
504	8.96	0.0028	0.16	0.6848
528	8.96	0.0028	0.16	0.6848
552	8.96	0.0028	0.16	0.6848
576	8.96	0.0028	0.16	0.6848
600	8.96	0.0028	0.16	0.6848
624	8.96	0.0028	0.16	0.6848
648	8.96	0.0028	0.16	0.6848
672	8.96	0.0028	0.16	0.6848

* indicates a significant difference at $p < 0.005$

response and $CdCL_2$ concentration at each tested exposure duration (96-672 hours) across which chi-square and p values ranged from chi-square = 36.54 ($p < 0.0001$) at 96 h of exposure to chi-square = 237.09 ($p < 0.0001$) at the 384 h exposure (Table 3.24). Egg hatching EC_{50} values in the median 50th sample SL quantile decreased from 10.5665 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±1.6052) at an exposure periods ranging from 384-672 h to 0.0809 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±0.06224) at 96 h (Table 3.25, Fig. 3.14 C). Logistic regression analysis also revealed a significant relationship between sample egg hatching response and $CdCL_2$ concentration at each tested exposure duration (96-672 hours) in the 75th SL quantile across which chi-square and p values ranged from chi-square = 29.25 ($p < 0.0001$) after an exposure of 96 h to chi-square = 147.99 ($p < 0.0001$) after an exposure period of 264 h (Table 3.24). Egg hatching EC_{50} values for this quantile decreased from 22.2657 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±4.4189) at exposure times ranging from 384-672 h to 0.3687 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±0.3516) at 96 h (Table 3.25, Fig. 3.14 D).

Efficacy of egg mass response was investigated by analyzing standard survival 96 h probit LC_{50} values versus multiple egg mass EC_{50} values. Egg hatching over a 72 h exposure period EC_{50} values were much lower than the 96 h survival LC_{50} values estimated from probit regression across all SL analyses. Egg hatch EC_{50} values ranged from 6.8032 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±1.1220) for median SL = 6.7 mm to 23.4612 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±4.5525) for median SL = 11.1 mm, while 96 h LC_{50} values ranged from 1504.57 (s.e. = ±39.96) for median SL =6.00 mm to 1628.26 μg $CdCl_2 \cdot L^{-1}$ (s.e. =±37.65) for median SL = 8.3 mm (Table 3.27).

Wald analysis comparing maximum likelihood probit estimations of 96 h LC_{50} to the 432 h EC_{50} for egg hatching over a single 72 h observation period without SL as a covariate yielded a $W = 2366.38$, $p = 0$. All Wald analyses comparing maximum likelihood probit regression

Table 3.27 Comparison of 50 % sample mortality (LC_{50}), 50 % sample reproductive egg mass response (EC_{50}) and 50 % sample reproductive hatch response (EC_{50}) estimated by maximum likelihood logistic regression determinations for samples of *Physa acuta* exposed to various concentrations of cadmium chloride ($CdCl_2$). In LC_{50} determinations, samples (n = 150) were exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 – 5000 µg $CdCl_2.L^{-1}$, for egg mass EC_{50} determinations, samples (n=30) were exposed to media with concentrations ranging from 0-2000 µg $CdCl_2.L^{-1}$, while for hatch EC_{50} determinations, samples (n = 927) were exposed to media with concentrations ranging from 0-2000 µg $CdCl_2.L^{-1}$. Values of LC_{50} for 96 h exposure periods are provided for analysis of the entire sample without shell length (SL) as a covariate and with SL as a covariate for individuals in the 25th SL quantile (SL = 6.0 mm), 50th SL quantile (median SL = 7.1 mm) and 75th SL quantile (SL = 8.3 mm). Values of egg mass EC_{50} for a 432 h exposure period are provided for the entire sample without shell length (SL) as a covariate and with SL as a covariate for individuals in the 25th SL quantile (SL = 6.7 mm), 50th SL quantile (median SL = 8.7 mm) and 75th SL quantile (SL = 11.1 mm). Values of EC_{50} for a 432 h exposure period are provided for the entire sample without shell length (SL) as a covariate and with SL as a covariate for individuals in the 25th SL quantile (SL = 7.1 mm), 50th SL quantile (median SL = 8.7 mm) and 75th SL quantile (SL = 11.1 mm).

	Survival LC_{50}			**Reproduction Egg Mass EC_{50}**			**Reproduction Hatch EC_{50}**		
Analysis	**SL (mm)**	**96 h LC_{50}**	**s.e.**	**SL (mm)**	**432 h EC_{50}**	**s.e.**	**SL (mm)**	**432 h EC_{50}**	**s.e.**
Without SL as a Covariate	N.A.	1589.26	±27.51	N.A.	199.302	±48.285	N.A.	11.4797	±2.1029
25th SL quantile	6.0	1499.32	±33.53	N.A.	N.A.	N.A.	6.7	6.3180	±1.0676
Median 50th SL quantile	7.1	1577.21	±27.42	8.7	127.292	±27.739	8.7	10.5665	±1.6052
75th SL quantile	8.3	1658.42	±32.04	N.A.	N.A.	N.A.	11.1	22.2657	±4.4189

estimates of 96 h LC_{50} and 432 h EC_{50} estimates for egg hatching over a single 72 h observation period with SL as a covariate proved significant as follows: for the 25th SL quantile, W = 1454.43, p = 0.001; for the median 50 th SL quantile, W = 2322.37, p = 0.001; and for the 75th SL quantile sample SL, W = 1896.89, p = 0.0.

Egg hatching oviposition over a 72 h exposure period for logistic regression EC_{50} values were much lower than the 96 h survival logistic regression LC_{50} values across al SL analyses. Egg hatching EC_{50} values ranged from 6.3180 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±1.676) for SL = 6.7 mm to 22.2657 μg $CdCl_2 \cdot L^{-1}$ (s.e. = ±4.4189) for SL = 11.1 mm, while 96 h LC_{50} values ranged from 1499.32 (s.e. = ±33.53) for median SL =6.00 mm to 1658.42 μg $CdCl_2 \cdot L^{-1}$ (s.e. =±32.04) for median SL = 8.3 mm (Table 3.27).

Wald analysis comparing maximum likelihood logistic estimations of 96 h LC_{50} to the 432 h EC_{50} for egg hatching over a single 72 h observation period without SL as a covariate yielded a W = 3367.20, p < 0001. All Wald analyses comparing logistic regression estimates of 96 h LC_{50} and 432 h EC_{50} estimates for egg hatching over a single 72 h observation period with SL as a covariate proved significant as follows: for the 25th SL quantile, W = 2034.52, p < 0.0001; for the median 50th SL quantile, W = 3320.35, p < 0.0001; and for the 75th SL quantile, W = 2704.58, p < 0.0001.

In both probit and logistic regression analyses (see above), the low egg hatching EC_{50} values recorded in the first 312 h of exposure in all three tested SL quantile groups resulted from eggs requiring at least this amount of time to fully develop to the hatching stage in *P. acuta* (Tables 3.21 and 3.25, Figs. 3.13 B, C and D, 3.14 B, C and D). Lack of hatching in egg masses observed before 312 h of exposure, thus included many egg masses in which hatching had not yet occurred but which would occur at a later observation period. This resulted in higher

fractions of egg masses being recording as having no hatched eggs in observations made prior to 312 h, thus reducing the estimated EC_{50} value. Thus, whether the data were analyzed by probit or logistic regression analyses, egg hatching response testing would yield the most accurate results if egg-hatching data was collected after at least 312 h of exposure (Figs. 3.13 B, C and D, 3.14 B, C and D).

Both probit and logistic regression analyses indicated that egg hatching EC_{50} values in response to $CdCl_2$ exposure increased significantly with increasing ovipositing adult size as SL in *P. acuta* (Tables 3.21 and 3.25, Figs. 3.13 B, C and D, 3.14.B, C, and D). This result suggested that larger ovipositing adult specimens of *P. acuta* produced egg masses in which egg development to a hatched juvenile was less inhibited than in egg masses produced by smaller ovipositing adults. This result may be associated with the fact that large adult specimens of *P. acuta* oviposite significantly larger egg masses containing greater numbers of eggs than smaller adults. When general linear regression analysis with ovipositing adult SL as a cofactor was applied to the number of eggs oviposited·mass^{-1} at sequential 72 h observation periods throughout the entire 672 h exposure to $CdCl_2$ concentrations ranging from 0-2000 μg $CdCl_2 \cdot L^{-1}$, there was both a significant increase in mean eggs oviposited·mass^{-1} with increasing ovipositing adult SL (chi-square = 15.62, $p < 0.0001$) and a significant interaction between ovipositing adult SL and $CdCl_2$ concentration (chi-square = 8.85, $p = 0.0029$). The adjusted mean eggs oviposited·mass^{-1} values yielded by this analysis for the control exposure of 0 μg $CdCl_2 \cdot L^{-1}$ were 14.965 eggs ·mass^{-1} (s.e. = ±1.2151) for the 25th sample SL quantile (SL = 6.7 mm), 19.483 eggs·mass^{-1} (s.e. = ±1.6582) for the median 50th sample SL quantile (median SL = 8.7 mm) and 22.824 eggs·mass^{-1} (s.e. = ±4.1730) for the 75th SL quantile (SL = 11.1 mm) (Table 3.28).

Table 3.28 Sub-lethal mean number of eggs·eggmass^{-1} estimated by a generalized linear model analysis for individuals of *Physa acuta* ovipositing egg masses over a 672 h (28 d) observation period at 72 h intervals when exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 - 2000 µg $CdCl_2 \cdot L^{-1}$. Mean egg values are provided for analyses of the entire sample without shell length (SL) included as a covariate, and with inclusion of SL as a covariate for individuals in the lower 25th SL quantile (SL = 6.7 mm), median 50th SL quantile (median SL = 8.7 mm) and 75th SL quantile (SL = 11.1 mm).

Exposure Concentration (µg $CdCl_2 \cdot L^{-1}$)	**Mean SL not a Covariate**	**s.e.**	**Mean SL = 6.7 mm**	**s.e.**	**Mean SL = 8.7 mm**	**s.e.**	**Mean SL = 11.1 mm**	**s.e.**
0	17.490	±1.3587	14.965	±1.2151	19.483	±1.6582	22.824	±4.1730
5	14.716	±0.5354	10.062	±0.3493	13.891	±0.4611	16.857	±1.1650
10	13.666	±0.3671	8.488	±0.2319	12.017	±0.2880	14.804	±0.6593
50	11.499	±0.2076	5.707	±0.1339	8.568	±0.1562	10.933	±0.2181
100	10.667	±0.1982	4.803	±0.1182	7.396	±0.1448	9.584	±0.1749
150	10.219	±0.2019	4.352	±0.1112	6.800	±0.1420	8.888	±0.1669
200	9.906	±0.2075	4.051	±0.1066	6.398	±0.1405	8.416	±0.1656
250	9.675	±0.2131	3.837	±0.1032	6.109	±0.1396	8.075	±0.1662
300	9.490	±0.2183	3.671	±0.1005	5.882	±0.1389	7.806	±0.1675
350	9.329	±0.2233	3.529	±0.0981	5.688	±0.1382	7.574	±0.1690
400	9.200	±0.2277	3.418	±0.0962	5.535	±0.1376	7.391	±0.1705
450	9.082	±0.2319	3.318	±0.0944	5.397	±0.1370	7.226	±0.1719
600	8.804	±0.2425	3.089	±0.0901	5.078	±0.1353	6.842	±0.1756
800	8.543	±0.2531	2.883	±0.0859	4.788	±0.1334	6.491	±0.1792
1000	8.335	±0.2621	2.724	±0.0826	4.562	±0.1316	6.216	±0.1820
1200	8.176	±0.2691	2.606	±0.0799	4.393	±0.1300	6.009	±0.1839
1400	8.045	±0.2750	2.511	±0.0777	4.256	±0.1286	5.841	±0.1853
1600	7.926	±0.2804	2.425	±0.0757	4.133	±0.1272	5.690	±0.1865
2000	7.741	±0.2888	2.298	±0.0725	3.946	±0.1248	5.459	±0.1880

Even though the mean number of eggs oviposited·mass^{-1} declined relative to control values with exposure to increasing $CdCl_2$ concentration, the same relative differences in mean eggs·mass^{-1} were observed over the entire 5-2000 μg $CdCl_2 \cdot L^{-1}$ exposure concentration range (Table 3.28). Bonferroni pair-wise comparison testing indicated that estimates of mean values of eggs oviposited·egg mass^{-1} were significantly different from each other at all tested $CdCl_2$ concentrations ($p < 0.0001$). The larger egg masses oviposited by larger individuals of *P. acuta* may have reduced the rate at which Cd^{2+} ions diffused into the egg mass. Reduced penetration of larger egg masses by Cd^{2+} ions could increase the chances of eggs in larger egg masses to develop to hatching of functional juveniles, thus explaining the tendency for egg hatching $CdCl_2$ EC_{50} values to be elevated in larger specimens of *P. acuta* which oviposite relatively larger egg masses than smaller individuals (Table 3.28).

Delay in Egg Hatching

General linearized model analysis for poisson distributions without ovipositing adult SL included as a covariate, indicated that increased $CdCl_2$ concentration (0 - 450 μg $CdCl_2 \cdot L^{-1}$) (chi-square = 43.54, $p < 0.0001$) significantly extended development time from newly oviposited eggs to newly hatched juveniles in *P. acuta* based on an initial 96 h observation, followed by 24 h interval observations from 168-672 h (Table 3.29 Fig. 3.15). At 450 μg $CdCl_2 \cdot L^{-1}$ no eggs developed to hatching during the 672 h observation period (Table 3.21). Time to hatching increased with increasing $CdCl_2$ concentration, being least at 8.1602 days (s.e. = ±0.0269) in control media of 0 μg $CdCl_2 \cdot L^{-1}$ and greatest at 10.5730 days (s.e. = ±0.0995) in 400 μg $CdCl_2 \cdot L^{-1}$ (Table 3.29, Fig. 3.15).

A generalized linear model analysis for poisson distributions with SL included as a covariate revealed that ovipositing adult size measured as SL was significantly related to time to

Table 3.29 Sub-lethal development time (days) to hatch mean values estimated by a generalized linear regression analysis for a poisson distribution for individuals of *Physa acuta* resulting in successful hatch over a 672 h (28 d) observation period at 72 h intervals when exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 - 450 µg $CdCl_2 \cdot L^{-1}$. Mean time to hatch values are provided for analyses of the entire sample without shell length (SL) included as a covariate, and with inclusion of SL as a covariate for individuals in the 25th SL quantile (SL = 6.7 mm), 50th SL quanitle (median SL = 8.3 mm) and 75th SL quantile (SL = 11.1 mm).

Exposure Concentration (µg $CdCl_2 \cdot L^{-1}$)	**Mean SL not a Covariate**	**s.e.**	**Mean SL = 6.7 mm**	**s.e.**	**EC_{50} SL = 8.3 mm**	**s.e.**	**EC_{50} SL = 11.1 mm**	**s.e.**
0	8.1602	±0.0269	8.3449	±0.0361	8.0087	±0.0314	7.5427	±0.1352
5	8.7486	±0.0178	8.9109	±0.0471	8.6606	±0.0287	8.2393	±0.0763
10	9.0136	±0.0184	9.1651	±0.0760	8.9559	±0.0401	8.6013	±0.0571
50	9.6635	±0.0348	9.7868	±0.2191	9.6849	±0.1085	9.5091	±0.0394
100	9.9605	±0.0501	10.0701	±0.3218	10.0200	±0.1616	9.9330	±0.0495
150	10.1343	±0.0616	10.2356	±0.3937	10.2168	±0.1998	10.1839	±0.0620
200	10.2622	±0.0713	10.3573	±0.4524	10.3618	±0.2314	10.3696	±0.0745
250	10.3603	±0.0795	10.4506	±0.5007	10.4732	±0.2577	10.5128	±0.0862
300	10.4412	±0.0868	10.5276	±0.5429	10.5652	±0.2809	10.6314	±0.0974
350	10.5137	±0.0936	10.5965	±0.5824	10.6477	±0.3027	10.7379	±0.1084
400	10.5730	±0.0995	10.6528	±0.6160	10.7152	±0.3214	10.8253	±0.1183
450	0.0000.	±0.0000	0.0000.	±0.0000	0.0000.	±0.0000	0.0000	±0.0000

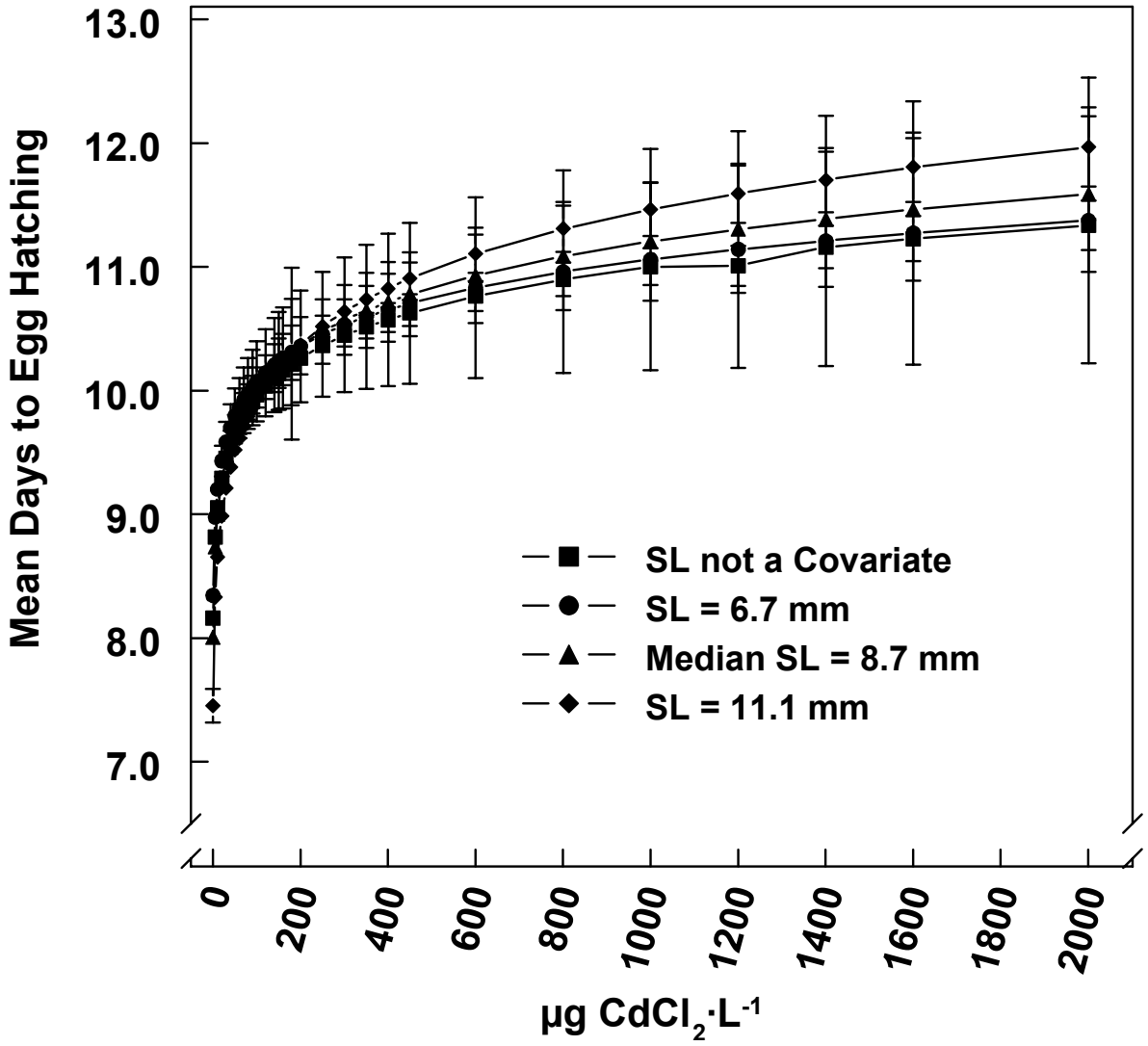


Fig.3.15 Estimated mean number of days to first eggs hatching to juvenile snails in individual egg masses (vertical axis) oviposited by isolated adult individuals of *Physa acuta* (n = 30-33) exposed to concentrations of cadmium chloride ranging from 0-2000 µg $CdCl_2 \cdot L^{-1}$ (horizontal axis). Egg masses were observed for hatching at 96 h, 192 h and, thereafter, every 24 h after oviposition over a total exposure period of 672 h (i.e., 28 d). Estimates of mean days to first hatching were determined by poisson regression analysis of $CdCl_2$ concentration versus days to first egg hatching as the dependent variable. Mean values were computed for the entire sample without shell length (SL) of ovipositing adults as a covariate (solid squares) and with inclusion of ovipositing adult SL as a covariate for individuals in the 25th (SL = 7.1 mm, solid circles), 50th (median SL = 8.7 mm, solid triangles), and 75th (SL=11.1 mm, solid diamonds), respectively. Error bars around estimatedmeans represent standard errors of the means.

hatching (chi-square = 4.08, p = 0.0433). In contrast there was no interaction between $CdCl_2$ concentration and SL (chi-square = 1.89, p = 0.1688). Chi-square and p values for the relation between hatching time and ovipositing adult SL were chi-square = 9.41 (p = 0.0002) for the 25th sample SL quantile (SL = 6.7 mm), chi-square value = 22.87 (p <0.0001) for the median 50th sample SL quantile (median SL = 8.3 mm) and chi-square = 29.57 (p <0.0001) for the 75th sample SL quantile. In the 25th sample SL quantile adjusted mean days to hatching increased from 8.3449 days (s.e. = ±0.0361) at an exposure concentration of 0 μg $CdCl_2 \cdot L^{-1}$ to 10.6528 days (s.e. = ±0.6160) at 2000 μg $CdCl_2 \cdot L^{-1}$ (Table 3.29, Fig. 3.15). In the 50th sample SL quantile (median SL = 8.7 mm), adjusted mean days to hatching increased from 8.0087 (se = ±0.0314) at 0 μg $CdCl_2 \cdot L^{-1}$ to 10.7152 (s.e. = ±0.3214 at 2,000 μg $CdCl_2 \cdot L^{-1}$ (Table 3.29, Fig. 3.15). In the 75^{th} sample SL quantile (SL = 11.1 mm), adjusted mean days to hatching increased from 7.5427 days (s.e. = ±0.1352) at 0 μg $CdCl_2 \cdot L^{-1}$ to 10.8253 eggs oviposited·$individual^{-1}$ (s.e. = ±0.1183) at 2000 μg $CdCl_2 \cdot L^{-1}$ (Table 3.29, Fig 3.15). For all SL analyses, Bonferroni pair-wise comparison testing indicated that estimates of mean times to egg hatching were significantly different from each other at all tested $CdCl_2$ concentrations (p < 0.00001).

Interestingly, time to egg hatching was not consistent among the tested sample SL quantiles across the range of $CdCl_2$ concentrations tested (0-2000 μg $CdCl_2 \cdot L^{-1}$). The slope of the response curve between time to hatching and $CdCl_2$ concentration appeared to become steeper with increasing size such that the 75th sample SL quantile had the lowest adjusted mean time to hatching at 0 μg $CdCl_2 \cdot L^{-1}$ and the greatest mean time to hatching value at the highest tested concentration of 450 μg $CdCl_2 \cdot L^{-1}$ (Fig. 3.15). Because the slope of the $CdCl_2$ concentration – time to hatching response curve progressively declined in the median 50th and

25th sample SL quantiles, the 25th sample SL quantile had the longest adjusted mean time to hatching at value 0 μg $CdCl_2 \cdot L^{-1}$ and the shortest mean time to hatching at 400 μg $CdCl_2 \cdot L^{-1}$μg $CdCl_2 \cdot L^{-1}$ (Fig. 3.15).

Effect of Cadmium Chloride on Egg Diameter

In addition to the standard reproductive endpoints of number egg masses and number of eggs oviposited per individual, this study examined egg diameter in *P. acuta* as a new sub-lethal endpoint parameter for toxicity studies. Application of least squares mixed model regression analysis without inclusion of SL as a covariate to data on the diameter of eggs oviposited by specimens of P. acuta revealed that egg diameter decreased significantly with exposure of ovipositing individuals to increasing $CdCl_2$ concentrations ranging from 0-300 μg $CdCl_2 \cdot L^{-1}$ (chi-square = 312.00, $p < 0.0001$). Adjusted means for egg diameter ranged from 0.8966 (s.e. = ±0.005169) at a concentration of 0 μg $CdCl_2 \cdot L^{-1}$ to 0.6461 (s.e. = ±0.005169) at a concentration of 300 μg $CdCl_2 \cdot L^{-1}$ (Table 3.30, Fig 3.16 A). Bonferroni pair-wise comparison testing indicated that mean egg diameter values at 0 μg $CdCl_2 \cdot L^{-1}$ was greater than at all tested concentrations of $CdCl_2$ (Table 3.31, Fig. 3.16 A).

A linear mixed model analysis of the effect of $CdCl_2$ concentration on egg diameter with inclusion of ovipositing adult SL as a covariate revealed that adult SL was significantly related to egg diameter across the range of $CdCl_2$ concentrations tested (chi-square = 5.19, $p = 0.0228$). There was also a significant $CdCl_2$ concentration-shell length interaction (chi-square = 6.05, $p = 0.0140$). This result allowed analysis of the effect of $CdCl_2$ exposure concentration of ovipositing adults on egg diameter for the 25th sample SL quantile (SL = 7.5 mm), median 50th SL quantile (median SL = 8.7 mm) (chi-square = 309.93, $p < 0.0001$) and 75th sample SL quantile (SL = 9.

Table 3.30 Sub-lethal reproduction egg diameter mean values estimated by a linear mixed model regression analysis for individuals of *Physa acuta* ovipositing eggs with an initial observation period of 72 h (3 d) observation period and then at intervals of 72 h intervals when exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0-300 µg $CdCl_2 \cdot L^{-1}$. Mean egg diameter values are provided for analyses of the entire sample without shell length (SL) included as a covariate, and with inclusion of SL as a covariate for individuals in the 25th (SL = 7.5 mm), 50th (median SL = 8.7 mm) and 75th (SL=9.9 mm) SL quantiles.

Exposure Concentration (µg $CdCl_2 \cdot L^{-1}$)	**SL not a Covariate**	**s.e.**	**SL = 7.5 mm**	**s.e.**	**SL = 8.7 mm**	**s.e.**	**SL = 9.9 mm**	**s.e.**
0	0.8966	±0.005169	0.8987	±0.005289	0.8954	±0.005136	0.8920	±0.005339
5	0.6917	±0.005169	0.6908	±0.005577	0.6876	±0.005289	0.6844	±0.005312
10	0.7125	±0.005350	0.7150	±0.005393	0.7119	±0.005319	0.7089	±0.005526
100	0.6592	±0.005169	0.6584	±0.005150	0.6583	±0.005154	0.6582	±0.005159
200	0.6969	±0.005169	0.6967	±0.005329	0.6998	±0.005169	0.7030	±0.005317
300	0.6461	±0.05169	0.6435	±0.005643	0.6499	±0.005179	0.6562	±0.005935

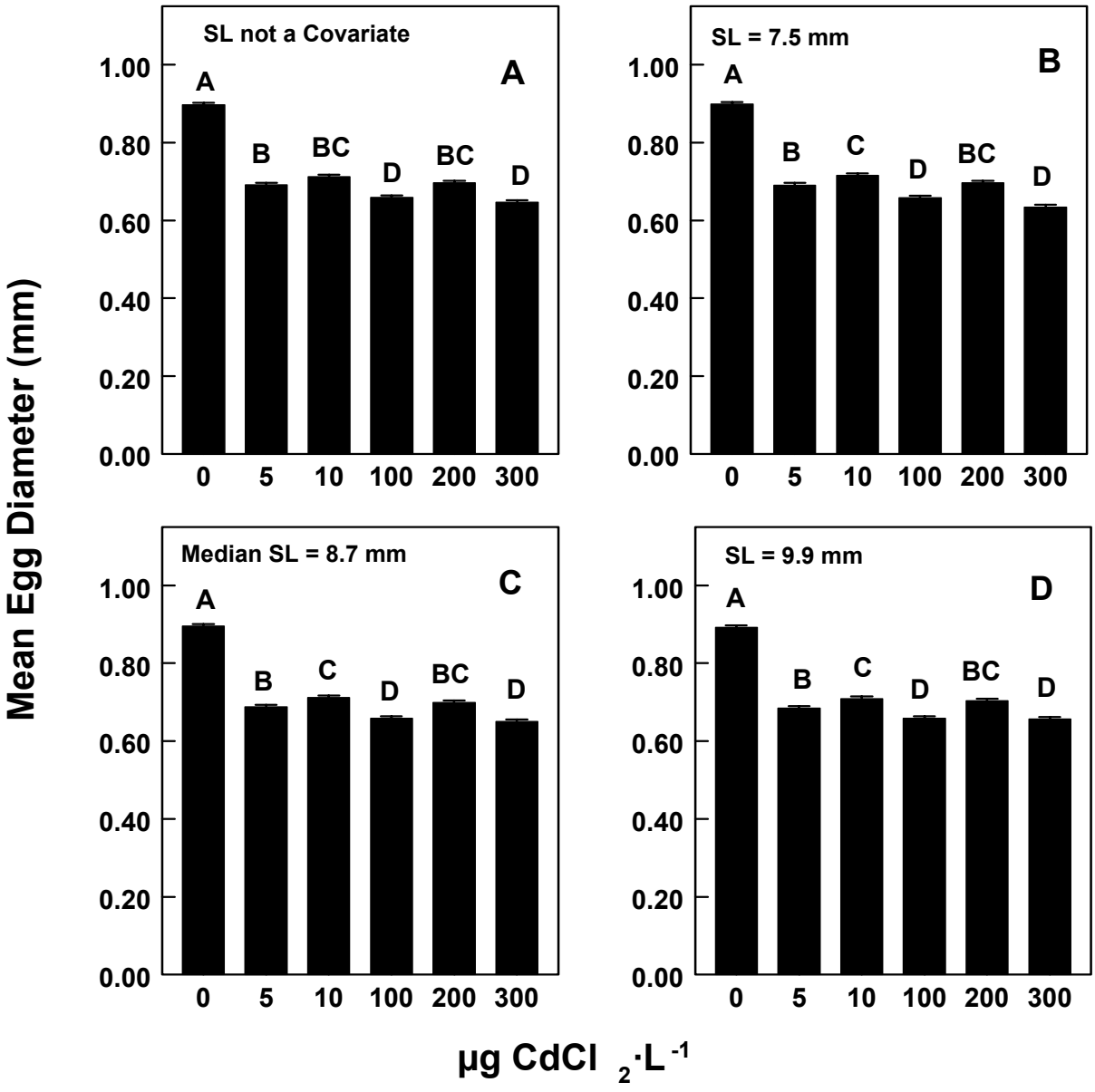


Fig.3.16 Effect of exposure of samples of *Physa acuta* to various concentrations ranging from 0-300 µg $CdCl_2 \cdot L^{-1}$ (horizontal axis) on mean diameter of eggs (n = 20) oviposited by adult individuals within an exposure period of 72 h (vertical axis) as estimated from a linear mixed model analysis. Mean values were computed without shell length (SL) of ovipositing adults as a covariate (A) and with inclusion of ovipositing adult SL as a covariate for individuals in the 25th (SL = 7.1mm) (B), 50th (median SL = 8.7 mm) (C) and 75th (SL=9.9 mm) SL quantiles (D), respectively. Error bars above histograms represent standard error of the means. Different letters above histograms indicate that mean values are significantly different ($p < 0.05$).

Table 3.31 Pair-wise comparisons of the mean diameter of eggs (n = 20) oviposited by adult individuals of *Physa acuta* exposed for up to 72 h to concentrations of cadmium chloride ranging from 0-300 µg $CdCl_2 \cdot L^{-1}$ (horizontal axis) as estimated from a linear mixed model regression analysis of log transformed data. Mean values were computed for the entire sample without shell length of ovipositing adults as a covariate (No SL covariate) and with inclusion of ovipositing adult SL as a covariate for individuals in the 25th SL quantile (SL = 7.5 mm), 50th (median SL = 8.7 mm) and 75th SL quantile (SL = 9.9 mm), respectively. P-values marked with an asterisk (*) indicate a significant difference in paired egg diameter values.

	No SL Covariate		**Median SL = 7.541 (mm)**		**Median SL = 8.731 mm**		**Median SL =9.922 mm**	
Concentration (µg $CdCl_2 \cdot L)^{-1}$	**Mean Egg Diameters (mm)**	**P**	**Mean Egg Diameters (mm)**	**P**	**Mean Egg Diameters (mm)**	**P**	**Mean Egg Diameters (mm)**	**P**
0-5	0.897-0.692	<0.0001*	0.899-0.691	<0.0001*	0.895-0.688	<0.0001*	0.895-0.684	<0.0001*
0-10	0.897-0.713	<0.0001*	0.899-0.715	<0.0001*	0.895-0.712	<0.0001*	0.895-0.709	<0.0001*
0-100	0.897-0.659	<0.0001*	0.899-0.658	<0.0001*	0.895-0.658	<0.0001*	0.895-0.658	<0.0001*
0-200	0.897-0.697	<0.0001*	0.899-0.697	<0.0001*	0.895-0.670	<0.0001*	0.895-0.703	<0.0001*
0-300	0.897-0.646	<0.0001*	0.899-0.635	<0.0001*	0.895-0.650	<0.0001*	0.895-0.656	<0.0001*
5-10	0.692-0.713	0.0773	0.691-0.715	0.00204*	0.688-0.712	0.0187*	0.684-0.709	0.0172*
5-100	0.692-0.659	0.0001*	0.691-0.658	0.0004*	0.688-0.658	0.0013*	0.684-0.658	0.0057*
5-200	0.692-0.697	1.000	0.691-0.697	1.000	0.688-0.670	1.000	0.684-0.703	0.2475
5-300	0.692-0.646	<0.0001*	0.691-0.635	<0.0001*	0.688-0.650	<0.0001*	0.684-0.656	0.0109*
10-100	0.713-0.659	<0.0001*	0.715-0.658	<0.0001*	0.712-0.658	<0.0001*	0.709-0.658	<0.0001*
10-200	0.713-0.697	0.5416	0.715-0.697	0.2761	0.712-0.670	1.000	0.709-0.703	1.000
10-300	0.713-0.646	<0.0001*	0.715-0.635	<0.0001*	0.712-0.650	<0.0001*	0.709-0.656	<0.0001*
100-200	0.659-0.697	<0.0001*	0.658-0.697	<0.0001*	0.658-0.670	<0.0001*	0.658-0.703	<0.0001*
100-300	0.659-0.646	1.00	0.658-0.635	0.7003	0.658-0.650	1.000	0.658-0.656	1.000
200-300	0.697-0.646	<0.0001*	0.697-0.635	<0.0001*	0.670-0.650	<0.0001*	0.703-0.656	<0.0001*

mm) (chi-square = 295.51, p <0.0001). For the 25 th sample SL quantile, adjusted mean values for egg diameter decreased from 0.8987 mm (s.e. = ±0.005289) at an exposure concentration of 0 μg $CdCl_2 \cdot L^{-1}$ through 0.6435 mm (s.e. = ±0.005643) at 2000 μg $CdCl_2 \cdot L^{-1}$ (Table 3.30, Fig. 3.16 B). For the median 50 th sample SL quantile, adjusted mean values for egg diameter decreased from 0.8954 mm (s.e. = ±0.005136) at an exposure concentration of 0 μg $CdCl_2 \cdot L^{-1}$ to 0.6499 mm (s.e. = ±0.005179) at 2000 μg $CdCl_2 \cdot L^{-1}$ (Table 3.31, Fig. 3.16 C). For the 75 th sample SL quantile, adjusted mean values for egg diameter decreased from 0.8920 mm (s.e. = ±0.005339) at an exposure concentration of 0 μg $CdCl_2 \cdot L^{-1}$ to 0.6562 mm (s.e. = ±0.005935) at an exposure concentration of 2000 μg $CdCl_2 \cdot L^{-1}$ (Table 3.30, Fig. 3.16 D). For each analyzed sample SL quantile, a Bonferroni pair-wise comparison test indicated that adjusted mean egg diameters at all tested $CdCl_2$ concentrations were significantly lower than that recorded at the control concentration of 0 μg $CdCl_2 \cdot L^{-1}$ (Table 3.31). Beyond suppression of egg mass diameter by exposure to $CdCl_2$ there was no observable trend in mean egg diameter across all tested $CdCl_2$ treatments (range = 5-300 μg $CdCl_2 \cdot L^{-1}$) (Table 3.30, Figs. 3.16 B, C, D).

This investigation demonstrated that $CdCl_2$ exposures as low as 5 μg $CdCl_2 \cdot L^{-1}$ significantly decreased the diameter of eggs oviposited by specimens of *P. acuta* relative to those produced in control media with 0 μg $CdCl_2 \cdot L^{-1}$. Thus, egg diameter appears to be a highly sensitive endpoint parameter with which to assess the non-lethal toxic impacts of cadmium exposure and, perhaps, the impacts of exposure to other toxicants in this species.

Discussion

Comparison of Analytical Techniques

The present investigation analyzed the effect of cadmium chloride ($CdCl_2$) on reproduction as fecundity and embryonic development responses in *P. acuta* using different

analytical methodologies including maximum likelihood probit and logistic regression analysis without and with inclusion of shell length (SL) as a size covariate. To determine the effects sub-lethal exposure, longer exposure periods are required (Eaton and Klaassen, 2003). In order to overcome the difficulties associated with short-term toxicity testing, this study also included longer observation periods (672 h) at lower $CdCl_2$ concentrations allowing estimation of the minimum tolerated levels of *Physa acuta* to this toxin.

This study demonstrated a near equivalence in the calculated EC_{50}'s among the different regression techniques of maximum likelihood probit and logistical analysis for egg mass oviposition, multiple egg mass oviposition and egg hatching endpoint data over an extended 672 h exposure period. Both analyses demonstrated a decrease in reproductive response as $CdCl_2$ concentration increased. This result confirmed prior predictions made regarding the similarity of the outcomes generated by these two approaches (Ellersieck and LaPoint, 1995).

In addition to the 50 % sample effect concentration resulting from maximum likelihood analysis, a formal analysis for number of egg masses oviposited per individual, number of eggs oviposited per individual and number of days to egg hatching resulted in mean estimates, as well as comparing the addition of shell length (SL) to the analyses as a covariate. All estimated mean values for those three analyses were compared using a Bonferroni multiple pair-wise comparison, which resulted in significant pair-wise differences for all concentrations. This analysis allowed comparisons with previously published values for other freshwater species.

The fraction of eggs in an egg mass developing into embryos was analyzed by logistic regression. The estimated values over a 672 h observation period at 168 h intervals (i.e., 168, 336, 504 and 672 h) demonstrated a decrease in the fraction of eggs developing to embryos resulting from a time (week)-concentration interaction. A Bonferroni multiple pair-wise

comparison analysis revealed significant differences between concentration pairs. All the above analyses a demonstrated a significant effect of $CdCl_2$ on reproductive endpoints in *P. acuta.*

Effect of Size as Shell Length on Toxicity Determinations

The statistical techniques utilized in this study were somewhat unusual in that they allowed additional factors (i.e., shell length covariates) to be included in the estimation of response values. Past environmental toxicity studies have generally neglected to include the impacts of covariates such as size measured as shell length even though they are generally known to impact and likely to confound results if not statistically controlled (Newman and McIntosh, 1991). Results of reproduction experiments in this study showed that the addition of shell length (SL) as a covariate in analyses consistently demonstrated a profound influence of individual size on estimated $CdCl_2$ EC_{50} values for egg hatching and multiple egg mass oviposition in *P. acuta.* These results strongly suggest that individual size must either be controlled or accounted for in data analysis in order to make accurate predictions of the sub-lethal reproductive impacts of toxic substances on test species.

Efficacy of Egg Mass EC_{50}, Number of Egg Masses, Number of Eggs, Fraction of Eggs Developing into Juveniles and Hatching EC_{50}

The effects of $CdCl_2$ on fecundity and development in *P. acuta* were tested with a variety of methods including effect concentration (50 % sample response) for egg mass oviposition, egg hatching, and multiple egg mass oviposition, generalized linear regression model testing of the number of egg masses ovipositied per individual, number of eggs oviposited per individual, the number of days to egg hatching and the fraction of eggs developing into embryos in an egg mass and least squares testing of egg diameter. All toxicity tests from this study required a lower number of animals per experiment (30 individuals/concentration) compared to survival testing (150 individuals per concentration, see Chapter 2). It also required a lower number of $CdCl_2$

concentrations be tested to provide accurate results. The range of $CdCl_2$ concentrations required for survivorship testing was 0-5000 µg $CdCl_2 \cdot L^{-1}$ while it was only 0-300 µg $CdCl_2 \cdot L^{-1}$ for egg diameter analysis and 0 – 2000 µg $CdCl_2 \cdot L^{-1}$ for the other tested reproductive endpoints. Sub-lethal reproductive endpoint testing revealed significant effects of $CdCl_2$ exposure at the lowest concentration tested (i.e., 5 µg $CdCl_2 \cdot L^{-1}$) on all reproductive endpoints, making it a much more sensitive predictor of $CdCl_2$ toxicity in *P. acuta* than survivorship testing in which the overall 96 h LC_{50} was 1572 µg $CdCl_2 \cdot L^{-1}$ (see Chapter 2) which was 314 times less sensitive than any reproductive endpoint. Similarly 50 % sample mortality could not be achieved in chronic exposure testing at $CdCl_2$ concentrations less than 800 µg $CdCl_2 \cdot L^{-1}$ making it 160 times less sensitive than reproductive endpoint testing. Even the highly sensitive sub-lethal crawl-out response determined in this study (overall CO_{50} = 295 µg $CdCl_2 \cdot L^{-1}$, see Chapter 2) was 59 times less sensitive than any of the reproductive endpoints analyzed.

Both maximum likelihood probit and logistic regression analyses demonstrated a significant effect of concentration on egg mass EC_{50} for all observation periods whether SL was or was not included as a covariate. All analyses also resulted in a concentration-response effect ranging from the highest EC_{50} in the initial 72 h exposure period to the lowest EC_{50} at 360 h of exposure (Table 3.3 and 3.6). Egg hatching (EC_{50}) demonstrated a similar concentration-response effect without and with SL as a covariate, decreasing from 384 h to 96 h for both maximum likelihood probit and logistic regression analysis (Table 3.21 and 3.25). The probit and logistic regression analyses of egg hatching with addition of SL as a covariate also showed a significant effect of concentration across all observation periods (96-672 h), but no significant concentration-SL interaction ($p > 0.05$) across all observation periods (Table 3.22 and 3.26). Egg mass and egg hatching effect concentration (EC_{50}) values (after 432 h of exposure) with SL as a

covariate at 188 µg $CdCl_2 \cdot L^{-1}$ and 12 µg $CdCl_2 \cdot L^{-1}$, respectively, were both for more sensitive than the overall standard acute 96 h LC_{50} estimated values for both maximum likelihood probit (1572 µg $CdCl_2 \cdot L^{-1}$) and logistic regression (1572 µg $CdCl_2 \cdot L^{-1}$) (Table 3.23 and 3.27).

Analyses for number of egg masses oviposited per individual and number of eggs oviposited per individual significantly declined with increasing $CdCl_2$ concentration over an exposure period of 672 h whether analyzed with or without inclusion of SL as a covariate (Table 3.16, 3.19). Similarly mean number of days to hatching increased significantly with increasing $CdCl_2$ concentration (Table 3.29). Bonferroni multiple pair-wise comparisons demonstrated that, in all three tests concentration pairs were different, resulting in a significant effect even at the lowest tested concentration of 5 µg $CdCl_2 \cdot L^{-1}$, making it a much more sensitive test of $CdCl_2$ toxicity in *P. acuta* than the standard acute 96 h mortality toxicity test (probit without SL as a covariate = 1572 µg $CdCl_2 \cdot L^{-1}$ and logistic = 1589 µg $CdCl_2 \cdot L^{-1}$) (Table 3.23 and 3.27).

Exposure to $CdCl_2$ significantly decreased the fraction of eggs in an egg mass that developed into embryos in *P. acuta* (Table 3.19). Logistic regression analysis revealed a significant effect of time of exposure, and a significant time-concentration interaction. However, unlike number of egg masses oviposited and number of eggs oviposited per individual, there was no effect of size as SL. The analyses revealed a significant depression in fraction of eggs hatching per mass relative to the control treatment at all tested $CdCl_2$ concentrations from 5-800 µg $CdCl_2 \cdot L^{-1}$ across all four weeks of exposure making it a much more sensitive predictor of $CdCl_2$ toxicity than either acute LC_{50} or chronic LT_{50} survivorship values.

Species Comparisons of Reproductive Mean Values

One issue that has been of concern in the toxicity testing literature is variation in estimated response values within species resulting from differences in experimental procedures (Marke and Solbe, 1998). Experimental procedures vary in water hardness, age and size of tested individuals, chemical species utilized and duration of exposure to test chemicals. In addition (Jensen and Forbes, 2001) demonstrated that different clones of *Potamopyrgus antipodarum* had different tolerances of $CdCl_2$ exposure indicating a genetic influence on tolerance estimates. They (Jensen and Forbes, 2001) also used calcium sulfate ($CaSO_4$) instead the more commonly used calcium carbonate ($CaCO_3$) to maintain hardness in their test media (Table 2.23).

The effect of cadmium chloride ($CdCl_2$) on fecundity in *P. acuta* computed as mean number of egg masses oviposited and mean number of eggs oviposited per individual as analyzed by a general linearized model regression analysis without size as a covariate for *P. acuta* in this study was compared to values previously published for other aquatic animal species (Table 3.32). Estimated means for eggs per mass oviposited in this study ranged from 17.490 (s.e. = ±1.3587) at 0 μg $CdCl_2 \cdot L^{-1}$ to 7.741 (s.e. = ±0.2888) at 2000 μg $CdCl_2 \cdot L^{-1}$ (Table 3.32). Estimates of the mean eggs·mass^{-1} value for the freshwater pulmonate snail, *Lymnea stagnalis*, vary from 45.5 (s.d. =18.4) at 0 μg $CdCl_2 \cdot L^{-1}$ to 9.1 (s.d. = 7.8) at 400 μg $CdCl_2 \cdot L^{-1}$ (Gomot, 1998) which are higher than the mean values of 17.490 at 0 μg $CdCl_2 \cdot L^{-1}$ to 7.741 at 2000 μg $CdCl_2 \cdot L^{-1}$ reported for adult *P. acuta* in this study (Table 3.32). The greater number of eggs per mass recorded for *L. stagnalis* may be a result of its much larger adult body size than *P. acuta*.

It is also of value to compare the $CdCl_2$ reproductive endpoint values determined for adult *P. acuta* in this study with that of other non-molluscan aquatic species, particularly those commonly

Table 3.32 Comparison of toxicity of different freshwater animal species to cadmium (Cd) measured as fecundity or development for reproduction in toxicity tests.

Species	Conditions	**Exposure Concentration ($\mu g\ CdCl_2 \cdot L^{-1}$)**	**Hardness ($mg\ Ca \cdot L^{-1}$)**	**Mean ± s.e ($\mu g\ CdCl_2 \cdot L^{-1}$)**	**Literature Citation**
Freshwater Gastropods					
**Physa acuta*	Mean Number of eggs ·mass^{-1}. 25 ± *2* °C for 672 h	0	50.0	17.490 (s.e. =.±1.3587)	This study
“	“	5	“	14.716 (s.e. =.±0.5354)	“
“	“	10	“	13.666 (s.e. =.±0.3671)	“
		50		11.499 (s.e. =.±0.2076)	
“	“	100	“	10.667 (s.e. =.±0.1982)	“
“	“	150	“	10.219 (s.e. =.±0.2019)	“
“	“	200	“	9.906 (s.e. =.±0.02075)	“
“	“	250	“	9.675 (s.e. =.±0.2131)	“
“	“	300	“	9.490 (s.e. =.±0.2183)	“
“	“	350	“	9.329 (s.e. =.±0.2233)	“
“	“	400	“	9.200(s.e. =.±0.2277)	“

Table 3.32 Continued

Species	Conditions	Exposure Concentration ($\mu g\ CdCl_2 \cdot L^{-1}$)	Hardness ($mg\ Ca \cdot L^{-1}$)	Mean ± s.e ($\mu g\ CdCl_2 \cdot L^{-1}$)	Literature Citation
"	"	450	"	9.082 (s.e. =.±0.2319)	"
"	"	600	"	8.804 (s.e. =.±0.2425)	"
"	"	800	"	8.543 (s.e. =.±0.2531)	"
"	"	1000	"	8.335 (s.e. =.±0.2621)	"
"	"	1200	"	8.176 (s.e. =.±0.2691)	"
"	"	1400	"	8.045 (s.e. =.±0.2750)	"
"	"	1600	"	7.926 (s.e. =.±0.2804)	"
"	"	2000	"	7.741 (s.e. =.±0.2888)	"
		Freshwater Snails			
Lymnaea stagnalis	Mean Number $eggs \cdot mass^{-1}$	0		45.5 (s.d.18.4)	Gomot, 1998
"	"	25	"	54.7 (s.d. 20.1)	"
"	"	50	"	55.5 (s.d. 15.5)	"
"	"	100	"	45.2 (s.d. 18.3)	"
"	"	200		33.9 (s.d. 19.1)	"
"	"	400		9.1 (s.d. 7.8)	"
Lymnaea stagnalis	Mean Hatch percent per week (week 2-7)	0		18.18 (s.d. 11.54)	Gomot, 1998
"	"	25		15.34 (s.d. 11.15)	"
"	"	50		20.65 (s.d. 14.24)	"

Table 3.32 Continued

Species	Conditions	Exposure Concentration ($\mu g\ CdCl_2 \cdot L^{-1}$)	Hardness ($mg\ Ca \cdot L^{-1}$)	Mean ± s.e ($\mu g\ CdCl_2 \cdot L^{-1}$)	Literature Citation
"	"	100		8.13 (s.d. 9.46)	"
"	"	200		0.41 (s.d. 0.64)	"
"	"	400		0 (s.d. 0)	"
		Freshwater Cladocerans			
Daphnia magna	Net reproductive rate EC_{50} Clone SK-6		N.A.	254 (95 CI =191-336)	Muyssen and Janssen, 2004
Freshwater Fish					
Oryzias latipes	Percent Hatch	0	N.A.	98.00(s.e. =.±2.00)	Gonzalez-Doncel *et al*., 2003
"	"	2500	N.A.	92.50(s.e. =.±4.79)	"
"	"	5000	N.A.	92.22(s.e. =.±2.61)	"
"	"	10000	N.A.	90.00(s.e. =.±4.08)	"
"	"	20000	N.A.	87.50(s.e. .±2.50)*	"
"	"	40000	N.A.	70.83(s.e. .±6.58)*	"
"	"	80000	N.A.	65.49(s.e. .±7.92)*	"
"	Mean Time to Hatch (days)	0	N.A.	10.60(s.e. =.±0.20)	Gonzalez-Doncel *et al*., 2003
"	"	2500	N.A.	10.91(s.e. =.±0.12)	"
"	"	5000	N.A.	10.49(s.e. =.±0.30	"
"	"	10000	N.A.	10.43(s.e. =.±0.16)	"
"	"	20000	N.A.	10.53(s.e. =.±0.14)	"
"	"	40000	N.A.	10.09(s.e. =.±0.25)	"
"	"	80000	N.A.	10.62 (s.e. .±0.71)	"

utilized in standard aquatic toxicity testing. However, a search of the literature proved that such data is rare. For the freshwater cladoceran, *D. magna*, no consistant impact of $CdCl_2$ was recorded on fecundity over 0-150 μg $Cd^{2+}\cdot L^{-1}$ (Muyssen and Jansen, 2004) suggesting that reproductive endpoints at 5 μg $CdCl_2\cdot L^{-1}$ in *P. acuta* may be much more sensitive than in the common test species *D. magna*. For the freshwater fish, *Oryzias latipes*, percent egg hatching hatching decreased from 98 % at 0 μg $CdCl_2\cdot L^{-1}$ to 65 % at 8000 μg $CdCl_2\cdot L^{-1}$ (González-Doncel *et al.*, 2003) (Table 3.32). In this study a significant reduction in egg hatching was recorded at 20 μg $CdCl_2\cdot L^{-1}$, four times greater than the 5 μg $CdCl_2\cdot L^{-1}$ effect recorded for *P. acuta* in this study. Unlike reported for *P. acuta* in this study where exposure to 5 μg $CdCl_2\cdot L^{-1}$ significantly effected time to egg hatching, time to egg hatching in *O. latipes* was not significantly affected by exposure to 0-8000 μg $CdCl_2\cdot L^{-1}$. Thus, while available comparative data is limited, reproductive endpoints in *P. acuta* generally appear to be much more sensitve than they are in other aquatic species making it an extremely strong candidate as a model testing species for aquatic toxicity testing.

Efficacy of Egg Diameter Response as a Measure of $CdCl_2$ Sensitivity

The egg diameter portion of this study demonstrated that egg diameter was also a highly sensitive endpoint for determination of $CdCl_2$ sensitivity/toxicity in *P. acuta* with and without inclusion of SL as a covariate. This test required a smaller concentration range of $CdCl_2$ (0-300 μg $CdCl_2\cdot L^{-1}$) to elicit a significant response as estimated by a mixed model ordinary least squares (OLS) regression analyses. Regression analysis yielded a mean egg diameter range from 0.8966 mm (s.e. = ±0.05169) at 0 μg $CdCl_2\cdot L^{-1}$ to 0.6461 mm (s.e. = ±0.05169) at a concentration of 300 μg $CdCl_2\cdot L^{-1}$ without SL as a covariate. Due to the significant shell length

(SL) effect, three analyses were conducted based on three sample SL quantiles. All three analyses demonstrated decreased adjusted means with increased $CdCl_2$ concentration. With SL as a covariate, OLS regression analysis resulted in an adjusted mean egg diameter that ranged from 0.0.8987 mm (s.e. = ±0.005289) at 0 µg $CdCl_2 \cdot L^{-1}$ to 0.6435 mm (s.e. = ±0.005643) at 300 µg $CdCl_2 \cdot L^{-1}$ for the 25th sample SL quantile, from 0.8954 mm (s.e. = ±0.005136) at 0 µg $CdCl_2 \cdot L^{-1}$ to 0.6499 mm (s.e. = ±0.005179) at 300 µg $CdCl_2 \cdot L^{-1}$ for the 50th sample SL quantile and from 0.8920 mm (s.e. = ±0.005339) at 0 µg $CdCl_2 \cdot L^{-1}$ to 0.6562 mm (s.e. = ±0.005935) at 300 µg $CdCl_2 \cdot L^{-1}$ for the 75th sample SL quantile (Table 3.30).

Thus, a significant effect of $CdCl_2$ on egg diameter was demonstrated in *P. acuta,* which could be used to quantify its sensitivity to this toxin. The results of this study indicated that egg diameter response measured after an initial observation of 72 h period, then at successive 24 h intervals through 192 h were able to detect the deleterious effects of $CdCl_2$ with a 192 h exposure period similar to that required for a standard 192 h LC_{50} determinations (Tables 2.3 and 2.5). Egg diameter determinations also required a smaller number of organisms (15 versus 150 snails per test) and lower toxicity test concentrations than snail survival. The egg diameter response was more accurate and the endpoint more readily and accurately identifiable than snail death defined as immobility in this study. It was also much more sensitive that survival endpoint testing in *P. acuta* with a significant reduction in egg diameter over controls being recorded at 5 µg $CdCl_2 \cdot L^{-1}$ compared to overall 96 h LC_{50} values estimated by either probit (1572 µg $CdCl_2 \cdot L^{-1}$) or logistic regression analyses (1589 µg $CdCl_2 \cdot L^{-1}$) (Table 3.23, 3.27).

Conclusions

This study met the criteria of demonstrating an effect of the toxin ($CdCl_2$) on *P. acuta* as a model organism for toxicity testing (Purchase *et al.,* 1998). Thus, *P. acuta* appears to be highly suitable as a new test species that could replace use of vertebrate species in toxicity testing. Use of *P. acuta* in $CdCl_2$ toxicity testing allowed information to be produced more rapidly, more accurately and less expensively in terms of direct costs and/or labor time than existing tests utilizing standard species. This study meet an additional requirement of providing additional information for prediction of toxicity through the introduction of a new toxicity testing methodology (Purchase *et al.*, 1998) based on the egg diameter response of *P. acuta* to $CdCl_2$ exposure.

The effect of cadmium chloride was demonstrated as a significant effect on the freshwater snail *P. acuta* using various fecundity and developmental endpoints in addition to the standard toxicity tests of acute and chronic survival. Significant reproductive endpoints included EC_{50} values for egg mass oviposition, sample multiple egg mass oviposition and fraction of eggs hatched per mass. A comparison between maximum likelihood probit and logistic regression analysis for estimated of EC_{50} values demonstrated essentially similar results, allowing comparison to previous studies and the conclusion that there was essentially no difference in the results of these two analyses. The affects of exposure to $CdCl_2$ on mean number of egg masses oviposited per individual, number of eggs oviposited per individual and the hatch delay (number of days to egg hatching) were estimated by the relatively new generalized linear models regression with responses fitting the poisson distribution.

In addition, this study demonstrated that a new endpoint, the egg diameter response was highly correlated to $CdCl_2$ concentration when analyzed by linear mixed model regression. This

study allowed analysis of this effect using a smaller sample sizes than standard survival methods (15 versus 150 snails), with a similar overall study duration (192 h). The egg diameter response endpoint was easier to identify than the more common survival endpoint of death identified as immobilization. This experimental procedure also had the advantage of providing a highly sensitive measurement in the laboratory, although the toxicity test procedures allowed the collection of egg masses in the field, without the necessity of moving and caring for adult snails in the laboratory.

CHAPTER 4

EFFECT OF CADMIUM CHLORIDE ON METABOLISM

Introduction

Purchase *et al*. (1998) have reviewed the requirements for development of new toxicity testing methodologies to replace or supplement existing standard methodologies. They state that a basic requirement for development of new environmental toxicity test methodologies is that they must be capable of supplying useful information not produced by existing tests and/or produce the information more rapidly, accurately and/or less expensively in terms of direct costs and/or labor than do existing standard methodologies. In addition, any newly proposed test methodology should be able to be integrated with existing toxicity tests since no single test can provide all required information. Information resulting from a new methodology should also be capable of being converted into predictive models that allow decision makers to resolve issues more quickly or accurately or to use less information than required from traditional test results when making decisions. Thus, data generated from any new test format must be capable of being linked to that produced by existing standard tests. Any newly developed testing methodology must also be readily and inexpensively transferable among laboratories and produce accurately repeatable results among different laboratories (Purchase *et al*., 1998). In addition, Purchase *et al.* (1998) highly recommend that any new methodologies allow replacement of vertebrate test species with invertebrate species whenever possible to avoid both the paperwork and public concerns associated with vertebrate toxicity and mortality testing. Finally, statistical analyses utilized to analyze data produced by any new toxicity testing methodology should allow reduction of error leading to more accurate predictions of

environmental toxicity and, thus, better decision-making on the part of regulators and industry (Purchase *et al*., 1998).

This section describes a study of the utility of utilizing changes in the oxygen consumption rate of adults of the common pulmonate pond snail, *Physa acuta*, as a new testing methodology to examine the toxicity of $CdCl_2$. The impacts of $CdCl_2$ concentration on acute oxygen uptake rates (Mo_2) at near air saturation with oxygen and Mo_2 in response to progressive hypoxia were assessed in *P. acuta*, using Analysis of CoVariance (ANCOVA). Oxygen consumption rate was chosen for investigation as a possible sub-lethal indicator of toxicity because it has previously been shown to be impacted by exposure to low levels of two molluscicides in the freshwater bivalves zebra mussels (*Dreissena polymorpha*) and Asian clam (*Corbicula fluminea*) (Moeller, 1993). Heavy metal impacts on oxygen consumption rates have also been demonstrated in marine gastropods and bivalves. The Mo_2 of specimens of the intertidal blue mussel, *Mytilus edulis L*., and intertidal snail, *Littorina rudis*, *we*re determined for specimens collected from 13 seashore sites of varying copper concentration along a 60 km copper pollution gradient extending from Avoca to Dublin on eastern coast of Ireland (Wilson and R. McMahon, 1981). While there was no correlation between environmental Cu exposure and Mo_2 in *M. edulis,* a strong positive correlation between environmental Cu concentration and Mo_2 occurred among samples of *L. rudis* suggesting that environmental copper pollution impacts metabolic rate in this snail species (Wilson and R. McMahon, 1981). Similarly, exposure to low Cd^{2+} concentrations (5-10 μg $Cd^{2+} \cdot L^{-1}$) depressed Mo_2 in the freshwater cladoceran, *Daphnia magna* (Barber *et. al.,* 1994). In contrast, short-term feeding of cadmium-impregnated food did not significantly affect Mo_2 in juvenile rainbow trout (*Oncorhynchus mykiss*) (Hollis *et al.,* 1999, 2000), nor did it impact the Mo_2 of rainbow trout hepatocyte cell cultures even though it was

impacted by exposure to copper ions (Manzel *et al.,* 2003). Although the results of previous investigations are mixed for respiratory response to $CdCl_2$ exposure, the Mo_2 responses of *P. acuta* and other freshwater pulmonate snails are known to respond to many environmental cues (R. McMahon, 1985).

In their review of the lethal and sub-lethal impacts of heavy metal toxicity, Newman and McIntosh (1991) note that animal body size impacts tissue metal concentration. Level of toxicity and size (measured as SL) was demonstrated in this study to be highly correlated with the impacts of $CdCl_2$ on the survivorship, behavioral response and reproduction of *P. acuta*. In spite of the widely accepted fact that there is an allometry between body size/age and toxicity tolerance, size/age of test specimens is rarely considered in aquatic toxicity testing (USEPA, 2001). In this section, oxygen consumption values were estimated with size measured as dry tissue weight (DTW) as a covariate on acute oxygen consumption rate at full air O_2 saturation and oxygen consumption rate in response to progressive hypoxia after a 48 h exposure to **l**ethal concentrations of $CdCl_2$.

Methodology

Impact of Cadmium Chloride on Oxygen Uptake Rates at Near Air O_2 Saturation

Once acute and chronic cadmium tolerance determinations had been completed, the short-term metabolic responses of *P. acuta* to a range of lethal and sub-lethal cadmium concentrations were investigated as a potential means for rapid determination of Cd toxicity. Such short-term testing of toxic effects allows avoidance of time consuming and labor intensive determination of LC_{50} and LT_{50} values in which mortality is the endpoint. Development of such short-term, non-lethal toxicity testing based on impairment of function was a priority for the U.S.

Environmental Protection Agency (USEPA, 2001) was much less expensive in terms of organism maintenance and the time (48 h) involved determining endpoints.

Oxygen uptake rates of individuals or groups of individuals of *P. acuta* depending on individual size (the SL of grouped individuals fell within 0.5 mm of each other) were determined with YSI model 53 Clark-Type silver-platinum, polarographic oxygen electrodes (Clark, 1956) using a methodology previously published for this species (R. McMahon, 1985). Snails were acclimated to 25 °C in artificial pond water on the three-day fish-flake feeding regime described above for at least three weeks prior to Mo_2 determinations. Prior to Mo_2 determinations, snails were held in control or test media without feeding for 48 h. This starvation period allowed avoidance of error in Mo_2 determinations due to increased metabolic demand (i.e., specific dynamic action) occurring immediately after food consumption.

Glass respiration chambers (2 cm internal diameter by 7 cm high) were filled with 5 ml of test or control media prior to Mo_2 measurements and brought to the test temperature of 25 °C by holding them in a transparent, water-jacketed bath through which water at 25 °C (± 0.1°C) was circulated from a Lauda K2/R® refrigerated constant temperature circulator. The jacketed holding bath holds four chambers (i.e., two chambers with snails in treatment media, one with a snail in control media and a blank control chamber containing only distilled water).

Prior to placing individuals in respiration chambers, air retained in the mantle cavity was expelled by gently prodding the snail's foot with a small paintbrush while holding it under water with the aperture directed upwards. Thereafter, snails were placed in the respiration chamber with 5 ml of treatment media of specific $CdCl_2$ concentration or in control artificial pond water media (see General Methodology) with an additional molar $CaCl_2$ concentration equivalent to that of the $CdCl_2$ test medium, compensating for any medium osmotic concentration effects on

Mo_2. Another chamber contained 5 ml of distilled water without a snail. A small amount of streptomycin was dissolved in the chamber water to suppress bacterial growth and gas exchange during Mo_2 measurements. Plastic snap rings were placed below the medium surface in each chamber to support a fully submerged circular magnetic stirrer that continuously circulated water past the electrode face. After seating stirrers in chambers, chamber water was stirred while remaining exposed to the atmosphere for greater than 15 min allowing snails to habituate to chamber conditions (i.e., pedally reattach and resume normal locomotion), O_2 concentration in the distilled water control to attain equilibrium with the external atmosphere and chambers containing snails to become well oxygenated (usually > 95 % of full air O_2 saturation [Po_2 = 160 Torr]) (R. McMahon, 1985).

During snail habituation and chamber equilibration, the oxygen electrode and plunger were held above the water's surface in one of the chambers containing snails in order to allow it to equilibrate to the experimental temperature (25 °C). After temperature equilibration, the electrode was placed in and moved downward into the distilled water control chamber expelling all chamber air and completely immersing the electrode membrane in the chamber medium. After, stabilization in the distilled water blank chamber, the electrode was calibrated to read 100 % O_2 concentration on both the electrode amplifier and strip chart recorder transcribing the electrode output allowing continuous recording of chamber medium O_2 concentration during individual Mo_2 determinations.

After calibration, the electrode was placed in a chamber containing a snail in control media and chamber water oxygen concentration continuously monitored until chamber O_2 concentration has been reduced by 10 % or for 30 min if a 10 % reduction in chamber O_2 concentration is not achieved within that time period. Mo_2 was similarly determined for snails in

the two remaining chambers containing treatment media of specific $CdCl_2$ concentration. Atmospheric pressure was determined with a mercury barometer at the beginning of each Mo_2 determination.

This methodology was used to determine Mo_2 for six individuals exposed to previously tested $CdCl_2$ dosages utilized in chronic mortality testing in the following progressive order: 10, 50, 100, 150, 200, 250, 300, 350, 400, 450, 600, 800, 1000, 1200, 1400, 1600, and 2000 μg $CdCl_2 \cdot L^{-1}$. Testing was carried out at increasingly higher concentrations until a significant impact on snail Mo_2 was observed. Testing occurred after an initial 48 h exposure of treated individuals to media at the test $CdCl_2$ concentration. Control individuals were held in artificial pond water media with an additional molar $CaCl_2$ concentration equivalent to that of the $CdCl_2$ added to the corresponding treatment media.

After completion of Mo_2 determinations, snails were removed from chambers and placed in 10 % nitric acid by volume to dissolve the mineral portion of the shell. The remaining shell soft tissue and proteinaceous periostracum shell covering were then rinsed in three separate 5 min washes of distilled water to remove any remaining nitric acid. The periostracum was then separated from the body tissues and the tissues wet weighed to the nearest 0.01 mg on an electronic balance. Tissue dry weight was then determined to the nearest 0.01 mg after attaining constant weight at 65 °C (>24 h).

The volume of oxygen consumed by each snail was determined by determining the amount of oxygen initially in the chamber based on chamber water volume and air O_2 saturation at the 25 °C experimental temperature and partial pressure of oxygen (Po_2) recorded during the Mo_2 determination corrected for vapor pressure (McMahon, 1985). The volume of oxygen consumed by each snail in μl O_2 at standard temperature and pressure (STP) was adjusted for any

O_2 consumption recorded in the blank chamber without a snail and divided by the time over which it was recorded to determine the individual Mo_2 values as μl $O_2 \cdot animal^{-1} \cdot hr^{-1}$. Where several grouped smaller individuals were used in the Mo_2 determination, group Mo_2 was divided by the number of individuals in the group to yield the μl $O_2 \cdot animal^{-1} \cdot hr^{-1}$ value. Total hourly Mo_2 for single and grouped snails was then divided by their dry tissue weight to yield the weight-specific Mo_2 rate in μl $O_2 \cdot mg^{-1} \cdot hr^{-1}$.

Impact of Cadmium Chloride on Respiratory Response to Progressive Hypoxia

In order to analyze the impact of $CdCl_2$ exposure on respiratory response to hypoxia in *P. acuta*, some snails used in Mo_2 determinations were allowed consume O_2 from chamber media until Po_2 in the chamber is reduced to a level at which no further O_2 is consumed. Testing occurred under exposures to the same concentrations of $CdCl_2$ utilized in testing Mo_2 response at near full air O_2 saturation (see above). Chamber Po_2 was continuously monitored on a strip-chart recorder throughout this process, allowing Mo_2 to be determined for each replicate sample at each tested $CdCl_2$ concentration at each 5 % decrease in chamber O_2 concentration from full air O_2 saturation to the O_2 concentration at which O_2 uptake ceased. Mo_2 recorded at each successive 5 % decrease in chamber O_2 concentration was then expressed as a percentage of the maximum Mo_2 recorded across the tested range of Po_2. Thus, the maximum standard Mo_2 value was assigned a value of 100 % and all other Mo_2 values assigned a percentage of that maximum value. The "Percent O_2 Regulation" or "Ro_2" value for an individual was then calculated as the integrated sum of standard percent Mo_2 values across the tested Po_2 range in progressive 5 % concentration increments and expressing that sum as a percentage of that which would have been recorded for a perfect oxygen regulator. For example, an individual displaying perfect oxygen regulation would have an R_{O2} of 100 %, and an animal exhibiting strict oxygen conformity

(where oxygen uptake declines in direct proportion to the decline in Po_2) would have a Ro_2 of 50 %. Ro_2 values greater than 50 % are indicative of oxygen regulation, with higher values indicating progressively greater regulatory ability. Ro_2 values below 50 % indicate that the metabolic rate declines exponentially in response to a progressive decline in Po_2.

Data Analysis

The effect of cadmium chloride ($CdCl_2$) on *P. acuta* was analyzed using an *a priori* model with ANCOVA using the computer program Statistica. Mo_2 data at full air O_2 saturation was analyzed by ANCOVA with $CdCl_2$ concentration as a treatment and individual size expressed as sample mean dry weight as the covariate. Pair-wise comparisons of mean Mo_2 at different $CdCl_2$ concentrations were provided by the Scheffé test.

Values of "Ro_2" reflecting the O_2 regulatory ability of test individuals exposed to progressive hypoxia were analyzed by ANCOVA with $CdCL_2$ concentration as a treatment and individual size expressed as sample mean dry weight as the covariate. Treatment differences in sample mean Ro_2 values were determined by Scheffé pair-wise comparison test. Significant increases or decreases in Ro_2 values from control samples will indicate that exposure to $CdCl_2$ concentrations had an impact on O_2 regulatory ability in *P. acuta*.

The response of snail Mo_2 and Ro_2 to $CdCl_2$ exposure was compared to data on acute and chronic tolerance of $CdCl_2$ exposures to determine the level of correlation between these measures and the sensitivity of respiratory rate and oxygen regulatory ability in predicting the non-lethal toxic effects of $CdCl_2$ on *P. acuta*.

Results

Acute Oxygen Consumption

An *a priori* regression of concentration and size, measured as dry tissue weight, was used to determine the effect of cadmium chloride ($CdCl_2$) on acute O_2 consumption. ANCOVA regression analyses for acute O_2 consumption provided estimates for determinations of samples of *Physa acuta* exposed to media with concentrations of cadmium chloride ranging from 0-1200 μg $CdCl_2 \cdot L^{-1}$. A significant effect of concentration for acute oxygen consumption was demonstrated by Type III Sum of Squares ($F = 3.886$, $p = 0.0136$) provided for individuals with a covariate of dry tissue weight (DTW). This analysis also revealed a significant relationship between acute oxygen consumption and dry tissue weight, ($F = 16.782$, $p = 0.0007$). Log mean values estimated from ANCOVA ranged from 1.3023 (s.d. 0.0597) at concentration of 1200 μg $CdCl_2 \cdot L^{-1}$ to 2.0344 (s.d. 0.0578) at concentration 200 μg $CdCl_2 \cdot L^{-1}$ (Table 4.1) A pair-wise comparison of concentration means resulted in significant differences at $\alpha = 0.05$ based on the Scheffé pair-wise comparison for the following pairs: 0-1200, 200-1200, 400-1200, and 800-1200 (Table 4.2, Fig. 4.1).

Progressive Hypoxia Response

The effect of percent air saturation on fractional oxygen consumption for concentrations ranging from 0-1000 μg $CdCl_2 \cdot L^{-1}$ was determined. The percent O_2 regulation value (PR_{O2}), the percentage of oxygen consumption rate maintained across all levels of hypoxia from 100-0 % of full air O_2 saturation relative to maintenance of O_2 consumption rate, ranged from 74.12 % at 1000 μg $CdCl_2 \cdot L^{-1}$ to 80.88 % at 200 μg $CdCl_2 \cdot L^{-1}$. The curves resulting from the decrease in air saturation demonstrated the similarity between concentrations (Fig. 4.2).

Table 4.1 Adjusted log_{10} mean values of oxygen consumption rates in µl O_2·individual^{-1}·h^{-1} estimated by ANCOVA analysis with dry tissue weight as a covariate for samples of *Physa acuta* (n = 6) exposed at 25 °C to media with cadmium chloride ($CdCl_2$) concentrations ranging from 0 - 1200 µg $CdCl_2$·L^{-1}.

		Ml O_2·individual^{-1}·h^{-1}	
µg $CdCl_2$·L^{-1}	**N**	**Log_{10} Adjusted Means**	**Standard Deviation**
0	6	1.7989	0.0581
200	6	2.0344	0.0578
400	6	1.9417	0.0579
600	6	1.7643	0.0617
800	6	1.9391	0.0597
1000	6	1.8118	0.0586
1200	6	1.3023	0.0597

Table 4.2 Pair-wise comparisons of acute adjusted mean oxygen consumption rates (μL O_2·Individual^{-1}·h^{-1}, indicated by values in parentheses) estimated by Analysis of Covariance (ANCOVA) with dry tissue weight (DTW) as a covariate and Scheffé post-hoc testing for samples of *Physa acuta* (n = 6) exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 - 1200 μg $CdCl_2$·L^{-1}. Values in the body of the table represent differences between O_2 uptake rates at different $CdCl_2$ concentrations with significant differences indicated in italic font and marked by an asterisk (*).

μg $CdCl_2$·L^{-1} (μL O_2·In^{-1}·h^{-1})	**Mg $CdCl_2$·L^{-1} (μL O_2·In^{-1}·h^{-1})**					
	0 (6.043)	**200 (7.648)**	**400 (6.971)**	**600 (5.838)**	**800 (6.953)**	**1000 (6.121)**
0 (6.043)						
200 (7.648)	1.605					
400 (6.971)	0.928	-0.677				
600 (5.838)	-0.205	-1.810	-1.133			
800 (6.953)	-0.090	-1.695	-1.018	0.115		
1000 (6.121)	0.078	-1.527	-0.850	0.283	0.168	
1200 (3.678)	*-2.365**	*-3.970**	*-3.293**	*-2.160**	*-2.275**	*-2.443**

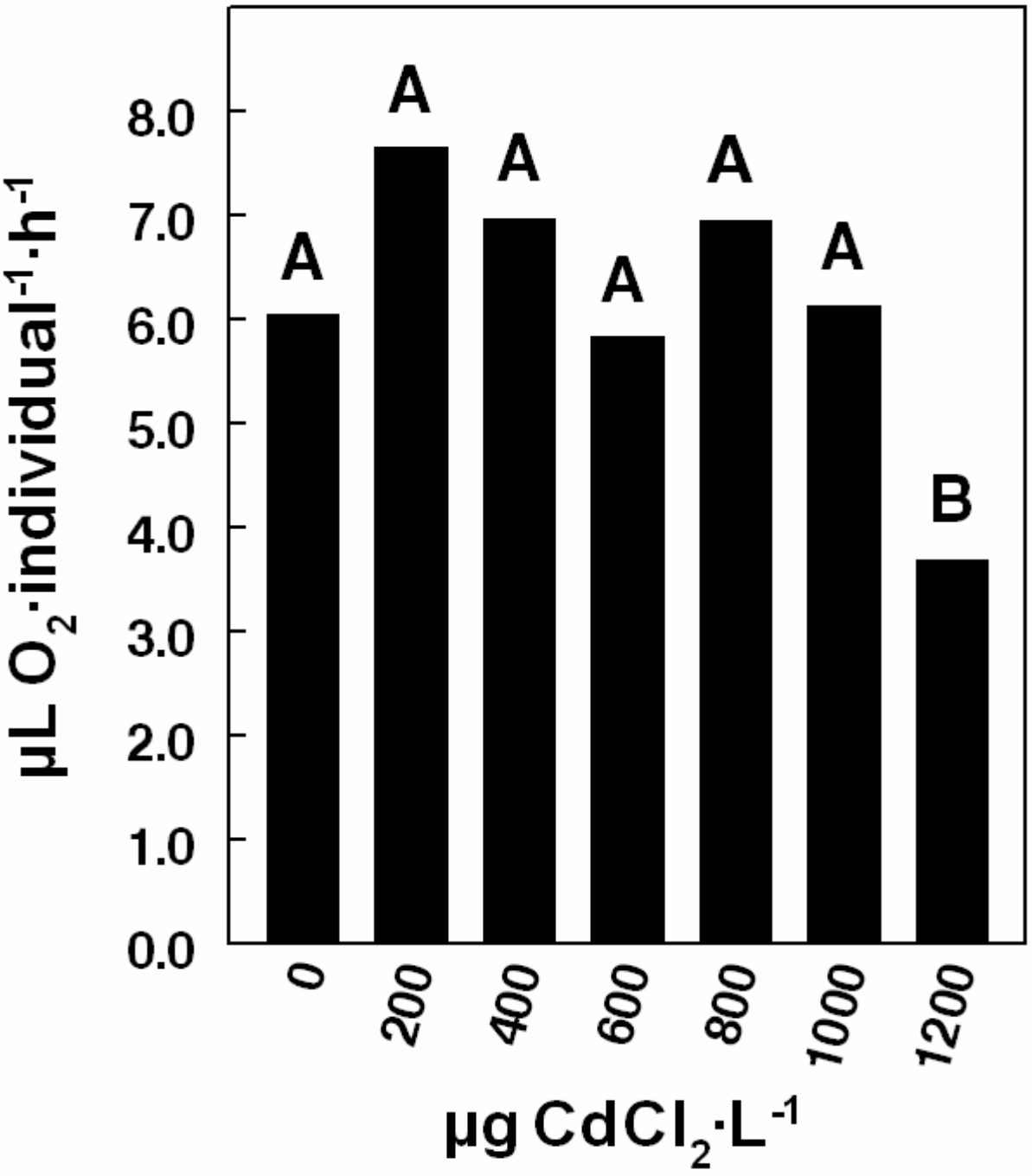


Fig.4.1 Adjusted mean oxygen consumption rates (n = 6) in micro-liters of O_2 per individual per hour (vertical axis) of *Physa acuta* acutely exposed to concentrations of cadmium chloride for 48 h ranging from 0 – 1200 µL $CdCl^{-1}\cdot L^{-1}$. Different letters above histograms indicate that mean values are significantly different as estimated by Scheffe pair-wise comparison ($p < 0.05$).

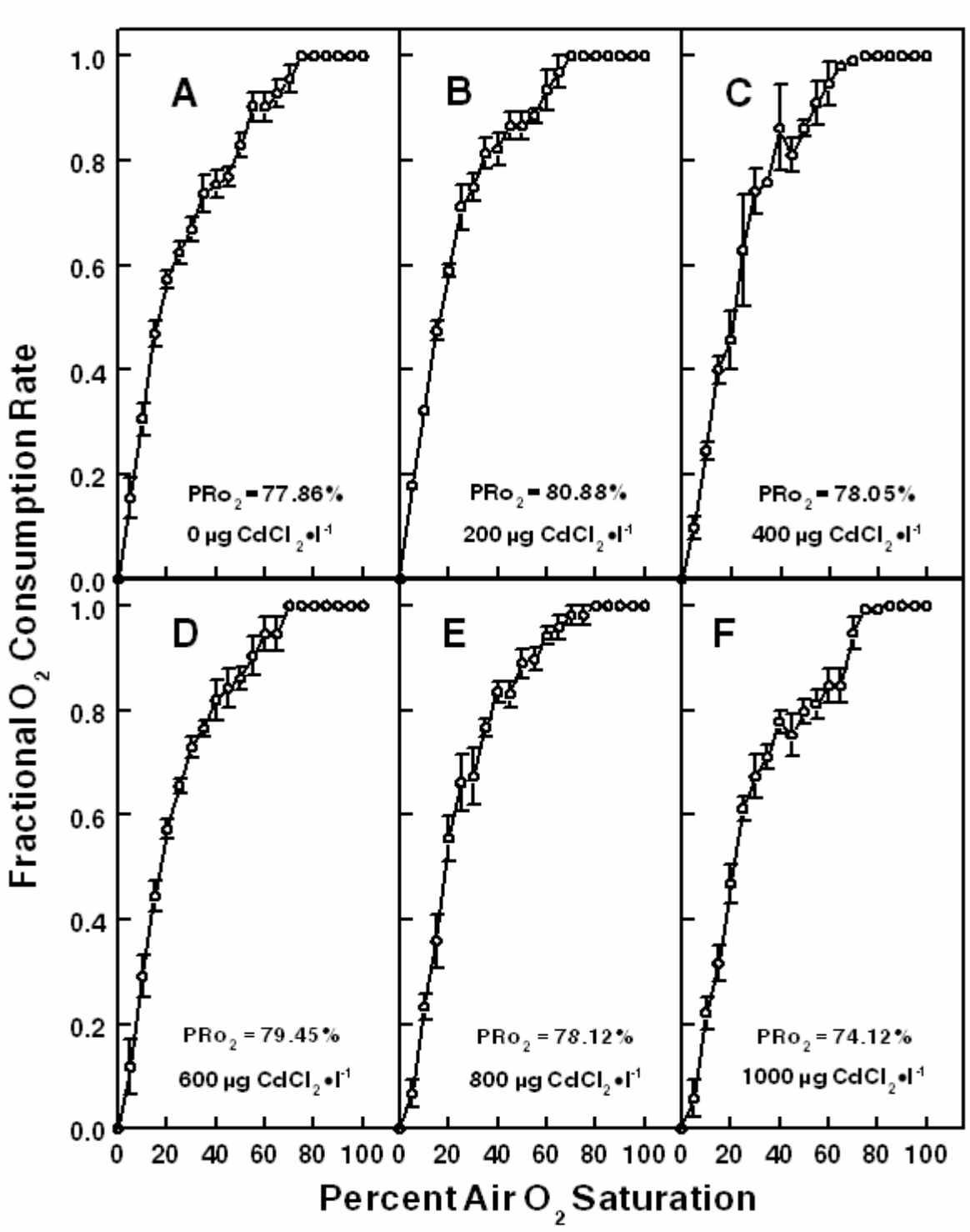


Fig.4.2 Fractional O_2 consumption rate values (vertical axis) expressed as the fraction of the oxygen consumption rate at full air O2 saturation (Po_2 = 159.068 torr) depicting the respiratory response to progressive hypoxia of samples of 4-6 individuals of *Physa acuta* acutely exposed to concentrations of cadmium chloride for 48 h ranging from 0-1000 µg $CdCl_2 \cdot L^{-1}$.

In order to determine the effect of cadmium chloride ($CdCl_2$) on low oxygen environments the *a priori* regression of concentration and size, measured as dry tissue weight (DTW), was used. ANCOVA regression analyses for reponse to hypoxia provided estimates for determinations of samples of *Physa acuta* exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0-1000 μg $CdCl_2 \cdot L^{-1}$. No significant effect of concentration on response to progressive hypoxia was revealed by Type III Sum of Squares ($F = 2.25$, $p = 0.1705$). This analysis also revealed no significant relationship between response to hypoxia and dry tissue weight, ($F = 4.31$, $p = 0.0766$). Adjusted mean values estimated from ANCOVA ranged from 71.99 % (s.d = ±1.81) at concentration of 1000 μg $CdCl_2 \cdot L^{-1}$ to 82.48 % (s.d. = ±1.77) at a concentration of 200 μg $CdCl_2 \cdot L^{-1}$ (Table 4.3, Fig. 4.2). A pair-wise comparison of concentration means resulted in no significant differences at $\alpha = 0.05$ based on the Scheffé pair-wise comparison (Fig. 4.3).

Discussion

Subjects not being allowed to acclimate to handling and experimental conditions; decreasing oxygen levels too quickly; nutrition; and size/age were reported to influence response to hypoxic conditions (B. McMahon 2001). The present study addressed these issues in the experimental design through allowing subjects to acclimate 48 h to the test concentration and to the test chamber. Oxygen levels started at 100 % saturation and were reduced through respiration by the subject. All experimental subjects were not fed for the duration of the 48 h test period. Size as dry tissue weight was recorded for each snail, with no juvenile subjects being utilized in this study.

Table 4.3 Adjusted mean percent O_2 regulation values estimated by ANCOVA analysis with dry tissue weight (DTW) as a covariate for samples of *Physa acuta* (n = 4-6) exposed to media with concentrations of cadmium chloride ($CdCl_2$) ranging from 0 – 1000 µg $CdCl_2 \cdot L^{-1}$. See text for an explanation of the derivation of the percent O_2 regulation (PRo_2) value.

µg $CdCl_2 \cdot L^{-1}$		**Percent O_2 Regulation (PRo_2)**	
	n	**Adjusted Mean**	**Standard Deviation**
0	6	76.66%	1.50
200	4	82.48%	1.77
400	4	79.23%	1.74
600	4	79.02%	1.73
800	4	75.27%	1.87
1000	4	71.99%	1.81

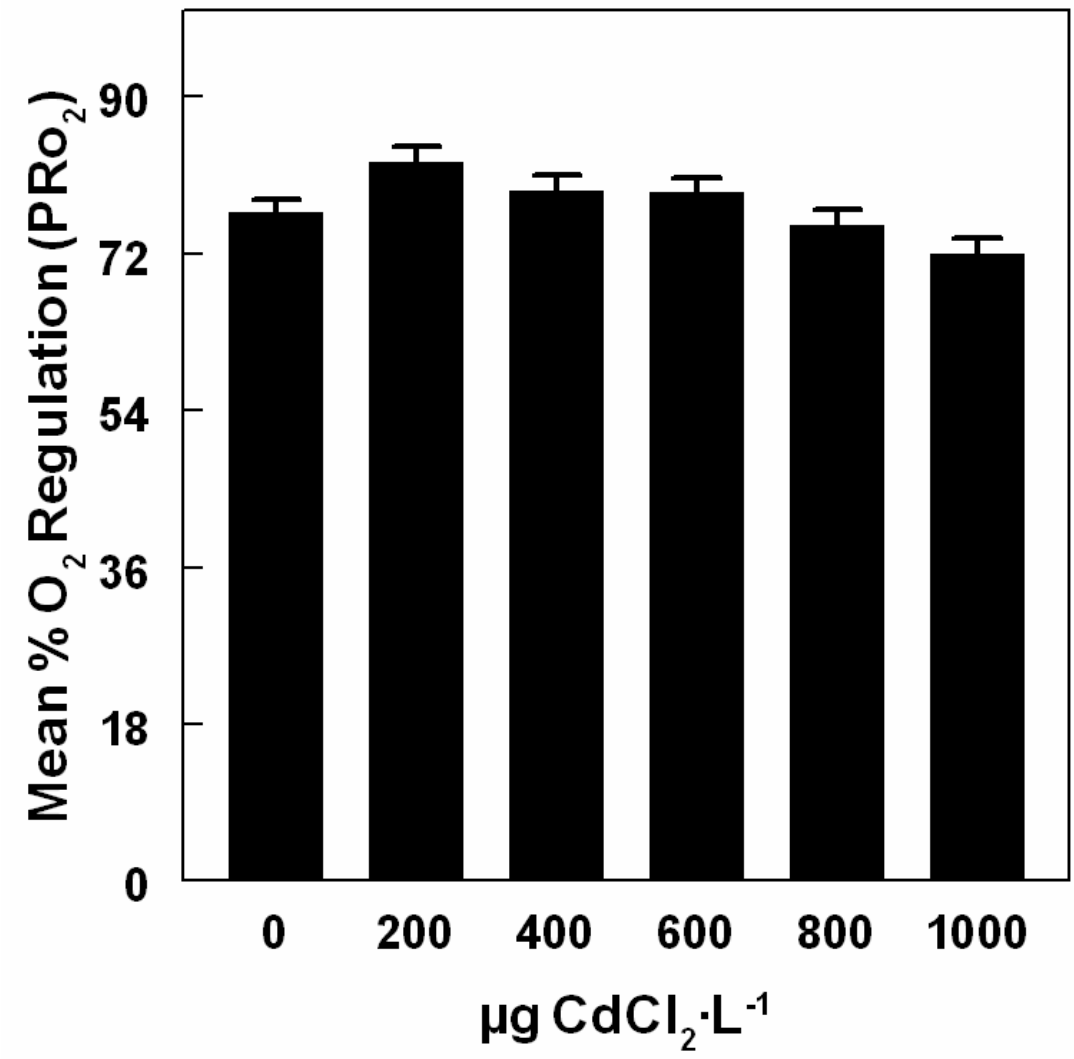


Fig.4.3 The impact of an acute, 48 h, exposure to cadmium chloride ($CdCl_2$) on the mean percent O_2 regulation values (PRo_2) representing respiratory response to progressive hypoxia in specimens of *Physa acuta*. PRo_2 values (vertical axis) are the percentage of oxygen consumption rate maintained across all levels of hypoxia from 100-0 % of full air O_2 saturation relative to maintenance of O_2 consumption rate equivalent to that at recorded at full air O_2 saturation from 100 % to 0 % media O_2 concentration. Histograms represent mean PRo_2 values for samples of *P. acuta* exposed to concentrations of $CdCl_2$ ranging from 0-1000 µg $\cdot L^{-1}$ (horizontal axis). Vertical bars above histograms represent standard errors of the means.

Comparison of Experimental Techniques for Acute Oxygen Consumption and Hypoxia Responses

The impacts of covariates such as size, as dry tissue weight, utilized in this study are usually considered in oxygen consumption studies because they are generally known to greatly impact and likely to confound results if not statistically controlled. Determinations of the effect of cadmium chloride ($CdCl_2$) on both acute oxygen consumption and hypoxia responses resulted from *a priori* ANCOVA regression analyses with dry tissue weight as a measure of size.

The hypoxia response determinations utilized the same experimental equipment, but required a greater observation period to derive the estimated value, which would increase the cost of this determination in terms of time and handling. The total exposure duration (48 h) was the same for both acute oxygen consumption and hypoxia responses, with the acute oxygen consumption study ending after 30 minutes or recording a 10 % reduction in oxygen concentration in the chamber. In contrast, observations for hypoxia response determinations continued until no further O_2 was measured in the chamber.

Species Comparisons of Acute Oxygen Consumption Values

The effect of cadmium chloride ($CdCl_2$) on acute oxygen consumption computed by ANCOVA regression analysis with size as a covariate for *P. acuta* in this study was compared to the effect for other aquatic animal species in the published literatures. Estimated means from the significant ANCOVA Regression Analysis for *Physa acuta* in the acute oxygen study ranged from 3.68 (s.d. = ±1.06) at 1200 μg $CdCl_2 \cdot L^{-1}$ to 7.65 (s.d. = ±1.06) at 200 μg $CdCl_2 \cdot L^{-1}$ (Table 4.4). There was a significant difference between mean values of acuteoxygen consumption for a concentration of 1200 μg $CdCl_2 \cdot L^{-1}$ and all other concentrations.

Table 4.4 Comparison of toxicity of different freshwater animal species to cadmium (Cd) measured as mean oxygen consumption in flow-through toxicity tests.

Species	Conditions	**Exposure Concentration ($\mu g\ CdCl_2 \cdot L^{-1}$)**	**Hardness ($mg\ Ca \cdot L^{-1}$)**	**Mean ± s.d. ($\mu l\ O_2 \cdot mg^{-1}\ DTW \cdot h^{-1}$)**	**Literature Citation**
Freshwater Gastropods					
**Physa acuta*	25 ± *2* °C. 48 h acclimation. Subjects not fed. 30 mins measurement.	0	50.0	6.04 (s.d.±1.06)	This study
"	"	200	50.0	7.65 (s.d. ± 1.06)	"
"	"	400	50.0	6.97 (s.d. ±1.06)	"
"	"	600	50.0	5.84 (s.d. ±1.06)	"
"	"	800	50.0	6.95 (s.d. ±1.06)	"
"	"	1000	50.0	6.12 (s.d. ±1.06)	"
"	"	1200	50.0	3.68 (s.d. ±1.06)	"
Freshwater Cladocerans					
Daphnia magna	Clone S-1. 20 ± 2 °C. 24 h acclimation. Subjects not fed. 1 h measurement.	0	N.A.	5.28 (±0.25)	Barber *et al*., 1994
"	"	1	"	4.03 (±0.09)	"
"	"	5	"	4.04 (±0.12)	"
"	"	20	"	3.87 (±0.49)	"
"	Clone F. 20 ± 2 °C. 24 h acclimation. Subjects not fed. 1 h measurement.	0	N.A.	5.53 (±0.49)	"
"	"	1	"	4.91 (±0.30)	"
"	"	5	"	5.36 (±0.51)	"
"	"	20	N.A.	5.93 (±1.03)	

Table 4.4 Continued

Species	Conditions	Exposure Concentration (μg $CdCl_2 \cdot L^{-1}$)	Hardness (mg $Ca \cdot L^{-1}$)	Mean ± s.d. (μl $O_2 \cdot mg^{-1}$ $DTW \cdot h^{-1}$)	Literature Citation
		Freshwater Fish			
Oncorhynchus mykiss	Juvenile Cd(NO3) O2 measurement conducted 30 d exposure period. 2 h after feeding.	0	140	4.27 (±0.10)	Hollis *et al*., 1999
"	"	3	"	3.91 (±0.14)	"
"	"	10	"	4.30 (±0.54)	"
Oncorhynchus mykiss	Juvenile $Cd(NO3)_2$	0	20	6.4 (±0.1)	Hollis *et al*., 2000
"	"	0.07	"	7.6 (±0.2)	"
"	"	0.11	"	6.7 (±0.4)	"
Ctenopharyngodon idella	Juvenile. $Cd(NO3)_2$ 26 °C. 96 h acclimation. Subjects fed.	0	140	0.86 (95% CI ±0.40)	Espina *et al.,* 2000
"	"	500	"	1.07 (95% CI±0.47)	"
"	"	1000	"	1.31 (95% CI±0.41)	"

It is valuable to compare the effect of $CdCl_2$ determined for adult *P. acuta* in this study with that of other non-molluscan aquatic species, particularly those commonly utilized in standard aquatic toxicity testing. Thus, under starvation conditions the effect of cadmium on oxygen consumption of two clones of the freshwater cladoceran, D*aphnia magna* demonstrated contrasting effects. The clone most tolerant to cadmium, S-1, demonstrated a significant decrease of oxygen consumption at 20 μg $Cd \cdot L^{-1}$. The S-1 clone mean oxygen consumption values ranged from 3.87 μl mg^{-1} dry weight h^{-1} (s.d. = ±0.49) at 20 μg $Cd \cdot L^{-1}$ to 5.28 μl O_2 mg^{-1} dry weight h^{-1} (s.d. = ±0.25) at 0 μg $Cd \cdot L^{-1}$ (Barber *et al.,* 1994). Clone F, more sensitive to cadmium exposure, demonstrated oxygen consumption values that ranged from 4.91 μl O_2 mg^{-1} dry weight h^{-1} (s.d. = ±0.30) at 1 μg $Cd \cdot L^{-1}$ to 5.93 μl O_2 mg^{-1} dry weight h^{-1} (s.d. = ±1.03) at 20 μg $Cd \cdot L^{-1}$.

In contrast with the above studies, experiments with freshwater fish involved feeding of test species during the acclimation and observation periods. A significant effect of cadmium nitrate was demonstrated on the grass carp, *Ctenopharyngodon idella* (Espina *et al.,* 2000). Oxygen consumption values ranged from 0.86 μl $O_2 \cdot mg^{-1} \cdot hr^{-1}$(95 % C.I. = ±0.40) at 0 μg $Cd(NO_3)_2 \cdot L^{-1}$ to 1.31 μl $O_2 \cdot mg^{-1} \cdot hr^{-1}$ (95 % C.I. = ±0.41) at 1000 μg $Cd(NO_3)_2 \cdot L^{-1}$ In contrast, no significant effect was revealed through analysis of cadmium nitrate on acute oxygen consumption for the juvenile rainbow trout, *Oncorhynchus mykiss* in either hard or soft water (Hollis *et al*., 2000). Cadmium demonstrated no effect on acute oxygen consumption for rainbow trout (*Oncorhynchus*

mykiss) hepatocytes, although copper revealed a significant increase on acute oxygen consumption (Manzl *et al*., 2003).

Species Comparison of Hypoxia Response Values

The effect of cadmium chloride ($CdCl_2$) on response to hypoxia computed by ANCOVA regression analysis with size as a covariate for *P. acuta* in this study was compared to the effect for other aquatic animal species in the published literature on several species. No significant effect of concentration on response to hypoxia was revealed in Type III Sum of Squares ($F = 2.25$, $p = 0.1705$) for *Physa acuta*. This analysis also revealed no significant relationship between hypoxia response and dry tissue weight, ($F = 4.31$, $p = 0.0766$). Similar effects were reported for other species such as water flea (*Daphnia magna*) (Pirow et al. 1999), burrowing crayfish (*Cambarus fodiens*) (B. McMahon and Hankinson, 1993).

Efficacy of Acute Oxygen Consumption and Hypoxia Response as a Measure of $CdCl_2$ Sensitivity

This study demonstrated a significant effect of $CdCl_2$ on acute oxygen consumption (M_{O2}) in *P. acuta,* which could be used to quantify its sensitivity to this toxin. The Mo_2 response observed on exposure to $CdCl_2$ was similar that reported to occur in the *Daphnia magna* clone F. The results of this study indicated that acute oxygen consumption response measured as Mo_2 values was able to detect the deleterious effects of $CdCl_2$ exposure more quickly (48 h) than acute 96 h survival LC_{50} determinations (1563.96 µg $CdCl_2 \cdot L^{-1}$, s.e. ±32.78). Values for M_{O2} determinations also required a smaller number of organisms (42 versus 150 snails per test). However, the cost of this study was greater than the cost of the acute survival study due to equipment

and due to time needed to observe the response. For the acute oxygen response, subjects were observed at 48 h for immobility during the acclimation period, and then each animal was observed for a period of 30 minutes while measuring the oxygen consumption. In contrast, one observation at 96 h was needed to determine mortality for the $LC_{50.}$

The results from the response to hypoxia study demonstrated no significant effect of cadmium chloride ($CdCl_2$) or size, as dry tissue weight (DTW) on the response to low oxygen environments, so this test was not more sensitive than standard 96 h aquatic toxicity survival test.

Conclusion

Both the acute oxygen consumption and hypoxia responses provided new information on the effect of cadmium chloride ($CdCl_2$) on the freshwater snail, *P acuta*. There was a significant effect of concentration adjusted for size, as dry tissue weight, on acute oxygen consumption. In contrast, there was no significant effect of concentration or size, as dry tissue weight, for the progressive hypoxia response study. Due to the high concentration (1200 μg $CdCL_2 \cdot L^{-1}$) needed to produce an effect for the acute oxygen consumption study and cost of equipment, this toxicity test is not an efficient replacement for the 96 h LC_{50} of 1563.96 μg $CdCL_2 \cdot L^{-1}$ (s.e. ±32.78). The hypoxia response toxicity test is not an efficient replacement for the acute 96 h survival LC_{50} because there was no significant response for concentration adjusted for dry tissue weight for *P. acuta* in this study.

CHAPTER 5

CONCLUSION

An investigation was conducted to determine if using the common, North American, freshwater, pulmonate snail, *Physa acuta,* as a model organism for testing acute and chronic toxicological effects of environmental pollutants was a viable method of screening chemical toxins. Several criteria were considered in evaluating the utility of the proposed new toxicological test methodology or organism (Purchase *et al*., 1998). This study investigated the use of *Physa acuta*, for alternative toxicological screening methods for determination of lethal concentrations of toxic chemicals as proposed by Purchase *et al.* (1998). Benefits of utilizing an invertebrate for toxicological testing rather than vertebrates include a reduction in space needed for testing and reduced cost of animal acquisition and care. An additional benefit was the reduced time necessary to complete sub-lethal toxicity tests.

The presence of physid snails is an indicator of poor water quality and nutrient rich conditions (U.S. Environmental Protection Agency [USEPA], 2001), therefore increasing the advantage of selecting *P. acuta* as the organism for toxicity testing. Freshwater snails are common and widespread, readily obtainable and easy to maintain in the laboratory, making them an ideal organism for low cost toxicity and pollution testing (Cheung and Lam, 1998, Melo *et al.,* 2000). Based on the literature, endpoints

such as size (shell length - SL) (McMahon, 1985), fecundity (Cheung and Lam, 1998), metabolic rate (oxygen consumption rate) (McMahon, 1985) and behavior (avoidance) (Alexander and Covich, 1991, McCarthy and Fisher, 2000) were readily quantified in *Physa.* The ability to measure such endpoints made *Physa* species ideal candidates for toxicology testing.

In contrast to short-term, acute, lethal toxicity testing, chronic toxicity determinations measure long-term, often sub-lethal, as well as lethal responses of test organisms to prolonged exposure to low levels of toxin. Because of the complexity, long durations and elevated expense of such chronic tests, they are conducted less frequently than static tests (US EPA, 2001). There is no standard measure for chronic effects. In addition to the LC_{50} endpoint of sample mortality after a specified period of toxin exposure utilized in acute toxicity testing, endpoints for chronic testing included the time period required to achieve a specific level of mortality (usually greater than 50 % mortality). Other endpoints utilized in chronic toxicity testing include relative changes in reproductive, developmental, growth, metabolic, and behavioral responses over extended exposure periods (USEPA, 2001). This study demonstrated the benefit of investigating several endpoints in toxicity testing to determine the most sensitive responses or to determine the most efficient toxicity testing method for decision-makers to resolve problems.

The current study introduced methodology to allow formal analysis of sub-lethal responses, behavior and reproductive endpoints, capable of supplying useful information not previously available for predictive models. The present study provided

information to increase the capability of comparing multiple toxicity endpoints and exposure times to other species as suggested by Mark and Solbe (1998). The new procedures allowed comparison between endpoint responses (survival, behavior and reproduction) based on the same statistical technique, to provide a more useful tool for determining which endpoint provided the most efficient response. In addition, this study provided multiple analytical methods to increase the capability of comparison with previous studies.

All toxicity tests in this study allowed the addition of covariates, such as shell length, to increase the efficiency of the predictive models. All tests demonstrated a size effect with the exception of egg mass EC_{50} and response to progressive hypoxia. Number of egg masses, number of eggs oviposited and delay to egg hatch analyses introduced in this study for reproductive measures provided estimates of adjusted means based on shell length as a covariate in regression analyses.

This study provided analyses for the sub-lethal responses, CO_{50} and reproductive $EC_{50,}$ to compare to survival LC_{50} based on comparable analytical techniques. The crawl-out response analyzed as the concentration needed to produce an effect in 50 % of the sample will allow comparisons to other analyses that utilize similar analytical techniques such as the survival LC_{50} and reproductive effect concentration analyses (EC_{50}). All effect concentration analyses, LC_{50} or EC_{50}, were estimated using both probit and logistic maximum likelihood regression to allow comparison between the techniques. The results from this study demonstrated the similarity of these

techniques, which increases the ability to compare present and future research to previously published studies.

Allometric Impacts on Toxicity Testing

Several factors influence the relationship between body size and tissue metal concentration. Metabolic rate, respiratory rate and relative surface area demonstrate allometric relationships with body burdens of toxic metals. Temporal factors including age, growth and exposure duration have been demonstrated to influence metal body burden. Age related changes in physiology, cytology and biochemistry are associated with maturation and reproduction impact size-dependent body metal concentrations (Newman and McIntosh, 1991).

This study demonstrated the effect of including size, as shell length, in each toxicity test. SL demonstrated a significant effect on acute and chronic survival and crawl-out responses. Both exposure concentration and exposure time revealed a significant effect of SL on survival response for acute and chronic analyses. Reproductive endpoints demonstrating a significant SL effect included number of egg masses, number of eggs, egg hatching $EC_{50,}$ delay in egg hatching and egg diameter.

Common Endpoints

Survival

Survival is the most common endpoint of toxicity testing (Phipps and Holcombe, 1985). Acute survival in the freshwater pulmonate *Aplexa hypnorum* to cadmium chloride has been investigated and a 96 h LC_{50} of 152 μg $CdCl_2 \cdot L^{-1}$ (88-261 μg $CdCl_2 \cdot L^{-1}$) determined (Holcombe *et al.,* 1984). Acute toxicity testing has been

conducted for *Physa acuta* (Cheung and Lam, 1998). The acute 24 h LC_{50} for juveniles is 1320 µg $CdCl_2 \cdot L^{-1}$ (1130-1540 µg $CdCl_2 \cdot L^{-1}$) and the 48 h LC_{50} was 1050 µg $CdCl_2 \cdot L^{-1}$ (810-1360 µg $CdCl_2 \cdot L^{-1}$).

The effect of cadmium chloride was demonstrated as a significant effect on the freshwater snail *P. acuta* using the standard toxicity tests of acute and chronic survival. Significant survival endpoints included the concentration that resulted in 50 % sample mortality, LC_{50}, and the time that resulted in 50 % sample mortality, LT_{50}. A comparison between maximum likelihood probit and logistic regression analysis for estimations of LC_{50} demonstrated essentially similar results, allowing comparison to previous studies. Time to death analysis was included to provide mortality estimates without excluding data outside the selected fixed time interval (Newman and Aplin, 1992). Values of LT_{50} in response to $CdCl_2$ exposure were estimated by the relatively new discrete logistic failure time model regression (Hicks *et al*., 2000).

Behavior

While crawl-out behavior has been investigated in regard to chemical cues associated with predators in freshwater snails, to date, no studies of crawl-out behavior to avoid aquatic pollutants have been performed for any marine, estuarine or freshwater gastropod species.

Previous studies demonstrate partial or weak avoidance behaviors in response to adverse conditions in freshwater snails (Alexander and Covich, 1991). Gastropod avoidance behaviors include: crawling to the waterline, crawling out of the water and altering crawling speed (McCarthy and Fisher, 2000). Physid snails respond to

chemical cues through the use of avoidance behaviors such as crawling to the waterline or out of the water (Dewitt *et al.,* 1998; McCarthy and Fisher, 2000).

McCarthy and Fisher (2000) tested the responses of *Physella heterostropha pomila* to four levels of predation risk. The treatments based on risk level vary from the control with no predation cues (low), cues from non-foraging crayfish (intermediate) to cues from crushed snails (high) and from foraging crayfish (high). "Crawl-out behavior" is defined as the snail being completely above the waterline, "surfacing" is defined as the snail floating or in contact with surface water and "exposed" is defined as snails attached below the water line to the sides or bottom of the aquaria. Crawl-out behavior is analyzed as the proportion of snails completely out of the water of those that moved to the surface or crawled out. Prior to performing an ANOVA, the data is arcsine transformed. The results indicated that weakest crawl-out response occurred in controls, an intermediate response in snails exposed to media from non-foraging crayfish, and the strongest response from media exposed to crushed snails or foraging crayfish. The ANOVA revealed a significant interaction for population x crayfish x injured snails ($F= 4.520$, $p= 0.043$) for crawl-out behavior.

In addition, this study demonstrated that a new endpoint, the crawl-out behavioral response was highly correlated to $CdCl_2$ concentration when analyzed by logistic regression. This study allowed analysis of this effect using smaller sample sizes than standard survival methods (90 versus 150), and a shorter overall study duration (12 h versus the standard acute exposure of 96 h). The crawl-out response endpoint was easier to identify than the more common survival endpoint of death identified as

immobilization. This experimental procedure also had the advantage of portability, allowing analyses to be conducted in the field.

Reproduction and Embryonic Development

Reproductive and developmental responses to cadmium exposure in snails have been investigated in several studies (Cheung and Lam, 1998; Gomot, 1998; Holcombe *et al.,* 1984). The effect of exposure to cadmium as $CdCl_2$ on the development of embryos of *P. acuta* was investigated at test concentrations of 0, 1000, 2000, 3000, and 4000 μg $CdCl_2$ L^{-1} over an exposure period of 48 h (Cheung and Lam, 1998). The acute 24 h LC_{50} for the embryonic development is 1270 μgL^{-1} $CdCl_2$ (1130 - 1420) and the 48 h LC_{50} was 850 μg $CdCl_2$ L^{-1} (710 - 1010 μg $CdCl_2$ L^{-1}) (Cheung and Lam, 1998). A chronic toxicity test was conducted at exposure concentrations of 100, 200, 300, 400 and 500 μg $CdCl_2$ L^{-1} in order to compare the number of embryos hatching after exposures of 8 and 28 days. Eggs not hatching after 28 days were considered unhatchable. ANOVA indicated a significant decrease in percent hatch with increasing $CdCl_2$ concentration. Hatching was completely inhibited at concentrations greater than 210 μg L^{-1} $CdCl_2$ L^{-1}.

The toxic effects of cadmium on sub-lethal responses of egg-laying and embryo development have been studied in the freshwater snail *Lymnaea stagnalis* (Gomot, 1998). Snails were fed lettuce supplemented with fish food. Egg masses were collected twice a week during water change and placed in Petri dishes with the same cadmium concentration as the adult. The number of egg masses and number of eggs per mass were counted and embryo development recorded weekly. Length of incubation and

number of hatched juveniles were recorded for each treatment. At the end of seven weeks, the mean number of eggs per mass for each concentration was determined and the percentage of eggs hatched in each treatment was compared to the control. The results demonstrated no significant difference between controls and concentrations of 0, 25, 50 and 100 μg Cd^{+2} (as $CdCl_2$) L^{-1} for number of egg masses and number of eggs per mass. At a concentration of 200 μg Cd^{+2} (as $CdCl_2$) L^{-1} there was a significant difference for number of eggs per mass relative to controls, but not in number of masses oviposited. At a concentration 400 of μg Cd^{+2} (as $CdCl_2$) L^{-1} there was a significant difference in both number of masses oviposited and number of eggs per mass relative to controls.

This study demonstrated more frequent and more deleterious effects as Cd concentration increased. The control group exhibited hatching response in 12-14 days over a two-day period. Treatment groups of 25 and 50 μg Cd^{+2} (as $CdCl_2 \cdot L^{-1}$) started hatching 4-5 days later over a 10-day period. A hatching rate of 8 % was recorded at 100 μg Cd^{+2} (as $CdCl_2$) L^{-1} which was significantly reduced relative to the 15-21 % hatching rate recorded at 0, 25 and 50 μg Cd^{+2} (as $CdCl_2$) L^{-1}. Time to hatching, number of eggs per mass and hatching rate (0.4 %) were all significantly reduced at 200 μg Cd^{+2} (as $CdCl_2$) L^{-1}. There was also a decrease in the size of embryos compared to controls. The study also revealed an inhibition concentration gradient within egg masses such that hatching success was reduced among eggs at the edge of the egg mass and hatching increased among eggs towards the center of the mass. This hatching gradient suggested that the gelatinous material surrounding the eggs provided protection from the inward

diffusion of external toxins from the surrounding medium. The egg envelope may have also inhibited inward diffusion of toxins, allowing the embryo to survive to an advanced stage of development. Prior to oviposition, maternal tissues may have provided an initial level of protection from toxins, so that eggs were not fully exposed to cadmium until after being oviposited in contaminated water (Gomot, 1998).

Exposure to heavy metals also has negative sub-lethal impacts on reproduction in other freshwater snails. In a 26 day reproductive study using individuals of the freshwater pulmonate, *Aplexa hypnorum,* no oviposition occurred at cadmium exposure concentrations that significantly impacted survival determined from a 96 h LC_{50} of 152 μg $CdCl_2 \cdot L^{-1}$ (88-262 μg $CdCl_2 \cdot L^{-1}$) (Holcombe *et al.,* 1984). Over a 26 d embryo development study, eggs were added to cadmium concentrations ranging from 0 - 21.6 μg $CdCl_2 \cdot L^{-1}$. Eighty four percent of all eggs developed normally after four days of chronic exposure to these cadmium concentrations. Hatching occurred after 6-9 days only at concentrations of 7.82 μg $CdCl_2 \cdot L^{-1}$ and below. No hatching occurred at higher concentrations (>11.71 μg $CdCl_2 \cdot L^{-1}$) after 10 days of exposure, but 11 embryos survived to the end of the study in the 12.46 μg $CdCl_2 \cdot L^{-1}$ treatment and 3 embryos in the 19.12 μg $CdCl_2 \cdot L^{-1}$ treatment. At 26 days, no embryos hatched in the 21.07 μg $CdCl_2 \cdot L^{-1}$ treatment, although one embryo hatched in the 21.56 μg $CdCl_2 \cdot L^{-1}$ treatment. There was a significant difference between the control and treatment levels of 7.82, 11.71, 12.46 and 21.56 μg $CdCl_2 \cdot L^{-1}$ in the number of embryos surviving to the end of the toxicity test period (Holcombe *et al.,* 1984).

The effect of cadmium chloride was demonstrated as a significant effect on the freshwater snail *P. acuta* using reproduction as an endpoint in addition to the standard toxicity tests of acute and chronic survival. Significant reproductive endpoints included the concentration that resulted in 50 % sample egg mass oviposition EC_{50}, 50 % sample multiple egg mass oviposition at 72 h observation intervals and 50 % sample hatch per mass EC_{50}. A comparison between maximum likelihood probit and logistic regression analysis for estimatations of EC_{50} demonstrated essentially similar results, allowing comparison to previous studies. Effect of exposure to cadmium chloride ($CdCl_2$) on mean number of egg masses, number of eggs and number of days to egg hatching were estimated by the relatively new general linear models regression with responses fitting the poisson distribution.

In addition, this study demonstrated that a new endpoint, the egg diameter response, was highly correlated to $CdCl_2$ concentration when analyzed with a linear mixed model regression. This study allowed analysis of this effect using a smaller sample sizes than standard survival methods (15 versus 150 snails), with a similar overall study duration (192 h). The egg diameter response endpoint was easier to identify than the more common survival endpoint of death identified as immobilization. This experimental procedure also had the advantage of providing a highly sensitive measurement in the laboratory, although the toxicity test procedures allowed the collection of egg masses in the field, without the necessity of moving and caring for adult snails in the laboratory.

Respiration

Exposure to heavy metals has been demonstrated to impact metabolic rates of molluscs measured as weight specific oxygen uptake rates (Mo_2). The dry tissue weighted oxygen consumption rates of specimens of the inter-tidal blue mussel, *Mytilus edulis L.*, and inter-tidal snail, *Littorina rudis*, were determined for specimens collected from 13 seashore sites of varying copper concentration along a 60 km copper pollution gradient extending from Avoca to Dublin along the eastern coast of Ireland (Wilson and McMahon, 1981). There was a significant negative correlation between environmental Cu exposure and dry tissue weight in *M. edulis*, but no correlation with Mo_2. In contrast, there was a strong positive correlation between environmental Cu concentration and Mo_2 among samples of *L. rudis* suggesting that environmental copper pollution impacted metabolic rate in this snail species.

Both the acute oxygen consumption and hypoxia responses provided new information on the effect of cadmium chloride ($CdCl_2$) on the freshwater snail, *P acuta*. There was a significant effect of concentration adjusted for size**,** as dry tissue weight, on acute oxygen consumption. In contrast, there was no significant effect of concentration or size, as dry tissue weight, for the respiratory reposnse to hypoxia. Due to the high concentration (1200 μg $CdCL_2 \cdot L^{-1}$) needed to produce an effect on acute oxygen consumption and the cost of equipment, this toxicity test is not an efficient replacement for the 96 h LC_{50} of 1563.96 μg $CdCL_2 \cdot L^{-1}$ (s.e = ±32.78). The hypoxia response toxicity test is not an efficient replacement for the acute 96 h survival LC_{50} because

there was no significant response in the adjusted dry weight oxygen regulatory response of *P. acuta* to $CdCl_2$ concentrations in this study..

Comparison of Toxicity Tests from This Study

The results of this study demonstrated a significant response to cadmium chloride for survival, crawl-out, fecundity and development as well as egg diameter toxicity tests for *P. acuta.* In contrast, no significant response was elicited for the progressive hypoxia response toxicity test. All toxicity tests demonstrating a significant response provided information with lower number of subjects and lower concentrations than the standard acute survival toxicity tests. The endpoints for the crawl-out response and the reproductive studies for fecundity and development provided measurements that were faster and easier to identify than immobility for the survival toxicity test. The reproductive toxicity tests resulted in the most sensitive measures, with all reproductive end points, egg masses, eggs and hatching, demonstrating differences between the control and 5 µg $CdCl_2 \cdot L^{-1}$, the lowest concentration tested.

Several toxicity tests from this study provided results faster than the standard 96 h survival test or the more common 504 h (21 d) reproductive tests. The crawl-out response demonstrated a significant effect in the shortest observation period, 12 h, in contrast to the standard 96 h acute survival toxicity test. The acute oxygen consumption toxicity test also elicited a significant response in 48 h, in contrast to the standard acute 96 h survival toxicity test.

The reproductive toxicity tests from this study resulted in shorter observation periods than the more common 504 h (21 d) studies. The egg mass, multiple egg mass oviposition over 72 h and hatch EC_{50} resulted in significant effects at 432 h (18 d).

Results from this study suggested that several toxicity endpoints would provide effective measurements in the field. The crawl-out response (CO_{50}) provided the most efficient method for use in the field due to the easily identifiable response, inexpensive and portable equipment and shorter 12 h observation period that resulted in a significant effect of cadmium toxicity. The egg mass and egg hatching effect concentration would also be efficient field measures for cadmium toxicity. Those tests provided easily identifiable end points and inexpensive and portable equipment, but require longer observation periods than the CO_{50} (432 h versus 12 h).

Species Comparison

The results from this study indicated that *Physa acuta* provided an effective model for toxicity testing based on published results for other species' responses to cadmium. The survival LC_{50} for *Physa acuta* ranged from 2960.49 (s.e. = ± 66.79) at 24 h to 79.3 (s.e. = ±10.58) at 672 h. LC_{50} values for freshwater snails ranged from 96 µg $CdCl_2 \cdot L^{-1}$ (Holcombe, 1985) to 35940 µg $CdCl_2 L^{-1}$ for *Brotia hainanensis (*Lam, 1996), with LC_{50} values for *P. acuta* falling into this range. *Daphnia magna* demonstrated a much more sensitive response to cadmium chloride than *P. acuta* (Guilhermino et al., 2000; Nebekeretal, 1986). The freshwater fish, *Oncorhynchus mykiss*, revealed the most sensitive response with values ranging from 1.5 $\mu g \cdot L^{-1}$ (Buhl and Hamilton, 1991) to 6.0 $\mu g \cdot Cd(NO_3)_2 \cdot L^{-1}$ (Kunada et al., 1980). LC_{50} values for the effect of cadmium, as

$Cd(NO_3)_2$ ranged from 1.5 µg $Cd(NO_3)_2 \cdot L^{-1}$ (Buhl and Hamilton, 1991) to 5080 µg·L^{-1}at 48 h for *Salvelinus fontinalis* (Holcombe et al., 1983). *P. acuta* LC_{50} values fall within the wide range of values demonstrated by freshwater fish.

Behavioral responses for *P. acuta* from this study revealed significant effects of cadmium chloride. Other studies demonstrated an effect of cadmium, as $Cd(NO_3)_2$, on freshwater fish. Changes in behavior demonstrated for *Ctenopharyngodon idellus* Val*.,* 1844 included changes in swimming speed and orientation and breathing at the surface (Yorulmazlar and Gül, 2003). Decreases in aggressive behavior, which affected the social dominance hierarchies, resulted from exposure to cadmium for *Oncorhynchus mykiss* (Sloman *et al*., 2003).

All reproductive toxicity tests from this study revealed an effect of cadmium chloride at a concentration of 5 µg $CdCl_2 \cdot L^{-1}$. *Daphnia magna* demonstrated inconsistent responses to cadmium chloride so *P. acuta* would provide a more effective model for reproductive endpoints (Muyssen and Janssen, 2004). *Physa acuta* demonstrated a decrease in egg hatching from 93.94 % at 0 µg $CdCl_2 \cdot L^{-1}$ to at 6.67 % at 350 µg $CdCl_2 \cdot L^{-1}$ over an exposure period of 672 h in this study. The freshwater fish, *Oryzias latipes,* demonstrated a decrease in egg hatching percent from 98 % for the control to 65 % for 8000 µg $Cd(No_3)_2 \cdot L^{-1}$ (Gonzalez-Doncel *et al.,* 2003). In contrast to the effect of cadmium chloride on delay in egg hatching for *P. acuta* revealed from this study, no effect on time to hatch was demonstrated for *O. latipes* (González-Doncel *et al.,* 2003).

Unlike the survival and reproductive experiments, few studies of the effect of cadmium on metabolism were found in the literature. This study revealed that $CdCl_2$ depressed oxygen consumption of *P. acuta* at 1200 $\mu g \cdot L^{-1}$, while $CdCl_2$ depressed oxygen consumption in *Daphnia magna* at 20 $\mu g \cdot L^{-1}$ (Barber *et al*., 1994). In contrast the grass carp, *Ctenopharyngodon idella*, demonstrated an increase in oxygen consumption at 1000µg $Cd(NO_3)_2 \cdot L^{-1}$ (Espina *et al*., 2000).

US Environmental Protection Agency Drinking Water and Effluent Standards

Amended drinking water standards set by US Environmental Protection Agency (USEPA, 2004) established a maximum limit of 2 $\mu g \cdot Cd \cdot L^{-1}$. Estimates from the maximum likelihood regression analyses for the acute 96 h LC_{50} (964.4µg of cadmium as $CdCl_2$) and chronic 672 h LC_{50} (48.6 µg of cadmium as $CdCl_2$) and 12 h crawl-out (CO_{50}) (181.2 µg of cadmium as $CdCl_2$) without the addition of SL resulted in values well above the drinking water quality standard.

Based on the maximum likelihood probit regression in this study, the fecundity and development toxicology tests demonstrated the greatest sensitivity to cadmium, as cadmium chloride. The 50 % sample multiple egg mass over a 72 h observation interval demonstrated values very similar to this standard. The analysis lacking SL resulted in the highest estimate of 10.4 µg of cadmium as $CdCl_2 \cdot L^{-1}$at 360 h. After the inclusion of SL as a covariate, the EC_{50} value for median SL = 8.7 mm resulted in 7.3 µg of cadmium as $CdCl_2$ at 360 h. Both the 432 h (18 d) 50 % sample egg hatching response (EC_{50}) without SL as a covariate (7.35 µg of cadmium as $CdCl_2 \cdot L^{-1}$) and with

SL median = 8.7 mm (6.95 µg of cadmium as $CdCl_2 \cdot L^{-1}$) resulted in values very close to the drinking water quality standard.

Size adjusted mean pair-wise comparisons for number of egg masses and number of eggs revealed significant differences between the control and all other concentrations from 3.07 – 1226.36 µg of cadmium as $CdCl_2 \cdot L^{-1}$. This demonstrated the effectiveness of using these endpoints as indicators for drinking water quality standards.

Results from this study were compared to point source pretreatment effluent standards set by 40.US CFR 437.11, which established a daily limit of 474 $\mu g \cdot Cd \cdot L^{-1}$ and a monthly limit of 96.2$\mu g \cdot Cd \cdot L^{-1}$. All acute 96 h LC_{50} survival toxicity tests for analyses with and without SL as a covariate resulted in values higher than the daily limit, indicating this toxicity response would not be an effective pretreatment effluent water quality screen. The chronic survival toxicity LC_{50} test approached the daily limit at 288 h (12 d) (LC_{50} = 479 $\mu g \cdot Cd \cdot L^{-1}$) for the analysis lacking SL as a covariate. The chronic LC_{50} at 288 h = 499 $\mu g \cdot Cd \cdot L^{-1}$ after adding median SL = 6.7 mm as a covariate. The chronic survival toxicity LC_{50} test approached the monthly limit at 552 h (23 d) (LC_{50} = 100 $\mu g \cdot Cd \cdot L^{-1}$) for the analysis lacking SL as a covariate. The chronic LC_{50} at 576 h = 103 $\mu g \cdot Cd \cdot L^{-1}$ after adding median SL = 6.7 mm as a covariate. Reproductive results demonstrated values effective in determining pretreatment effluent levels for fecundity and for development. The 672 h (28 d) 50 % sample egg mass response (EC_{50}) lacking SL as a covariate (115.4 µg of cadmium as $CdCl_2 \cdot L^{-1}$) resulted in EC_{50} values below the daily limit of 474 $\mu g \cdot Cd \cdot L^{-1}$, but slightly above the monthly limit of

96.2 µg·Cd·L^{-1} for pretreatment effluent. With the addition of SL as a covariate, EC_{50} values for estimates including SL as a covariate fall below the required limits for both daily (474 µg·Cd·L^{-1}) and monthly (96.2 µg·Cd ·L^{-1}) limits.

Multiple waste stream limits were established at 17.2 µg·Cd·L^{-1} for a daily maximum and 10.2 µg·Cd·L^{-1} for a monthly maximum (US 40 CFR). Estimates from the maximum likelihood regression analysis for the acute 96 h LC_{50} (964.4µg of cadmium as $CdCl_2$) and chronic 672 h LC_{50} (48.6 µg of cadmium as$CdCl_2$) and 12 h crawl-out (CO_{50}) (181.2 µg of cadmium as $CdCl_2$) without the addition of SL resulted in values remaining above the multiple waste stream daily and monthly limits for water quality standard.

The reproductive toxicity tests from this study resulted in the most sensitive values, as demonstrated through adjusted mean number of egg masses and eggs and fraction of eggs developing into embryos, with all tests demonstrating significant concentration pair-wise comparisons between the control and 3.07 µg of cadmium as $CdCl_2$. Both the 672 h (28 d) 50 % sample egg mass response (EC_{50}) without SL as a covariate (115.4 µg of cadmium as $CdCl_2$·L^{-1}) and the 50^{th} SL quantile (SL = 8.7 mm) (78.0µg of cadmium as $CdCl_2$·L^{-1}) resulted in EC_{50} values higher than that of the multiple waste stream standard. Both the 432 h (18 d) 50 % sample egg hatching response (EC_{50}) without SL as a covariate (7.34 µg of cadmium as $CdCl_2$·L^{-1}) and with 50^{th} SL quantile (SL = 8.7 mm) (6.95 µg of cadmium as $CdCl_2$·L^{-1}) resulted in values below standards for multiple waste stream water, indicating this toxicity test represented an efficacious methodology to evaluate multiple waste stream toxicity levels..

Acute survival toxicity tests from this study resulted in 96 h LC_{50} values above the limits designated by the federal government for drinking water, pretreatment effluent and multiple waste stream water quality standards, and therefore are not good water quality indicators for monitoring those pollution standards. In contrast, chronic survival toxicity tests for exposure periods of 576-672 h without SL as a covariate and for exposure periods of 600–672 h after the addition of SL as a covariate (50^{th} SL quantile = 6.7 mm) revealed values below the standard for pretreatment effluent water quality standards. Chronic values remained above the multiple waste stream and drinking water quality limits, and would not be efficient indicators for those standards. The crawl-out response with or without SL as a covariate resulted in values above the standard for drinking water limits, suggesting this response would not provide an acceptable indicator for drinking water quality. The crawl-out response provided values below the limit for daily pretreatment effluent and less than three times the value for the monthly limit and therefore may be used as a water quality indicator.

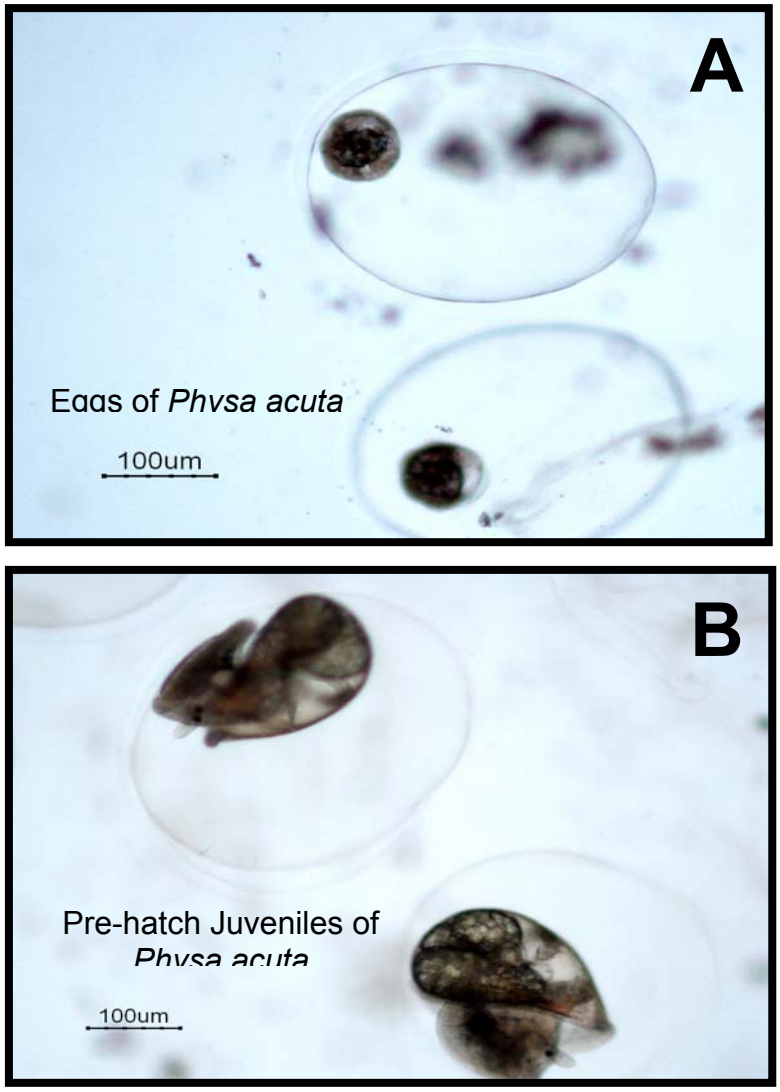
The results from the reproductive toxicity tests included in this study revealed that these toxicity tests for *Physa acuta* provided a good indicator of water quality utilizing the federal standards for drinking water, pretreatment effluent water quality and multiple wastewater stream sources. All reproductive toxicity tests estimating number of egg masses, eggs and days to hatch from this study revealed significant differences between the control and the lowest concentration tested (5 μg $CdCl_2 \cdot L^{-1}$). The egg mass EC_{50} would be an efficient indicator for pretreatment effluent water quality, but not for drinking water or multiple waste stream water quality. In contrast,

egg-hatching EC_{50} would make an efficient indicator for all three water quality standards.

APPENDIX A

ADULT AND EMBRYO STAGES OF *PHYSA ACUTA*

A.1: Pictured adult freshwater, pulmonate pond snail, *Physa acuta*



A.2: Pictured (A) Eggs in freshly oviposited egg masses and (B) fully formed juveniles just prior to hatching of the freshwater, pulmonate pond snail, *Physa acuta.*

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