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Histological and ultrastructural studies of larval and juvenile Lampsilis (Bivalvia) from the Upper Mississippi River

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Histological and ultrastructural studies of larval and juvenile
*Lampsilis* (Bivalvia) from the upper Mississippi River

Lasee, Becky Ann, Ph.D.
Iowa State University, 1991
Histological and ultrastructural studies of larval and juvenile *Lampsilis* (Bivalvia) from the Upper Mississippi River

by

Becky Ann Lasee

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

Department: Animal Ecology
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In Charge of Major Work

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For the Major Department

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For the Graduate College

Iowa State University
Ames, Iowa
1991
# TABLE OF CONTENTS

**GENERAL INTRODUCTION**  

SECTION 1. ULTRASTRUCTURAL STUDY OF THE LARVAL AND JUVENILE SHELL OF LAMPSILIS VENTRICOSA (BIVALVIA: UNIONIDAE) AND OBSERVATIONS OF PLEUROBEMA CORDATUM (BIVALVIA: UNIONIDAE)  

ABSTRACT  

INTRODUCTION  

MATERIALS AND METHODS  

RESULTS  

DISCUSSION  

LITERATURE CITED  

APPENDIX  

SECTION 2. POST-LARVAL DEVELOPMENT OF LAMPSILIS VENTRICOSA (BIVALVIA: UNIONIDAE) IN THE LABORATORY  

ABSTRACT  

INTRODUCTION  

MATERIALS AND METHODS  

RESULTS  

DISCUSSION  

LITERATURE CITED  

APPENDIX
GENERAL INTRODUCTION

Background

Freshwater unionids often dominate streambeds in terms of their biomass and numbers, and they show tremendous diversification. In North America there are about 50 nominal genera which include over 225 species and subspecies (Davis and Fuller 1981). Bivalves significantly impact ecological processes in freshwater ecosystems. As filter feeders, mussels convert plankton, a food source unavailable to many aquatic species, into an available food source. Many aquatic birds (Fuller 1974), mammals (Convey et al. 1989) and fish, including paddlefish, sturgeon, suckers, channel catfish and freshwater drum (Perry 1979), consume juvenile and adult mussels. Mussel shells also provide substrate for fish spawning and a surface for establishment of other aquatic invertebrate communities (Fuller 1974).

Declines in Mussel Populations

Human activities of the last century have severely impacted freshwater mussel stocks of the Upper Mississippi River (UMR). Navigation, development of the lock and dam system, hydraulic dredging, commercial harvest, siltation and pollution have all contributed to declines in mussel abundance and diversity (Fuller 1974; Thiel 1981; Perry 1979). Recent introduction of the exotic zebra mussel (Dreissena polymorpha) into the Great Lakes and surrounding watersheds is an additional threat to native mussels (Walker 1991).

Increased commercial pressure and the occurrence of extensive mussel die-offs are two additional concerns. The commercial value of mussel shells is substantial. Thousands of tons of shells are shipped annually to Japan and used as implant nuclei for cultured marine pearls. Presently, they also are used for the culture of freshwater pearls in the United States (Isom and Hudson 1982). The commercial value, during each of the years between 1983-1987, was estimated at more than one million dollars by the Upper Mississippi River Conservation Committee (UMRCC) (Fritz 1990). The UMRCC also reported sporadic episodes of mussel die-offs throughout the Mississippi River during the years 1982-1987 (Fritz 1990). No causative agents or pathogens were identified that would explain the die-offs.
Reproduction and Life History

The freshwater mussel, *Lampsilis ventricosa*, is found throughout the UMR (Perry 1979; Thiel 1981; Miller 1988), primarily in main channel border habitats (Holland-Bartels and Kammer 1989). The life histories of *L. ventricosa* and many of its North American relatives were studied extensively during the early 1900's (Lefevre and Curtis 1910; Surber 1912; Reuling 1920; Coker et al. 1921; Howard 1922). The life cycles of unionids are complex. Many adults, including the lampsiline mussels, are long term (bradytictic) breeders (Coker et al. 1921). Fertilization occurs in late summer or fall for bradytictic breeders, when sperm are released from the male and drawn into incurrent siphons of nearby females. Eggs are fertilized within specialized portions of the gills, called marsupia, where the embryos develop into a parasitic larva called a glochidium (Lefevre and Curtis 1910; Matteson 1948). Glochidia are held within marsupia until their release the following spring. At this time they are infective and fully formed (consisting primarily of two valves joined by a dorsal hinge, lined by larval mantle cells and held together by a single adductor muscle) (Lefevre and Curtis 1910; Wood 1974; Waller and Mitchell 1989). Released glochidia must contact and encapsulate within specific tissues, usually the gills, of a suitable fish host. After successful attachment, glochidia metamorphose (transform) to small juveniles and within a few weeks, excyst to begin a free-living existence in the substrate. The juvenile is equipped with a protractile foot, rudimentary gills, and presumptive digestive and circulatory organs (Churchill and Lewis 1924; Karna and Millemann 1978; Waller and Mitchell 1989).

Many aspects of the host-parasite relationship have been studied, including identification of hosts (Fuller 1974; Matteson 1948; Tedla and Fernando 1969; Isom and Hudson 1984; Zale and Neves 1982; Waller et al. 1988), distribution of glochidia on hosts (Dartnall and Walkey 1979; Threlfall 1986; Dudgeon and Morton 1983; Jansen 1991; Jansen and Hanson 1991) and host histopathogenesis associated with glochidiosis (Arey 1932; Futish and Millemann 1978; Karna and Millemann 1978; Waller and Mitchell 1989). Descriptions of glochidial development (Harms 1909; Lefevre and Curtis 1910, 1911; Wood 1974) and glochidial morphology (Giusti 1973; Kat 1984; Waller et al. 1988; Castilho et al. 1989) are numerous in the literature.
However, little is known about development and early life history requirements of unionid juveniles. Studies dealing specifically with the juvenile stage are complicated, primarily because of the small size of juveniles and problems with collecting and identifying juveniles in the field (Coker et al. 1921). Neves and Widlak (1987) noted that new information on juvenile habits, habitat and ecology is critically needed in order to form recovery plans that will adequately protect endangered and threatened mussel species in the United States.

Laboratory Culture

Juveniles of freshwater mussels have seldom been noted from field collections and unlike marine bivalves, are difficult to rear in the laboratory. For many unionid species, fish hosts have not been identified, and for others, a very specific or rare host species may be required. The use of artificial media to transform glochidia circumvents these problems. Ellis and Ellis (1926, 1927) were the first researchers to report successful transformations of freshwater mussel glochidia in physiological nutrient solutions. *Lampsilis fallaciosa* glochidia were transformed in a complex medium containing salts, dextrose and a mixture of amino acids. Unfortunately, neither the composition of the medium nor their methods were ever published. The next report describing in vitro methods was by Isom and Hudson (1982). They transformed large numbers of glochidia of six genera using a medium containing salts, amino acids, vitamins and fish plasma. More recently, Keller and Zam (1990) simplified the methods of Isom and Hudson by substituting a commercial medium and horse serum for the complex medium containing fish blood. The advantages of using artificial media for transforming glochidia include: (1) large numbers of juveniles can be produced from a single female and large-scale experiments (i.e. toxicity tests) can be conducted in the laboratory; (2) juvenile species whose host or hosts are unknown can be reared in the laboratory; and (3) the timing of juvenile release can be controlled (Keller and Zam 1990). One limitation is that transformation rates are variable, and largely depend on the species studied. For instance, Keller and Zam (1990) reported limited success in transforming *Lampsilis teres* and *Villosa lenosa* glochidia, compared to transformation rates reaching up to 96% for *Anodonta imbecilis*.

There has been only limited success in rearing juveniles in the laboratory. Hudson and Isom (1984) conducted numerous laboratory studies using continuous flow and static water systems to rear freshwater mussel juveniles. Of seven species studied,
maximum growth and survival was reported for *Anodonta imbecilis* juveniles. A maximum length of 5.1 mm was reached by *A. imbecilis*, from an initial size of 0.28 mm. In contrast, growth and survival were poor for juvenile *Fusconaia ebena, Ligumia recta, Pleurobema cordatum, and Carunculina moesta*. Hudson and Isom (1984) suggested that nutritional problems may account for low survival of these juveniles. At this time studies indicate *A. imbecilis* juveniles are well suited for laboratory studies (Hudson and Isom 1984; Keller and Zam 1990, 1991).

**Metal Effects on Bivalve Mussels**

Specific reasons for the decline in the health of mussel communities in the UMR are currently unknown. Past and continued, although reduced, influxes of metal pollutants, primarily from industrial and municipal discharges from the Minneapolis-St. Paul metropolitan area, may be a contributing factor. Boyer (1984) reported concentrations of arsenic, cadmium, copper and zinc in the upper pools of the UMR either approached or exceeded U. S. Environmental Protection Agency (EPA) water quality criteria for aquatic life. Sediment levels of arsenic, cadmium and chromium also exceeded criteria for low level contamination (refer to Boyer 1984). Rada et al. (1990) reported that even though water and sediment quality in Pools 2-4 have improved in the last several years, the surficial sediments still remain contaminated with metals, at levels that could potentially be a threat to aquatic biota. Resuspension of contaminated sediments, for example, by barge traffic, may also increase the bioavailability of metals to aquatic organisms. This is especially significant for benthic filter-feeding bivalve molluscs.

Adult bivalves can accumulate heavy metals at concentrations greatly exceeding those in the water and sediment (Anderson 1977; Heit et al. 1980; Pugsley et al. 1988). It is for this reason that adults are frequently used in biomonitoring and bioaccumulation studies. Most bioaccumulation studies have shown the kidney, gills, digestive gland and mantle of adult mussels to have higher concentrations of metal contaminants than either the digestive tract, shell or muscle (Manly and George 1977; Tessier et al. 1984; Evtushenko et al. 1986; Robinson and Ryan 1986; Herwig et al. 1989). Hemelraad et al. (1986a, 1986b, 1987) detailed cadmium accumulation in freshwater adult mussels. For *Anodonta cygnea*, cadmium uptake in whole animals, as well as for most organs, occurred in a biphasic manner. During the first four weeks, accumulation was linear, followed by a 2 week stagnation period. In the sixth week, cadmium uptake resumed at a high rate (second phase) and then leveled off again in the
eighth and tenth week of exposure. Accumulation in organ systems was: kidney > midgut gland > gills > whole animal > mantle = mantle edge > alimentary tract/gonadal complex > foot and adductor muscles. However, there was some variability in organ accumulation patterns during exposures. Hemelraad and Herwig (1988) used the histochemical sulphide-silver technique to evaluate cadmium localization in the tissues of *A. cygnea* and *A. anatina*. Their studies indicated that the mantle and gill are the primary accumulation sites and the kidney the end-accumulator. Similar accumulation studies have not been performed using juvenile mussels.

The sensitivities of different life stages of bivalves to metal exposures have been evaluated infrequently. Harrison et al. (1984) reported that copper sensitivity decreased for the freshwater clam, *Corbicula manilensis*, with successive developmental stages (based on LC50's at 24- and 96-h). Adults were very resistant to copper (LC50 > 2600 μg Cu/L). Similar studies were performed by Ringwood (1990) evaluating the toxicity of cadmium to different stages of the marine bivalve, *Isognomon californicum*. Based on 48-h LC50's, newly settled juveniles were less sensitive than embryonic or larval stages and adults were the least sensitive. The life cycles of unionid bivalves are different from either corbiculid or marine species; therefore, similar patterns of metal sensitivity for different life stages may not be expected. There are apparently no reported comparative studies of metal tolerances among embryonic, larval and juveniles stages of unionid bivalves.

Effects of metals on juvenile stages of the freshwater unionid *Anodonta imbecilis* were evaluated by Keller and Zam (1991). Their studies showed the following order of metal toxicity (48-h LC50) in soft water: cadmium > copper > mercury > nickel > chromium > zinc. Metal toxicities were significantly lower in hard water; however cadmium was still the most toxic metal. Keller and Zam (1991) also demonstrated that metal mixtures altered metal toxicity to *A. imbecilis* juveniles. For instance, mixtures containing nickel, zinc, mercury or copper were more toxic than were single metals. Conversely, cadmium was less toxic in mixtures with zinc and copper.

The effective toxicity of a metal contaminant is the result of an organism's ability to limit nonspecific binding to vulnerable sites. Freshwater mussels have the ability, at least initially, to regulate the distribution of metals at the tissue and cellular levels. Various detoxification and sequestration mechanisms have been identified, such as incorporation into calcium concretions (Pynnonen et al. 1987), binding to
metallothionein-like low molecular weight proteins (Accomando et al. 1990) and immobilization in phagolysosomes (Coombs and George 1978).

With continuous prolonged metal exposures nonspecific binding at susceptible sites may occur, possibly producing lesions. Reported histopathological effects of contaminants on adult bivalves include: 1) for gills, fusion of filaments, mucous hypersecretion, degeneration of collagenous connective tissue, lesions, hyperplasia, detachment of abfrontal cells from chitinous rods, increased vacuolization and loss of cilia (Sunila 1986, 1988; Marshall and Talbot 1979); (2) for digestive glands, changes in epithelial structure, vacuolization and accumulation of lipids in digestive and basophil cells (Lowe and Clarke 1989); (3) for the kidneys, ultrastructural changes including swollen mitochondria and reduced glycogen reserves (Hemeiraad et al. 1990); (4) neoplasia (Mix 1983; Gardner and Yevich 1988) and (5) acute and chronic inflammatory responses (Sunila 1988; Farley 1988). There are apparently no histopathological studies dealing with effects of metal contaminants on juvenile unionids.

Study Objectives

Objectives of this study were to: 1) describe the ultrastructure of the larval and post-larval shell of L. ventricosa and P. cordatum; 2) document the post-larval development of the digestive organs, gills, foot and nervous system of L. ventricosa reared in the laboratory; 3) determine acute toxicities of cadmium to larval and juvenile stages of L. ventricosa and 4) evaluate growth and histopathological effects of subchronic cadmium exposures on juvenile L. ventricosa.

Explanation of Dissertation Format

The dissertation uses alternate format and is presented in three sections. Each section is a separate manuscript that will be submitted for publication in a professional journal. Each section contains its own abstract, introduction, materials and methods, results, discussion and literature cited. An appendix follows section 1 and section 2. The figures included in appendices will not be used in publications. A general summary follows section 3. Literature cited in the general introduction follows the general summary.

Chapter 1 describes larval shell ultrastructure and early juvenile shell growth of L. ventricosa and observations of P. cordatum shell ultrastructure.
Chapter 2 outlines early growth and internal organ development of juvenile *L. ventricosa* reared in the laboratory. This was the first study of unionid post-larval development to use both light microscopy and electron microscopy.

Chapter 3 examines sensitivity of larval and juvenile *L. ventricosa* to acute and subchronic cadmium exposure. Growth and histopathological effects of cadmium on recently released juveniles were also determined.
SECTION 1. ULTRASTRUCTURAL STUDY OF THE LARVAL AND JUVENILE SHELL OF LAMPSILIS VENTRICOSA (BIVALVIA: UNIONIDAE) AND OBSERVATIONS OF PLEUROBEMA CORDATUM (BIVALVIA: UNIONIDAE)
Ultrastructural study of the larval and juvenile shell of *Lampsilis ventricosa* (Bivalvia: Unionidae) and observations of *Pleurobema cordatum* (Bivalvia: Unionidae)

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ABSTRACT

The larval and juvenile shell ultrastructure of *Lampsilis ventricosa* was studied using scanning electron microscopy. *Pleurobema cordatum* glochidia and juveniles were also examined to determine if similar shell developments occur in an unrelated species. Numerous pores covered lateral surfaces of the glochidial (larval) valves of both species. Pores extended through the inner calcareous shell layer but did not penetrate the outer periostracum (cuticle). Shell deposition began at drop off with formation of the juvenile shell at anterioposterior peripheral edges of the larval shell. Shell deposition into larval pores coincided with early growth of the juvenile shell. This pore-filling has not previously been described. By 7 days post-drop off, the juvenile shell of *L. ventricosa* was extended to ventral margins of the larval shell. Juvenile shell growth was greatest at the anterior end. After 21 days in laboratory culture, the juvenile shell of *L. ventricosa* exhibited significant growth, and concentric growth rings were evident.
INTRODUCTION

Freshwater unionid mussels exhibit complex life cycles which usually include an obligate parasitic stage on the gills or fins of fish. Parasitic glochidia require several weeks of development while encapsulated on their fish host in order to undergo metamorphosis (transformation) to the juvenile stage. Released juveniles fall to the bottom sediments and continue shell growth and internal organ development (Lefevre and Curtis 1910; Coker et al. 1921; Howard 1922; Matteson 1948).

Studies of the early life history of freshwater juvenile mussels have been limited by the small size of juveniles and the difficulty of collecting them in the field. With recent developments in the laboratory culture of freshwater bivalves, it is now possible to perform morphological studies using very young juveniles. To date, there are few descriptions of juvenile shell morphology in comparison to numerous studies describing the shell ultrastructure of glochidia (Giusti 1973; Calloway and Turner 1978; Atkins 1979; Rand and Wiles 1982; Waller et al. 1988; Castilho et al. 1989).

The purpose of the present work was to describe ultrastructural aspects of the growth of the juvenile shell of the unionid pocketbook mussel, *Lampsilis ventricosa*. Scanning electron microscopy (SEM) was used to describe larval shell pores and postmetamorphic growth of the juvenile shell. Glochidia and juveniles of the river pigtoe, *Pleurobema cordatum*, were also examined to determine if similar patterns of shell development occur in an unrelated species.
MATERIALS AND METHODS

Laboratory Culture

Gravid _Lampsilis ventricosa_ females were collected from the Upper Mississippi River (Pool 7, River Mile 702.5 to 714.3) by brailing and handpicking. _Pleurobema cordatum_ females were collected from the Green River (below Lock and Dam 5, Mississippi River). Glochidia were flushed from the marsupium of the female using a hypodermic syringe and needle. Viability of glochidia was determined by placing approximately 50-100 individuals into a 1-5% saline solution. Infective glochidia respond by closing their valves. Glochidia were then used to infect fish hosts. Largemouth bass (_Micropterus salmoides_), smallmouth bass (_M. dolomieui_) and walleye (_Stizostedion vitreum vitreum_) were used to transform _L. ventricosa_ and _Notropis_ spp. were used for artificial infections of _P. cordatum_. Fish were held in separate flow-through aquaria at 20-25 °C. Beginning approximately 20-21 days post-infection, tanks were siphoned daily, and contents were filtered through a 150 µm mesh screen. A dissecting microscope was used to collect juveniles from filtered contents. Juveniles were then transferred to 100 ml static culture chambers and fed a diet of artificially cultured phytoplankton (_Chlamydomonas, Scenedesmus, Selenastrum_ and _Ulothrix_) at a concentration of approximately 300,000-500,000 cells/ml. Water and algae were replaced in culture chambers daily or, occasionally, every other day.

Electron Microscopy

Representative _L. ventricosa_ juveniles were processed for SEM at 0, 1, 7, 14 and 21 days post-drop off. Fewer _P. cordatum_ juveniles were available for analysis; they were processed at 0 and approximately 3-6 days post-drop off. Representative glochidia of both species were examined.

Two methods were used to process glochidia and juveniles for SEM: (1) **Examination of internal shell features:** For bacterial removal of soft parts and organic tissue, live specimens were placed into small petri dishes containing tap water and left uncovered for 3 to 6 days (modified from Castilho et al. 1989). After organic decomposition, the shells were washed in distilled water and dehydrated through a graded ethanol series. (2) **Examination of external shell features:** Glochidia and juveniles were
fixed in 3% glutaraldehyde in 0.1 M Sorenson’s phosphate buffer (pH 7.2-7.4) and
post-fixed for 1-2 h in 1% osmium tetroxide. Juveniles were rinsed in buffer and
dehydrated through a graded ethanol series.

Specimens from both treatments were either air-dried in a desiccator or critical
point-dried using CO₂. There were no apparent morphological differences between air-
dried and critical point-dried specimens. After air drying some shells were fractured
using a razor blade. Specimens were mounted on disks with aluminum tape and silver
paint, sputter-coated with gold-palladium and examined with a JEOL JSM-35 scanning
electron microscope operated at 15 kV and 60-100 μA beam current.

Elemental Analysis

A Kevex Energy Dispersive X-ray System on a JEOL 1200EX scanning transmission
electron microscope was used to determine composition of material filling larval shell
pores. A Quantex computer program was used for acquiring X-ray spectra and elemental
identification. Spectra were collected with an average of 2100 cps, operating at low and
high kV’s. Spectra were obtained from three areas on shells: pore protuberance on
glochidial shell surfaces, glochidial shell surfaces adjacent to pores and recently formed
juvenile shell surfaces.
RESULTS

Glochidial Shell Morphology

Numerous pores were observed on the lateral external surfaces of the shells of *L. ventricosa* and *P. cordatum* glochidia (Figs. 1-4). Pore size ranged from 0.7-1.8 μm in diameter for *L. ventricosa* and 0.8-2.3 μm for *P. cordatum*. Examination of external shell surfaces at higher SEM magnifications revealed that pores extend through the calcareous layer (inner shell layer) but do not penetrate the outer periostracum or cuticle. The cuticle was slightly invaginated into the pore (Figs. 2, 4) and was visible with SEM only at appropriate specimen orientation (tilt) and focus. Additional micrographs showing glochidial shell ultrastructure are presented in the Appendix.

Juvenile Shell Morphology

External Shell Morphology

The external shell surface of juveniles at drop off was very similar to that of glochidia. The only difference was a slight increase in the size of the larval shell (8% for *L. ventricosa* juveniles) (Table 1, Fig. 7). Valvular pores were still evident and exhibited no change from the larval condition (Figs. 7-8).

Beginning as early as 1 day after drop off, the postmetamorphic (juvenile) shell became apparent with shell deposition at anterior and posterior edges of the glochidial valve (Figs. 5, 9). The thicker, nonporous juvenile shell of *L. ventricosa* and *P. cordatum* was sharply demarcated from the porous larval shell. Shell deposition into valvular pores of the larval shell coincided with postmetamorphic marginal shell development (Figs. 6, 10). The pore-filling continued until the periostracum was lifted up and a protuberance formed (Figs. 6, 10, 12). Calcareous material (Fig. 19) within protuberances was, at least initially, less dense in appearance than that of juvenile shell surfaces (Fig. 10). The spinous periostracum of *L. ventricosa* was readily apparent covering protuberances (Fig. 10, 12).

Shell growth was rapid for juvenile *L. ventricosa*. By 7 days, juveniles had increased nearly 50% in length (Table 1) and the juvenile shell had extended to ventral margins of the larval shell. There was no shell addition to the hinged dorsal surface. It also became apparent, for both unionid species, that shell growth was asymmetrical; shell deposition was greater at the anterior end of the larval valve (Fig. 5).
At 3 weeks of age, *L. ventricosa* juveniles averaged 418 μm in length, and there had been significant growth of the juvenile shell (Fig. 11, Table 1). Concentric growth lines were conspicuous (Fig. 11). Also at this stage, the electron density of shell protuberances more closely resembled that of nonporous shell areas (Fig. 12). Additional micrographs showing juvenile shell ultrastructure are shown in the Appendix.

Shell deposition into pores did not appear to be a uniform process. Pores located at the periphery of the shell, nearest the mantle edge, appeared to fill in sooner than pores located more dorsally, away from the mantle (Figs. 13-14).

### Table 1. Shell growth of *Lampsilis ventricosa* in laboratory culture

<table>
<thead>
<tr>
<th>Life Stage</th>
<th>n</th>
<th>Length (μm)</th>
<th>Height (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glochidia</td>
<td>15</td>
<td>209 ± 7 (197-221)</td>
<td>245 ± 7 (231-259)</td>
</tr>
<tr>
<td><strong>Juveniles:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 day</td>
<td>91</td>
<td>227 ± 16 (196-269)</td>
<td>253 ± 12 (207-279)</td>
</tr>
<tr>
<td>1 day</td>
<td>20</td>
<td>247 ± 12 (233-269)</td>
<td>247 ± 12 (233-269)</td>
</tr>
<tr>
<td>3 day</td>
<td>51</td>
<td>290 ± 17 (259-331)</td>
<td>274 ± 13 (248-300)</td>
</tr>
<tr>
<td>7 day</td>
<td>122</td>
<td>339 ± 26 (269-414)</td>
<td>306 ± 22 (259-362)</td>
</tr>
<tr>
<td>14 day</td>
<td>23</td>
<td>388 ± 37 (321-455)</td>
<td>347 ± 37 (285-440)</td>
</tr>
<tr>
<td>21 day</td>
<td>29</td>
<td>418 ± 49 (310-507)</td>
<td>367 ± 41 (310-466)</td>
</tr>
</tbody>
</table>

**Internal Shell Morphology**

Examination of the internal surfaces of bacterially treated and fractured shells revealed ostia or pore openings (Fig. 15). The pore canals were not completely filled.
The larval and juvenile shell commissure was also evident in internal shell observations (Fig. 16). The postmetamorphic shell was deposited inside and at the periphery of the larval valves. There was no additional shell deposition medially (Figs. 17-18).

**Elemental Analysis**

Examination of shell protuberances in 1-d old *L. ventricosa* by energy dispersive X-ray microanalysis disclosed the presence of high Ca concentrations (Fig. 19). Spectra acquired from juvenile shells (recently deposited) and shell surfaces adjacent to pores were nearly identical to that obtained from pore protuberances.
DISCUSSION

The present study is the first description of ultrastructural characteristics of the postmetamorphic shell of the freshwater bivalves, *Lampsilis ventricosa* and *Pleurobema cordatum*. A unique pore-filling process was reported for the first time.

Lillie (1895), Lefevre and Curtis (1912) and Coker et al. (1921) were among the earliest researchers to describe valvular pores in unionid glochidia. More recent studies using SEM confirmed the presence of larval shell pores and generated some debate as to whether or not pores perforated the outer periostracum or cuticle. Giusti (1973), Calloway and Turner (1978) and Castilho et al. (1989) described pores covered by the outer cuticle; whereas, Rand and Wiles (1982) observed valvular pores penetrating through the cuticle of *Anodonta cataracta* and *A. implicata*. Castilho et al. (1989) suggested that Rand and Wiles were observing shell artifacts produced by improper specimen processing, which resulted in dissolution of the cuticle. Our SEM examination of juveniles substantiated that the larval cuticle covers pores. This was especially apparent once pore-protuberances were formed.

Descriptions of shell pores are not limited to unionid bivalves. Mackie (1978) and Robertson and Coney (1979) described valvular pores, or what they defined as punctal canals or punctae, in larval and adult shells of the freshwater sphaerids, *Sphaerium occidentale* and *Musculium securis*, respectively. Their studies demonstrated, just as in glochidial shell pores, punctae protrude through calcareous shell layers, but do not penetrate the periostracum. Pore-filling does not occur in sphaerid bivalves.

The functional significance of shell pores is unknown. To date, descriptions of shell pores are limited to those freshwater bivalve species that brood their young within specialized gill chambers called marsupia. Therefore, it is possible that shell pores are a physiological adaptation for marsupial stages. Castilho et al. (1989) observed cytoplasmic extensions of the mantle epithelium extending into the pores of *Anodonta cygnea* and suggested that metabolic exchange between larva and parent was a most likely function. Robertson and Coney (1979) observed similar cytoplasmic extensions of the mantle into shell pores for *M. securis*, and they presented four potential functions of pores: (1) embryonic nourishment; (2) secretory function; (3) sensory function; and (4) absorptive or respiratory function. There is little support that pores have a nutritive function because pores are covered by a relatively dense cuticle, ranging from
1.2-18 μm in thickness for sphaerid bivalves (Mackie 1978) to approximately 1.4-1.9 μm for *L. ventricosa* glochidia (Lasee, unpublished data). Robertson and Coney (1979) suggested that if transperiostracum oxygen diffusion was possible, then respiration is a more reasonable function of the shell pores.

Valvular pores may also have some function in extramarsupial stages. Rand and Wiles (1982) suggested that shell pores would allow encapsulated glochidia to obtain nutrients from their fish hosts and may also be important in exchange of respiratory gases. Robertson and Coney (1979) proposed adult stages of *M. seciris* may utilize pores to monitor moisture. Sphaerids typically exhibit shell closure during dry environmental periods, and if shell pores are permeable to water molecules, it would allow the organism to detect increases in moisture content (Robertson and Coney 1979).

Examination of fractured shells did not completely resolve attachment morphology. Waller et al. (1988) described for lampsilin glochidia the peripheral edges of larval valves as turned inward to form a continuous shelf or lip. The thickened lip may provide additional surface area for juvenile shell attachment.

At this time it is not known how the concentric growth lines observed on juvenile shells correlate to environmental or intrinsic factors. Coker et al. (1921) described in detail the formation of growth bands in unionid bivalves. Simply stated, banding is the result of repeated retraction and reextension of the mantle. Low temperatures were believed to strongly influence growth interruptions (Coker et al. 1921). Because laboratory temperatures are fairly constant, it is possible that juvenile shell growth lines reflect other factors, such as semidiurnal or diurnal periodicities.

The filling of larval shell pores observed for juvenile mussels resembles descriptions of shell repair or shell regeneration in adult molluscs. Watabe (1983) defined shell regeneration as deposition of new shell material in damaged areas. He noted that this process is accomplished more readily at shell edges than in midshell regions. Additionally, for damaged areas away from the mantle edge, the repaired shell often differs from normal in structure, composition of the organic matrix and minerals and in crystal morphology (Watabe 1983). Similar observations were made for the pore-filling in juvenile *L. ventricosa*: pores in peripheral shell areas appeared to be filled in sooner than pores in dorsal shell areas. Pore-filling in juvenile unionids may be an excellent model for further investigations of molluscan biomineralization processes.
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Figure 1. Lateral view of *Lampsilis ventricosa* glochidium. Numerous pores are evident on external surfaces. Bar= 75 μm

Figure 2. Higher magnification of external surface of *Lampsilis ventricosa* glochidium showing pores (arrows) covered by the periostracum (p). Bar= 2 μm

Figure 3. Lateral view of *Pleurobema cordatum* glochidium showing numerous pores in shell valves. Bar= 50 μm

Figure 4. Higher magnification of external valvular surface of *Pleurobema cordatum* glochidium. Pores are covered by the periostracum. Bar= 4 μm

Figure 5. *Pleurobema cordatum* juvenile, 3- to 6-d old, lateral view. Arrows indicate postmetamorphic shell. Note shell surface is covered with protuberances formed by filling of the pores. Bar= 50 μm

Figure 6. Higher magnification of lateral shell surface of *Pleurobema cordatum* juvenile showing filled pores. Bar= 5 μm
Figure 7. Juvenile *Lampsilis ventricosa*, at drop off, lateral view. Note shell surface is covered with numerous pores, unfilled. At this time there is no growth of postmetamorphic shell. Bar = 50 µm

Figure 8. Enlarged view of external surface of *Lampsilis ventricosa*, at drop off. Note unfilled pores. Bar = 1.5 µm

Figure 9. Juvenile *Lampsilis ventricosa*, 1-d old, lateral view. Arrows indicate the postmetamorphic juvenile shell in early stages of growth. Note external shell surfaces are covered with numerous protuberances. Bar = 75 µm

Figure 10. Enlarged view of external surface of *Lampsilis ventricosa*, 1-d old. Note globular appearance of filled pores. The periostracum, with associated spines, covers shell protuberances. Bar = 1.5 µm

Figure 11. Juvenile *Lampsilis ventricosa*, 21-d old, lateral view. Concentric rings of growth are evident on the juvenile shell. Bar = 100 µm

Figure 12. Enlarged view of shell protuberance of *Lampsilis ventricosa*, 21-d old. The electron density of shell protuberance is similar to that of adjacent shell surfaces. Bar = 1.5 µm
Figure 13. External surface of *Lampsilis ventricosa* juvenile (1-d old) showing shell sculpturing at dorsal shell margin. Note presence of filled and unfilled pores. Bar= 35 μm

Figure 14. Same juvenile as in Fig. 13. Shell margins contain larger numbers of filled pores compared to dorsal shell regions. Bar= 35 μm

Figure 15. Internal shell surface of fractured juvenile *Lampsilis ventricosa* (7-d old) Ostia (arrows) of pores (p) are evident. Bar= 4 μm

Figure 16. Internal shell surface of 7-d old *Lampsilis ventricosa* showing adhesion of juvenile shell (J) to the glochidial shell (G). Bar= 5 μm

Figure 17. Fractured shell (internal surfaces) of 7-d old *Lampsilis ventricosa* showing denser juvenile shell (J) overlying the thinner glochidial shell (G). Bar= 5 μm

Figure 18. Fractured shell (internal surfaces) of 7-d old *Lampsilis ventricosa* showing juvenile shell (J) overlapping the glochidial shell (G). Bar= 10 μm
Figure 19. Energy dispersive X-ray spectra from shell protuberance of 1-d *Lampsilis ventricosa*. Note large calcium peaks ($K_{\alpha} = 3.691$ and $K_{\beta} = 4.012$ KeV). Elemental peaks were also generated from specimen disk (Cu, $K_{\alpha} = 8.047$ KeV) and specimen coating (Pd, $L_{\alpha} = 2.838$ and $L_{\beta} = 3.172$ KeV).
1-Oct-1990 10:44:30
Execution time = 8 seconds
SP1
Vert = 2043 counts Disp = 1
Quantex>

Preset = 100 secs
Elapsed = 100 secs

Range = 10.230 keV

Integral 0 = 116126
APPENDIX
Figure 20. *Lampsilis ventricosa* glochidia within marsupial conglutinate. Note larval mantle (m) and adductor muscle (a) in fractured specimens. Bar= 200 μm

Figure 21. Enlarged view of lateral surface of *Lampsilis ventricosa* glochidium. Shell pores are evident. Bar= 75 μm

Figure 22. *Lampsilis ventricosa* juvenile, 2-d old. Lateral view showing filled pores and juvenile shell (j). Bar= 75 μm

Figure 23. *Lampsilis ventricosa* juvenile, 21-d old. There has been significant growth of the juvenile shell. Bar= 125 μm
SECTION 2. POST-LARVAL DEVELOPMENT OF LAMPSILIS VENTRICOSA (BIVALVIA: UNIONIDAE) IN THE LABORATORY
Post-larval development of *Lampsilis ventricosa* (Bivalvia: Unionidae) in the laboratory

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ABSTRACT

Morphological and structural changes in the digestive organs, gills, foot, mantle, nervous system and sensory organs of post-larval *Lampsilis ventricosa* were examined using light and electron microscopy. Glochidia underwent metamorphosis (transformation) while encapsulated on the gills of fish hosts. A protractile foot, three pairs of gill papillae, presumptive digestive organs, a well developed nervous system and both a larval and definitive mantle were present before juvenile excystment. The digestive gland was filled with large concentrations of lipid bodies, ranging from 0.9 to 2.7 μm in maximum diameter. Excysted juveniles were reared in the laboratory for 56 days. At release juveniles averaged 227 μm in length and had undergone all the necessary ontogenetic changes needed for ingesting and processing phytoplankton. Most importantly, the stomach had differentiated into an anterior gastric portion (with ciliary sorting regions) and a posterior style sac (with a crystalline style, dorsal gastric shield and dense short cilia). Subsequent developments in the alimentary tract consisted of elongation of the intestine, pushing a loop into the pedal region and further elaboration of ciliary sorting areas and linings. The postmetamorphic alignment of the alimentary system was not altered until 56 days when the style sac shifted ventrally.

After 2 weeks, juvenile digestive glands were devoid of lipid bodies. This loss of lipids correlated with a rise in juvenile mortality. Diverticulation of the digestive glands began at 3 weeks of age; by 8 weeks numerous diverticula surrounded the stomach and projected ventrally into the foot. Gill filaments were added to the posterior end, by papillary outgrowths, beginning approximately 3 weeks after release. Frontal gill surfaces were lined by distinct lateral, frontal and laterofrontal ciliary tracts. At release the juvenile foot was well developed and exhibited differentiation in external morphology. Lateral dorsal foot surfaces were covered with a microvillar epithelium; whereas, ventral epithelial surfaces were covered with dense elongate simple cilia. By 8 weeks, the byssus complex was fully formed. Byssus glands had undergone extensive diverticulation and a ciliated groove (the gland opening), had formed along the ventral length of the foot. There was no evidence that a byssal thread had been elaborated by any of the juveniles examined.
INTRODUCTION

The Upper Mississippi River (UMR) supports one of the most diverse mussel faunas in North America. Thiel (1981) identified approximately 30 species from Pools 3-11 of the UMR and Miller (1988) reported 23 unionid species associated with wing dams in Pool 7. The diversity, abundance and distribution of unionid mussels are currently threatened by commercial harvest, pollution, commercial navigation, impoundments, hydraulic dredging, siltation and sedimentation (Fuller 1974; Perry 1979; Thiel 1981; Miller 1988). Freshwater mussels may be especially sensitive to habitat alterations because of their complex life cycles, benthic orientation and filter-feeding behavior.

The freshwater mussel, *Lampsilis ventricosa*, occurs throughout the UMR in main channel border areas (Holland-Bartels and Kammer 1989). Adults are long-term (bradytictic) breeders (Coker et al. 1921). Females release mature glochidia in the spring or early summer. The parasitic glochidium must contact and encapsulate on the gills of a suitable fish host, primarily centrarchid and percid species (Fuller 1974), in order to metamorphose (transform) to the juvenile stage. After several weeks, juveniles excyst from their fish host and fall to the bottom sediments. At this time, the juvenile is equipped with a protractile foot, rudimentary gills, sensory structures and definitive digestive organs. Glochidia and juveniles may represent vulnerable stages in the life cycle. Successful recruitment into the adult population is dependent on glochidia attaching to appropriate fish hosts and release of juveniles into suitable habitats. Early life history and habitat requirements of juvenile *L. ventricosa* are essentially unknown. Unfortunately, this is also true for most UMR unionid species (Neves and Widlak 1987).

There are few embryological and basic developmental studies dealing with freshwater unionids. Ontogenetic studies are limited to Lillie's (1895) detailed description of embryogenesis in *Anodonta* and *Unio* (species not indicated) and descriptions of glochidial development by Harms (1909), Lefevre and Curtis (1910a, 1911) and Wood (1974). Studies on post-larval development are also rare; however, several studies dealing primarily with fish gill histopathology resulting from glochidiosis include information on juvenile morphology and anatomy (Karna and Millemann 1978; Waller and Mitchell 1989). General descriptions of juvenile growth and development following the parasitic stage can be found in the extensive studies...
performed in the early 1900's as part of the U.S. Bureau of Fisheries mussel rehabilitation and propagation program (Lefevre and Curtis 1910b; Surber 1912; Reuling 1920; Coker et al. 1921; Howard 1922; Churchill and Lewis 1924). Howard (1922) described growth of the Lake Pepin mucket (Lampsilis radiata siliquoldea) reared in field cage-culture. General descriptions of juvenile internal anatomy were included in his study. Churchill and Lewis (1924) used histological methods to describe internal morphology and feeding habits of juvenile Lampsilis anodontoides [sic], ranging in size from 0.2 mm to over 12 mm. Because photomicrographic equipment was limiting at that time, their micrographs are not of high quality.

Declining health of mussel populations in the UMR has provided an impetus for conducting studies dealing with early life history stages of unionid bivalves. Freshwater mussels represent a valuable resource in the UMR, and if the mussels are to be properly managed, more information is needed on food requirements, habitats and early growth and development of juveniles. The objective of this study was to describe post-larval growth and development of Lampsilis ventricosa in laboratory culture. Light microscopy and electron microscopy were used to describe anatomy of the digestive organs, gills, foot, nervous system and sensory organs of developing juveniles.
MATERIALS AND METHODS

Laboratory Culture

Gravid *Lampsilis ventricosa* females were collected from Pool 7 of the Upper Mississippi River (River Mile 702.5 to 714.3) by brailing and handpicking. Glochidia were flushed from the marsupium of the female using a hypodermic syringe and needle. Viability of glochidia was determined by placing a representative sample of individuals into a 1-5% saline solution. Infective glochidia responded by closing their valves. Glochidia were then used to infect largemouth bass (*Micropterus salmoides*), smallmouth bass (*M. dolomieui*) or walleye (*Stizostedion vitreum vitreum*). Infected fish were held in flow-through aquaria at 20-25 °C. Beginning approximately 20-21 days post-infection, tanks were siphoned daily and contents filtered through a 150-μm mesh screen. A dissecting microscope was used to collect juveniles from filtered contents. Juveniles were then transferred to 100 ml static culture chambers (50-100 juveniles per chamber) and fed a diet of artificially cultured phytoplankton (*Chlamydomonas, Scenedesmus, Selanastrum* and *Ulothrix*) at a concentration of approximately 300,000-500,000 cells/ml. Hudson and Isom (1984) reported additions of silt to cultures improved growth and survival of laboratory-reared unionid juveniles. Silt (approximately 5-10 grams) was added to several of the *L. ventricosa* cultures. Water and algae were replaced in culture chambers daily or occasionally every other day. Some juveniles were transferred at 3 days of age to a recirculating culture system. This system consisted of a 10-gallon aquarium equipped with a power filter to circulate the water. Juveniles were maintained in 100 ml plastic beakers (fitted with 200 μm nitex screen at sides and bottoms) which were partially submersed in the water.

Growth and Survival Data

Length (maximum anterior to posterior distance) and height (maximum dorsal to ventral distance) measurements were made on representative juveniles from each culturing experiment using a calibrated ocular micrometer on a compound microscope. Measurements are reported in micrometers.
Because juveniles were removed periodically during culturing experiments, exact survival rates could not be determined. Approximate survival rates for a specified period were computed based on the formula: \( \frac{\text{number of juveniles recovered alive}}{\text{total number juveniles recovered} - \text{number of juveniles removed for microscopy}} \).

**Light and Electron Microscopy**

Juveniles were processed for light microscopy (LM), transmission electron microscopy (TEM) and scanning electron microscopy (SEM) at the following stages: 1) encapsulated (immediately prior to release); 2) 12-h to 2-d post-drop off; 3) 7- to 14-d post-drop off; 4) 21- to 28-d post-drop off and 5) 56-d post-drop off.

Specimens for LM, TEM and SEM were fixed in one of three fixatives: (1) 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate with 5.5% sucrose and 2 mM calcium chloride added, pH 7.2; (2) 2% paraformaldehyde, 3% glutaraldehyde in 0.2 M cacodylate buffer and (3) 3% glutaraldehyde in 0.067 M Sorenson's phosphate buffer with 5% sucrose added, pH 7.4.

After fixation, specimens for LM and TEM were washed in their respective buffers, and post-fixed in 1% osmium tetroxide (in appropriate buffer) for 1-2 hours. Specimens were dehydrated in a graded series of ethanols and embedded in Spurr's resin. Specimen blocks were sectioned with glass or diamond knives on a Reichert ultramicrotome. For LM, thick sections (0.5-1.0 \( \mu \text{m} \)) were cut and stained in either azure II/methylene blue or 1% toluidine blue in 1% borax (Dawes 1979). For TEM, thin sections (70-90 nm) were cut, stained with lead citrate and uranyl acetate (Dawes 1979) and examined with a JEOL 1200EX scanning/transmission electron microscope (STEM) operating at 80 kV.

Two additional treatments were used to alleviate problems from poor fixative infiltration and poor sectioning due to the hard calcified shell of juveniles. To improve fixation, juveniles were relaxed in an approximate 1:2 solution of 10% magnesium sulfate or magnesium chloride and water (modified from Hodgson and Burke 1988). Specimens were considered sufficiently relaxed when they did not respond to gentle prodding with a probe. To improve sectioning, juveniles were decalcified in 1-2% ascorbic acid following post-fixation (modified from Dietrich and Fontaine 1975). Ascorbic acid treatments ranged from 1 hour for thin-shelled 0- to 7-d juveniles to 8-10 hours for 56-d juveniles.
Specimens to be examined using SEM were rinsed in appropriate buffer, dehydrated in a graded ethanol series and critical-point dried in CO₂ or air-dried following treatment with hexamethyldisilazane (Nation 1983), which gave comparable results. Dried specimens were mounted on disks with silver tape and paint, sputter coated with gold-palladium and examined with a JEOL JSM-35 scanning electron microscope operated at 15 kV and 60-100 μA.
RESULTS

Encapsulated Juvenile (Figs. 1-10)

By 20-21 days post-infestation, encapsulated juveniles had nearly completed metamorphosis and almost all glochidial structures had been replaced by definitive juvenile organs (Figs. 1-9). Histopathogenesis associated with *L. ventricosa* infestations are shown in Figures 1-2 and in the Appendix (Figs. 66-70).

Presumptive digestive organs consisted of an anterior esophagus, stomach, intestine and accessory digestive glands (Figs. 2-5). The stomach was essentially undifferentiated, and the crystalline style was lacking. Paired digestive glands were located laterally and slightly dorsal to, and in close association with the stomach. Numerous lipid bodies ranging from 0.93-2.67 μm in diameter were concentrated within the digestive glands (Figs. 4, 7).

The foot (Figs. 1-3) was visible in the mid-section of the mantle cavity and was attached to the visceral mass dorsally. Ventral surfaces of the foot were covered with long simple cilia; whereas, lateral and dorsal muscular surfaces were convoluted and covered with a microvillar epithelium (Figs. 1-3, 8). Foot retractor muscles were also well developed (Fig. 8).

Three gill papillae (or filaments) occupied the posterior region of the mantle cavity (Figs. 5-6). These paired organs were located on each side of the foot and attached at their dorsal ends to the visceral mass (Fig. 5). Eventually, these will form the adult ctenidia of the inner gill (refer to Appendix, Figs. 62-65). Simple cilia lined the areas between the filaments (Figs. 6, 9).

The nervous system was well developed and all necessary ganglia required for controlling activities of the juvenile following release were present. These included: (1) paired pedal ganglia, located ventral to the stomach and in the dorsal regions of the foot (Fig. 1); (2) cerebropleural ganglia, located anteriorly, lateral to the esophagus (Fig. 3) and (3) visceral ganglion, located posteriorly, ventral to the posterior adductor (Fig. 3).

The statocyst anlagen was distinguishable and located dorsal to the pedal ganglia (Fig. 3). Cells bordering the narrow statocyst lumen were cuboidal and unciliated. The statolith had not been secreted.
Both the glochidial and definitive mantle were evident in light micrographs of encapsulated juveniles (Figs. 1, 3, 5). Autophagosomes, containing what appeared to be cellular debris, were evident in transmission electron micrographs of the larval mantle (Fig. 10). Lysosome-like bodies were also present in these cells (Fig. 10). This observation lends support to the hypothesis that the larval mantle has an reabsorptive role in juvenile metamorphosis.

Juvenile: 12-h to 2-d (Figs. 11-19)

Juvenile transformation rates in the laboratory were largely dependent on water temperatures, with warmer temperatures accelerating juvenile metamorphosis and drop off. Approximately 22 days were required for juvenile transformations at water temperatures of 22-23 °C. There was little variation in size of juveniles at drop off, with juveniles averaging 227 μm in length (Table 1). At this stage, all organs necessary for maintaining equilibrium, locomotion and feeding were present (Fig. 11).

Important ontogenetic changes occurred in the digestive system which would allow juveniles to begin ingesting and processing phytoplankton (Figs. 12-16). Gastric differentiation was complete with a slight constriction of the median portion of the stomach producing a posterior style sac stomach and an anterior gastric stomach into which enter the esophagus and digestive gland ducts (Figs. 13, 15, 16). Gastric differentiation coincided with development of the labial palps, anterior gastric ciliation, anterior intestinal ciliation, and formation of the gastric shield along the dorsal wall of the style sac (Fig. 16). The style, a transparent noncellular rod-shaped structure, was secreted and rotated (as visible in living specimens) by the dense short cilia of the style sac (Fig. 16). Rotation of food in the stomach was observed in living specimens. The intestine had been extended in length and now formed a looped structure (Fig. 16). The intestine opened as an anus on the side of the posterior adductor muscle. The digestive glands were unchanged from those observed in encapsulated forms and contained endogenous lipid bodies which filled large portions of the glands (Fig. 14).

The foot was well developed at metamorphosis (Figs. 11-13). By at least the first day following drop off, healthy juveniles became very active and were observed crawling on the substrate of culture chambers. The distinct morphological differences between lateral dorsal microvillar surfaces and ventral ciliated surfaces of the foot were best observed with SEM (Figs. 17-18).
Table 1. Shell growth of *Lampsilis ventricosa* in laboratory culture

<table>
<thead>
<tr>
<th>Juvenile Age</th>
<th>n</th>
<th>Length (μm) Mean±SD (min-max)</th>
<th>Height (μm) Mean±SD (min-max)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>Fed</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12-24 hours</td>
<td>91</td>
<td>227 ± 16 (196-269)</td>
<td>253 ± 12 (207-279)</td>
</tr>
<tr>
<td>2 days</td>
<td>20</td>
<td>247 ± 12 (233-269)</td>
<td>247 ± 12 (233-269)</td>
</tr>
<tr>
<td>7 days</td>
<td>122</td>
<td>339 ± 26 (269-414)</td>
<td>306 ± 22 (259-362)</td>
</tr>
<tr>
<td>14 days</td>
<td>23</td>
<td>388 ± 37 (321-455)</td>
<td>347 ± 37 (285-440)</td>
</tr>
<tr>
<td>21 days</td>
<td>29</td>
<td>418 ± 49 (310-507)</td>
<td>367 ± 41 (310-466)</td>
</tr>
<tr>
<td>28 days</td>
<td>16</td>
<td>527 ± 33 (466-569)</td>
<td>465 ± 46 (393-538)</td>
</tr>
<tr>
<td>35 days</td>
<td>10</td>
<td>726 ± 105 (590-938)</td>
<td>604 ± 80 (509-737)</td>
</tr>
<tr>
<td>42 days</td>
<td>11</td>
<td>795 ± 128 (536-992)</td>
<td>675 ± 125 (536-804)</td>
</tr>
<tr>
<td>48 days</td>
<td>5</td>
<td>841 ± 160 (589-1018)</td>
<td>713 ± 116 (563-884)</td>
</tr>
<tr>
<td>56 days</td>
<td>9</td>
<td>889 ± 162 (670-1139)</td>
<td>770 ± 130 (603-1005)</td>
</tr>
<tr>
<td><strong>Starved</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 day</td>
<td>5</td>
<td>231 ± 5 (228-238)</td>
<td>238 ± 6 (233-248)</td>
</tr>
<tr>
<td>3 days</td>
<td>5</td>
<td>293 ± 25 (264-316)</td>
<td>291 ± 11 (279-305)</td>
</tr>
<tr>
<td>7 days</td>
<td>5</td>
<td>297 ± 20 (279-321)</td>
<td>281 ± 23 (259-310)</td>
</tr>
<tr>
<td>14 days</td>
<td>1</td>
<td>310</td>
<td>310</td>
</tr>
</tbody>
</table>
The gill papillae remained rudimentary. Gills were covered with frontal and lateral cilia, and both were compressed against the papillae surfaces in what may be either a nonfeeding state or the result of fixation (Fig. 19). Although not evident in SEM micrographs, examination of LM sections indicated that laterofrontal cilia had also formed at this time (Fig. 13). Distal ends of adjacent papillae were connected by a ciliary membrane (Fig. 19).

Paired statocysts were fully formed at juvenile release (Figs. 12-13). In living juveniles it was possible to see the wall of the statocyst and, within this, the single statolith (Fig. 11). The statocysts function as gravity sensors and were essential if the juvenile was to orient its crawling with respect to gravity.

Also by this time, the renopericardial anlagen has given rise to a laterally elongated kidney and the pericardium, located posterior to the style sac (Fig. 15). When living specimens were examined from a dorsal aspect, the rhythmic contractions of the heart were apparent.

Juvenile: 7- to 14-d (Figs. 20-34)

By the seventh day following release, juveniles averaged 339 μm in length and by day 14 reached maximum lengths up to 455 μm (mean = 388 μm) (Table 1). There were no major changes in the morphology of the stomach or intestinal tract of juveniles (Figs. 20-23, 25, 27). The gastric and style sac regions of the stomach retained their same positions (Figs. 20-21); however, sorting regions of the gastric stomach appeared more specialized (Figs. 21, 23). Dense ciliation of the anterior intestine now reached the rectum. These cilia apparently functioned in transporting rejected food items along the intestine for expulsion from the anus (Figs. 20, 21).

The most significant change to the digestive system was a substantial loss of lipids from digestive glands. This loss may have resulted in an increase in the diameter of the digestive gland lumen and a thinning of the gland wall (Fig. 24). After the lipid loss, digestive glands more closely resembled those of adult bivalves (although adult glands are diverticulated). Two cell types were most common: basophil cells (Sumner 1966a, 1966b), with prominent dark nuclei, and digestive cells, the absorptive cells (Fig. 24).

The absence of lipids from digestive glands correlated with the sudden increase in mortality of laboratory-reared juveniles. Approximately 20-30% of juveniles survived beyond this period. Better growth of fed versus starved juveniles indicated that phytoplankton was supplying at least some of the nutritional requirements of
juveniles (Table 1). Variable sizes of algal cells were observed in stomachs, ranging from 2-3 μm in maximum diameter for \textit{Chlamydomonas} cells to approximately 8-10 μm for \textit{Selenastrum} cells (Figs. 21, 23). Both large and small cells were observed in the intestine and rectum (Figs. 20, 22, 27).

Musculature was more pronounced in older juveniles than in recently released juveniles. Anterior retractor muscles extended from their origin, posterior to the anterior adductor muscle and cerebropleural ganglion, to the forward dorsal edge of the foot (Figs. 20, 27). Conversely, posterior retractors originated anterior to the posterior adductor (Fig. 22) and extended to the trailing dorsal edge of the foot, ventral to the pedal ganglia and statocyst (Figs. 20, 25, 27-28). Insertions of the pedal retractors into the foot, ventral to the sytle sac, formed a characteristic cross-hatch of fibers (Fig. 20). Adductor muscles appeared to reach their definitive shapes; the posterior adductor was oblong (Fig. 25) and the anterior adductor was spherical.

The separate pedal ganglia had fused to form one large organ, approximately 45 μm in length (Figs 25-26, 28). The fused organ directly underlay the statocysts. The pedal ganglion medulla appeared to be a fiber tract network and the cortex a connective tissue sheath (Fig. 26). Commissures joining ganglia were difficult to trace. Figures 27-28 show the morphology of juvenile statocysts. Each statocyst was composed of a thin-walled sac, roughly spherical and approximately 26 μm in diameter (Fig. 28). The wall of the sac was formed from a single layer of cells of two types: ciliated cells (or hair cells) and nonciliated cells (Fig. 28). Within each statocyst was a single translucent unattached spherical statolith, approximately 5 μm in diameter (Fig. 28).

There was no further addition of filaments to the juvenile gills (Fig. 29); however, arrangement of ciliature was more typical of adult lamellibranch gills (Owen 1978; Way et al. 1989). Distinct lateral, frontal and laterofrontal tracts covered proximal gill surfaces (Figs. 30-34). The laterofrontal cirri were elongate and composed of cilia arranged in two parallel rows (Figs. 30-34). Individual cilia bended from the main axis at regular intervals along the length of the cirrus (Fig. 34).

Juvenile: 21- to 28-d (Figs. 35-44)

Juvenile \textit{L. ventricosa} achieved lengths of approximately 0.4 mm at 3 weeks and 0.5 mm at 4 weeks of age (Table 1). This represents average increases in length of 84% and 132%, respectively, compared to 12- to 24-h juveniles.
The digestive organs appeared to increase in size proportionately with that of the shell; the length of the stomach was nearly one-half of the shell length (Fig. 35). Even at 4 weeks, the gastric stomach and style sac retained their early postmetamorphic positions (Fig. 35). There was continued elongation of the intestine and the intestinal loop extended farther into the pedal region (Fig. 36). Diverticulation of digestive glands had occurred, producing major segments which extended dorsally, and especially ventrally (Figs. 35-37). These segments now completely surrounded the esophagus and gastric stomach.

Abundant algae and protozoans were observed in stomachs of juveniles (Figs. 35-38). Transmission electron micrographs of the stomach revealed two distinct structures, both of unknown origin, composition and function. First, several spherical granules (approximately 1 μm in length) were observed in the stomach lumen (Fig. 38). These granules consisted of concentric layers surrounding a dense core and resembled calcareous concretions (Pynnonen et al. 1987). Second, numerous crystals also were observed dispersed throughout the stomach lumen (Fig. 38). The crystals ranged from 1.7 to 2.4 μm (mean = 2.03 μm) in maximum diameter.

External foot morphology was essentially unchanged. Figure 35 shows the dense ciliation of the anterior ventral foot surfaces of a 21-d L. ventricosa juvenile. Food particles entrapped within the cilia appeared to be transported by the cilia towards the mouth.

The mantle and mantle cavity were well developed (Fig. 39). The inner mantle epithelium contained characteristic columnar cells covered with a microvillar surface (Figs. 39-41). Cells of this mantle epithelium were joined by well-defined junctions which consisted of a zonula adhaerens and a septate junction (Figs. 40-41). The septate junction was composed of parallel cell membranes, approximately 2-3 nm apart, with septa traversing the intercellular space (Figs. 40-41). Numerous blood cells were evident in the hemocoel (Fig. 39). Some of these cells resembled granulocytes described by Bubel (1989) for Mytilus edulis (Fig. 39).

The most notable morphological development at this stage was the formation of new gill papillae. New papillae were added from the posterior end during juvenile growth. As new filaments were added, the more anterior filaments increased in length. The rate at which individuals added gill papillae was highly variable. At 21 days, there was a minimum of 4 pairs of gill papillae. By 28 days, from 5-10 pairs were present. Ciliation of proximal gill surfaces was identical to that described for 7-d juveniles.
Scanning electron micrographs of the distal edge of the gill filament (dorsal to the food groove) showed a reduction in the laterofrontal cirri and presence of numerous mucous balls (Fig. 42). Abfrontal medial gill surfaces were composed of unciliated cells (Figs. 43-44). Their cell borders culminated in what appeared to be gland cell (possibly mucocytes) openings (Figs. 43-44).

Juvenile: 56-d (Figs. 45-61)

The number of juveniles that survived to this developmental stage was low (with survival of 6% and 7% in the two best culturing trials). Mean shell length of juveniles was 889 μm. The largest juvenile was over 1 mm in length, which is approximately a 400% increase in length when compared to recently excysted juveniles. The shell was darkly pigmented and no longer transparent; therefore, direct examination of internal organs was no longer possible. External shell surfaces were also heavily coated with decaying algae and detritus.

Major developments in the digestive system had occurred. Most notable was a shift in alignment of the primary digestive organs. The esophagus and gastric stomach retained their original position; however, the style sac had turned ventrally (Figs. 45-47). From this point on in the developmental process, the style sac could be considered a structure separate from the stomach, because it did not contain food (Figs. 45-46). The crystalline style was only in contact with contents of the stomach at its dorsal margin (Figs. 45-46).

Both digestive glands now had numerous diverticula which projected ventrally into the foot, as well as, numerous dorsal diverticula (Figs. 45, 47). Digestive cells contained many food vacuoles, which are characteristic of cells performing digestive processes (Fig. 49). Abundant granular endoplasmic reticulum was also associated with the digestive gland cells of *L. ventricosa* juveniles (Fig. 50).

There was a marked increase in the complexity of the juvenile foot. Between 4 and 8 weeks of development, a deep ventral invagination in the foot had produced the byssus complex (Figs. 51-56). Byssus glands were situated in the posterior regions of the foot, immediately posterior to the pedal ganglion. Extensive diverticulation of the byssus glands had occurred (Figs. 53-55). Diverticula extended dorsally to the edge of the digestive gland tubules and ventrally to the edges of the byssal groove (Figs. 53-55). The byssus glands of juvenile *L. ventricosa* apparently open along the ventral surface of the foot into the ciliated byssal groove (Fig. 54). Secretory granules of the byssus
glands ranged in diameter from 0.8 to 1.5 µm and had a moderately electron-dense, fine granular matrix (Figs. 55-56). There was no evidence that the byssal thread had been secreted by any of the juveniles examined.

Muscular and connective tissues of the foot contained numerous concretions (Figs. 57-58). Concretion dimensions varied from about 0.5 to 1.5 µm in maximum diameter with the larger spherules clearly exhibiting concentric layers (Fig. 58).

Formation of the ctenidia, by papillary outgrowths, apparently had continued. All juveniles examined had a minimum of 8-10 gill filaments (Fig. 59). Anterior filaments were more elongate than those of younger juveniles (Fig. 59). The distal ends of each filament were expanded, and adjacent filaments were connected by ciliary bridges (Fig. 59). Proximal frontal gill ciliation was similar to that of adults (Fig. 60-61).
DISCUSSION

Growth of Laboratory-Reared Juveniles

Growth of juvenile *Lampsilis ventricosa* in the laboratory was reduced in comparison to that of similar age groups from field cage-culture. Juvenile *L. ventricosa* reared in cages in the Black River (La Crosse Co., WI) reached lengths ranging from 1.1 to 3.7 mm (mean=1.9) by 46 days and 1.5 to 3.3 mm (mean=2.4) after 80 days (Zigler, S.J. 1991, pers. comm). The largest juvenile obtained from laboratory experiments reached only the lowest size range of field-cultured juveniles.

Slower growth of laboratory-reared juveniles may have resulted from an insufficient diet. Histological sections of the juvenile digestive tract revealed abundant phytoplankton in the alimentary tract; however, it is possible that either concentration of cells was inadequate or a diversity of food items was lacking. Hudson and Isom (1984) reported two conditions that improved growth and survival of *Anodonta imbecilis* and *Dysnoma triquetra* in laboratory culture: (1) use of naturally occurring plankton cultured in the laboratory and (2) addition of silt to cultures. Addition of silt to our cultures did not greatly improve growth or survival of juvenile *L. ventricosa*. Further investigations of optimal physical and chemical culture conditions, in addition to nutritional requirements, are needed to develop algal diets and culture techniques that will support maximum growth of *L. ventricosa* juveniles in the laboratory.

Organ Development

Juveniles of *L. ventricosa* exhibited significant postmetamorphic organ development in laboratory culture. This was the first study to utilize both light and electron microscopy in descriptions of unionid juvenile development.

Digestive System

Encapsulated juveniles, approaching excystment, possessed a well developed mouth, esophagus, intestine, anus and an undifferentiated stomach. Within the first several days following juvenile release, the digestive system had undergone the necessary modifications for assimilation of algae and other food items. Most importantly, the stomach had differentiated into an anterior gastric stomach (characterized by ciliary sorting regions) and a posterior style sac stomach (characterized by a large crystalline
Further developments in the alimentary tract were minor until 56 days following drop off. At this time the alignment of organs had been altered by the ventral shift of the style sac stomach. The style sac was devoid of food and should no longer be considered a stomach. The crystalline style was in contact with food only at its dorsal end.

Digestive glands of encapsulated and recently released juveniles were filled with numerous lipid bodies. Fraser (1989) and Gallager et al. (1986) reported that energy requirements of early larval stages of marine bivalves are largely met by lipid reserves, primarily in the form of triacylglycerol (TAG). The role of endogenous lipids in development of freshwater juveniles has not been evaluated. It appears that lipids may also be an important energy source for juvenile L. ventricosa, especially during the first few weeks.

Rapid increases in juvenile mortality occurring after two weeks in laboratory culture could be correlated with the loss of lipids from digestive glands. Similar mortality patterns were reported by Hudson and Isom (1984) for the unionid species Fusconaia ebana, Ligumia recta, Pleurobema cordatum and Carunculina moesta reared in the laboratory. Histological evaluations of these species would indicate if similar losses of lipids from digestive glands occur at this critical stage.

Diverticulation of the digestive glands of L. ventricosa juveniles began at 3 weeks. After 8 weeks, digestive gland diverticula completely surrounded the stomach and projected ventrally into the foot. At this stage, glands are morphologically similar to that of adult bivalves (Sumner 1966a, 1966b; Moore et al. 1978).

The functional significance or composition of crystals in the stomach lumen of juveniles is unknown. To my knowledge, this is the first report of such structures. It is possible that the crystals may somehow be involved in formation of the crystalline style or result from style dissolution. Judd (1987) studied the protein composition of the style from 12 bivalve species using gel electrophoresis. For most species, 20-40 distinct polypeptides were distinguished (of these 4-5 were major proteins and one was not glycosylated). From his studies, Judd (1987) concluded that the style was largely composed of mucin-type glycoproteins; however, he could not reach any conclusions on how the glycoproteins combined to produce a solid secretion. Two possible explanations include: (1) the crystals are composed of glycoproteins; or (2) the glycoproteins form the organic matrix that provide nucleation sites for formation and growth of crystals.
Further ultrastructural studies, along with X-ray microanalysis and X-ray diffraction, are needed in order to determine if these crystals play any role in crystalline style formation.

**Gills**

According to Raven (1966) the typical lamelliform gills of bivalves form in two fundamentally different ways: by papillary outgrowths, as in the unionid *Anodonta*, or by folding, as in the marine bivalve *Cyclas cornea*. The papillary type was exhibited by *L. ventricosa* juveniles. Gill rudiments of encapsulated juveniles consisted of a row of three papillae on each side of the foot. Gill papillae were added to the posterior end beginning approximately 3 weeks after release. As new filaments were added, the more anterior filaments increased in length. Gill formation exhibited by *L. ventricosa* juveniles paralleled the process described by Herbers (1913) for *Anodonta* (detailed by Raven 1966). Eventually the gill filaments will unite to form the definitive inner gill; the outer gill arises later by a similar process.

Gill ciliature of encapsulated juveniles consisted of a row of dense simple cilia lining lateral papillary surfaces. By day 7, frontal gill surfaces were covered with distinct lateral, frontal and laterofrontal ciliary tracts. Laterofrontal cirri were composed of 10-14 pairs of cilia arranged in parallel rows. According to Owen (1978) the number of cilia per laterofrontal cirrus is species-specific. Abfrontal gill surfaces were unciliated.

**Foot**

External surfaces of the juvenile foot were morphologically distinct. Lateral dorsal surfaces had a microvillar epithelial covering; whereas, ventral surfaces were densely covered with long simple cilia. Fiege (1990) described similar characteristics for the foot of juvenile pteropods and suggested that the ciliated surfaces function not only in locomotion but in funneling food items toward the mouth.

By the 8th week, the byssus complex of the foot had formed. Byssus glands were diverticulated and a ciliated groove (the gland opening) had formed along the ventral length of the foot. There is little information available on the morphology, ultrastructure or histochemistry of unionid byssus glands. Studies dealing with byssus glands of marine bivalves show these glands to be very complex (Tamarin and Keller
Based on histochemical properties, Cranfield (1973) identified nine different types of gland cells in the foot of *Ostrea edulis* pediveligers. There was no evidence that a byssal thread had been elaborated by any of the juveniles examined. Churchill and Lewis (1924) reported that the byssal thread of juvenile *Lampsilis luteola* does not appear until the mussel is over 1 mm in length.

**Mantle**

During metamorphosis, the larval organs (i.e. mantle and adductor muscles) degenerated and were replaced by rudimentary juvenile organs. The larval mantle persisted longer than other larval structures. Ultrastructure of the larval mantle revealed the presence of autophagosomes similar in appearance to those occurring in the kidney of *Anodonta cygnea* (Hemelraad et al. 1990). This observation tends to support the explanation presented by Arey (1932) and Wood (1974) that the larval mantle functions in digestion of degenerating larval tissues as well as host tissues.

**Nervous and Sensory Systems**

The nervous system was fully differentiated prior to juvenile excystment. Juvenile *L. ventricosa* possessed a bilateral nervous system characterized by the following ganglia: the cerebropleural ganglia, pedal ganglia and visceral ganglion. By day 14, the paired pedal ganglia had fused to form a single structure. Commissures which joined ganglia were not traced. The pedal ganglion remained in close contact with the statocysts throughout the developmental stages. According to Raven (1966) the cerebropleural ganglia innervate molluscan statocysts.

The statocysts originated and remained in the base of the juvenile foot. According to Cragg and Nott (1977), by being in the base of the foot, statocysts are less affected by erratic movements of the shell during crawling. The statocyst anlagen was composed of cuboidal cells surrounding a very small lumen. At juvenile release, the statocyst cavity had increased in diameter, the cells of the wall had narrowed, and the statolith had formed. Cilia also formed on the surfaces of some of the epithelial cells. The presence of ciliated or hair cells is a morphological feature of proven gravity receptors (Cragg and Nott 1977). The statolith increased in size with the growth of the juvenile and exhibited concentric growth zones.
Future Studies

The lack of comparative studies is a major drawback to the study of post-larval
development in unionid mussels. Further studies of juvenile organogenesis and
development may reveal structural differences between species. To date, there are few,
if any, methods for the identification of larval and juvenile stages of closely related
species. Further knowledge of internal morphology may be especially valuable for the
identification of juveniles of endangered species that cohabit with indistinguishable
congener species.
LITERATURE CITED


Figure 1. Longitudinal section of *Lampsilis ventricosa* encapsulated in largemouth bass gill tissues. The host capsule (HC) surrounds the metamorphosing juvenile. Arrowhead indicates the mouth. Definitive organs (DM=definitive mantle; E=esophagus; F=foot; G=gill; P=pedal ganglion; S=stomach) and glochidial mantle (GM) are evident. Bar= 50 μm

Figure 2. Encapsulated juvenile approaching excystment (note thin capsule wall at arrowhead). Arrow indicates fusion and hyperplasia of fish host gill lamellae. Foot (F) and digestive gland (D) of the juvenile are apparent. Bar= 50 μm

Figure 3. Longitudinal section through digestive and sensory organs of encapsulated juvenile (C=cerebropleural ganglion; E=esophagus; F=foot; G=gill; S=statocyst anlagen; V=visceral ganglion). Bar= 50 μm

Figure 4. Detail of the digestive gland of encapsulated juvenile showing numerous lipid bodies (I). Bar= 20 μm

Figure 5. Longitudinal view of rudimentary gill papillae of an encapsulated juvenile (arrowhead indicates middle papilla) (D= digestive gland; DM=definitive mantle; GM=glochidial mantle). Bar= 50 μm

Figure 6. Detail of gill filaments showing cilia (Ci) lining the area between adjacent filaments. Bar= 20 μm
Figure 7. Transmission electron micrograph of digestive gland tissue of an encapsulated *Lampsilis ventricosa* juvenile showing concentrations of lipid bodies (L) and characteristic granular endoplasmic reticulum (G) (GL=glycogen; N=nucleus; V=vacuole). Bar= 1.5 μm

Figure 8. Transmission electron micrograph of the lateral microvillar region of the juvenile foot. Retractor muscles (RM) are evident (m=mitochondrion; MV=microvilli; X=cross-hatch fibers of retractor muscles). Bar= 3 μm
Figure 9. Transmission electron micrograph of the gill filament of an encapsulated Lampsilis ventricosa juvenile showing abundant cilia (Ci). Arrowheads indicate location of ciliary basal bodies (m=mitochondrion; N=nucleus). Bar= 1.5 μm
Figure 10. Transmission electron micrograph of the glochidial mantle of *Lampsiliis ventricosa* showing a large autophagosome with lysosome-like bodies (L) and cellular debris (CD) (G=golgi complex; GL=glycogen; M=mitochondrion; MV=micr ovilli; N=nucleus; V=vacuole; ZA=zonula adhaerens). Bar= 2 μm
Figure 11. Light micrograph of living *Lampsilis ventricosa* (1-d old). Foot (f) is orientated ventrally and is very flexible. The statocyst (s) is evident in dorsal foot regions (d=digestive gland; g=gill papillae). Bar= 100 \( \mu \)m

Figure 12. Longitudinal section through a 1-d old juvenile (not decalcified). Arrowhead indicates the statocyst and statolith (f=foot; g=gill; m=mantle; p=periostracum of shell; s=stomach; sh=shell). Bar= 40 \( \mu \)m

Figure 13. Sagittal section of a 2-d old juvenile (decalcified) showing pedal ganglia (p) ventral to statocyst (c=cerebropleural ganglion; d=digestive gland; f=foot; g=gill; h=hinge ligament; m=mantle; p=pedal ganglia; s=stomach). Arrowhead indicates position of laterofrontal cilia of the gills. Bar= 50 \( \mu \)m

Figure 14. Light micrograph of the digestive gland of a 2-d old juvenile showing large concentrations of lipid bodies (l) (f=food particles, primarily algal cells in the stomach). Bar= 20 \( \mu \)m

Figure 15. Light micrograph showing arrangement of the juvenile (2-d old) digestive system (a=anterior adductor; e=esophagus; f=foot; g=gastric stomach containing algal cells; h=heart; i=intestine; k=kidney; lp=labial palp; m=mouth; p=posterior adductor muscle; ss=style sac stomach). Bar= 50 \( \mu \)m

Figure 16. Higher magnification of Figure 15 showing ciliation of esophagus (E), gastric stomach (G) and dense cilia (c) of style sac stomach. Arrowhead indicates two algal cells embedded within the matrix of the translucent crystalline style. The gastric shield (GS) is formed along the dorsal wall of the style sac stomach (a=algal cells; H=heart). Bar= 20 \( \mu \)m
Figure 17. Scanning electron micrograph showing external view of the foot of *Lampsilis ventricosa*, 1-d old (dorsal shell surfaces are to the left). Note dense simple cilia (c) on ventral surfaces and a microvillar (mv) convoluted surface covering dorsolateral foot surfaces (s=shell; m=mantle). Bar = 10 μm

Figure 18. Higher magnification of microvillar foot epithelium. Bar = 10 μm

Figure 19. Scanning electron micrograph of two adjacent gill filaments (buds) (gb) of a 1-d old juvenile. Lateral cilia (lc) and frontal cilia (fc) are compressed against gill surfaces, possibly indicating a non-feeding state. Note ciliary bridges connecting proximal ends of adjacent filaments (arrows). Bar = 15 μm
Figure 20. Longitudinal section of a 14-d old juvenile *Lampsilis ventricosa*. Arrowhead indicates area where pedal retractor muscles cross (cs=crystalline style; d=digestive gland duct; g=gastric stomach; h=hinge ligament; i=anterior intestine; p=pedal ganglion; pa=posterior adductor muscle; r=rectum). Bar= 50 μm

Figure 21. Detail of the juvenile stomach (14-d old). The style sac portion is characterized by a large mucoprotein style (CS), short stiff cilia (C) and a dorsally located gastric shield (arrow). Algal cells, including *Chlamydomonas* (ch) and *Selanastrum* (s) are located in the anterior gastric (G) stomach. Bar= 20 μm

Figure 22. Higher magnification of the intestinal tract showing algal cells (arrows) (C=cilia of style sac; PA=posterior adductor muscle; PR=posterior retractor muscles; R=rectum). Bar= 20 μm

Figure 23. Transmission electron micrograph of the gastric stomach of *Lampsilis ventricosa* (14-d old juvenile) showing lateral bands of cilia (C) that form the ciliary sorting region of the gastric stomach (ch=Chlamydomonas; s=Selanastrum; n=nucleus of epithelial cell; v=vacuole). Bar= 5 μm

Figure 24. Cross-section through the digestive gland of a 14-d old juvenile). Basophil (B) and digestive cells (DC) are the most abundant cells (L=lumen; CS=crystalline style). Arrow indicates a cytoplasmic granule, most likely a phagolysosome or residual body. Bar= 20 μm
Figure 25. Longitudinal section through a 14-d old *Lampsilis ventricosa* (ai=anterior intestine; cs=crystalline style; F=foot; G=gills; P=pedal ganglion; PR=posterior retractor). Bar= 40 μm

Figure 26. Detail of the pedal ganglion. Paired pedal ganglia of metamorphosed juveniles have fused to form a single large organ (Ci=cilia of style sac; C=cortex; M=medulla of pedal ganglion). Bar= 15 μm

Figure 27. Longitudinal section of 14-d old juvenile showing location of statocyst (S) below style sac stomach. Arrow indicates rejected algal cells in intestinal tract (cs=crystalline style; A=anterior retractor muscles; F=foot; G=gill papillae; PR=posterior retractor muscles). Bar= 40 μm

Figure 28. Enlarged view of statocyst, located dorsal to the pedal ganglion (P). The statocyst chamber is formed from a single layer of cells (arrow), consisting of ciliated (c) and nonciliated cells. A single unattached statolith (st) is located within the chamber lumen (lu). Bar= 15 μm
Figure 29. Longitudinal section through a 14-d old *Lampsilis ventricosa* juvenile showing digestive organs and cross-sections of three gill papilla (arrowhead indicates middle papillae) (cs=crystalline style; d=digestive gland; f=foot; M=mantle; S=statozyst). Bar= 50 µm

Figure 30. Enlarged view of gills, transverse section, showing laterofrontal cirri (arrows) and simple lateral cilia (LCi) (AF=abfrontal cell; BV=branchial vein; FC=frontal cell; LC=lateral cell). Bar= 15 µm

Figure 31. Longitudinal section through gill papilla showing arrangement of laterofrontal cirri (arrow). Bar= 15 µm

Figure 32. Transmission electron micrograph of a longitudinal section through a gill papilla showing laterofrontal cirri (arrow) (BB=basal body). Note each cirrus is composed of two parallel rows of cilia. Bar= 1.5 µm
Figure 33. Scanning electron micrograph of the frontal gill surface of 7-d old *Lampsilis ventricosa* juvenile (F=frontal cilia; L=lateral cilia; LF=laterofrontal cirri). Bar= 6 μm

Figure 34. Detailed view of the frontal cilia (F) and laterofrontal cirri (LF). Note microvillar (m) gill surface and basal origins (arrowhead) of frontal cilia. Bar= 3 μm
Figure 35. Longitudinal-section of a 21-d old *Lampsilis ventricosa* juvenile showing axial relationship of the gastric stomach (G) and style sac stomach (AR=anterior retractor muscles; CG=cerebropleural ganglion; CS=crystalline style; E=esophagus; F=foot; G=gill papilla; GS=gastric shield; I=intestine; L=labial palp; M=mantle cavity; P=pericardium; PA=posterior adductor muscle; PR=posterior retractor muscles; PG=pedal ganglion). Note dorsally located diverticulum (D) of the digestive gland. Cilia (C) of the foot appear to direct food particles toward the mouth (arrow). Bar= 75 μm

Figure 36. Cross-section through a 21-d old juvenile showing portions of the intestinal (i) loop in the foot (F) (cs=crystalline style; g=gastric stomach; D=digestive gland; S=statocyst; Sh=shell; St=statolith). Bar= 40 μm

Figure 37. Oblique section through the gill papillae (G) of a 21-d old juvenile (cs=crystalline style; e=esophagus; i=intestine; D=digestive gland; F=foot; M=mantle; PA=posterior adductor muscle). Bar= 60 μm
Figure 38. Transmission electron micrograph of portions of the gastric and style sac stomach of a 21-d old *Lampsilis ventricosa*. Numerous *Chlamydomonas* (Ch) and *Selenastrum* (S) algal cells are present in the stomach lumen. Protozoans (P) also may have been ingested. The gastric shield (GS) is composed of microvilli (mv) embedded in a matrix (possibly chitinous) (mx) (Ci=cilia of ciliary sorting region). Numerous crystals (arrows) and several calcium concretion-like structures (arrowheads) are also evident in the stomach lumen. The origin and function of both of these structures is unknown. Bar= 4 μm
Figure 39. Light micrograph of the inner mantle epithelium of a 21-d old juvenile *Lampsilis ventricosa*. Arrow indicates the microvillar surface of the columnar epithelial cells. Several blood cells in the mantle hemocoel resemble granulocytes (G) of adult bivalves (SH=shell). Bar= 15 µm

Figure 40. Transmission electron micrograph showing the microvillar border (in cross-section) of the columnar epithelium (21-d old juvenile) (M=mitochondrion; MV= microvilli; arrow= zonula adhaerens). Bar= 1 µm

Figure 41. Transmission electron micrograph (composite) of the mantle epithelium. Adjacent columnar cells are joined by a zonula adhaerens (ZA) and septate junction (SJ). Microvilli (MV) are cut in longitudinal-section (CW=cell web; M=mitochondrion; MVB=multivesicular body; N=nucleus). Bar= 50 nm
Figure 42. Scanning electron micrograph of the distal edge of the gill filament of a 28-d old *Lampsilis ventricosa* juvenile. Laterofrontal cirri (LF) are reduced and mucous balls are abundant (m) (F=frontal cilia; arrow=gland cell pore). Bar= 6 μm

Figure 43. Scanning electron micrograph of the abfrontal gill surfaces. Abfrontal cells are unciliated and covered with a microvillar epithelium. Grooves (arrowheads) delineate cell boundaries which culminate in what may be gland cell openings (GC) (L=lateral cilia; LF=laterofrontal cirri). Bar= 15 μm

Figure 44. Higher magnification of gland cell opening. Bar= 6 μm
Figure 45. Cross-section through the ventral visceral mass and dorsal foot region of a 56-d old juvenile *Lampsilis ventricosa*. The style sac (SS) has shifted ventrally to its adult position (I=intestine; D=digestive diverticulum; F=foot; G=gill). Bar= 75 μm

Figure 46. Detail of style sac showing large conical crystalline style (cs), dense cilia (Ci) and dorsal gastric shield (gs) (m=muscle of foot). Bar= 35 μm

Figure 47. Cross-section through dorsal visceral mass of a 56-d old juvenile showing ciliary sorting (CS) regions of the stomach. The digestive gland exhibits numerous diverticula (D). Bar= 75 μm

Figure 48. Detail of ciliary sorting region of the stomach (56-d old juvenile) (A=algal cells; Ci=cilia; De=debris attached to outer shell; E=columnar cells of epithelium). Bar= 15 μm
Figure 49. Transmission electron micrograph of the digestive cells of the digestive gland of a 56-d old *Lampsilis ventricosa*. Note abundance of digestive vacuoles (DV). Bar= 75 nm

Figure 50. Transmission electron micrograph of the cytoplasm surrounding digestive vacuoles occupied by well developed granular endoplasmic reticulum (GER) (M=mitochondria). Bar= 50 nm
Figure 51. Scanning electron micrograph of lateral and ventral surfaces of the foot of *Lampsilis ventricosa* (56-d old). Note dense ciliation of the foot with medial byssal groove (BG) (MV=microvillar foot surface; SH=shell). Bar= 50 μm

Figure 52. Cross-section of the foot showing paired statocyst (S) chambers located dorsal to the pedal ganglion (P). Arrow indicates the statolith. The ventral foot surfaces are covered by dense cilia (C) (D=digestive gland). Bar= 40 μm

Figure 53. Light micrograph of a major byssus duct (B) in cross-section (n=nucleus of digestive gland basophil cell; BGD=byssus gland diverticulum; D=digestive gland). Bar= 15 μm

Figure 54. Longitudinal section through the ciliated byssal groove (BGD=byssus gland diverticulum; D=digestive gland). Bar= 15 μm
Figure 55. Light micrograph showing numerous secretory granules of the byssus gland of *Lampilis ventricosa* (56-d old). Bar= 30 μm

Figure 56. Transmission electron micrograph of secretory granules (SG) of the byssus gland (N=nucleus of gland cell). Bar= 1 μm

Figure 57. Transmission electron micrograph of concretions (arrowheads) from the foot (56-d old juvenile) (m=mitochondrion; CT=connective tissue; M=muscle). Bar= 1 μm

Figure 58. Transmission electron micrograph of a large concretion (arrowhead) showing concentric layers (M=muscle; N=nucleus). Bar= 75 nm
Figure 59. Scanning electron micrograph of a juvenile *Lampilis ventricosa* (56-d old) with portions of the shell (SH) removed to show gill papillae. New papillae were added posteriorly (arrow). At the same time anterior papillae became elongated (F=foot). Bar= 50 μm

Figure 60. Scanning electron micrograph of the proximal frontal gill surfaces (m=mucous ball; F=frontal cilia; L=lateral cilia; LF=laterofrontal cirrus). Bar= 15 μm

Figure 61. Higher magnification of laterofrontal cirri. Each cirrus is fully extended and individual cilia (arrowheads) that compose the cirrus are apparent (F=frontal cilia). Bar= 5 μm
APPENDIX
Figure 62. Scanning electron micrograph of the posterior region of a *Lampsilis radiata siliculoidea* juvenile (80-d old, approximately 3 mm in length) reared in field cage-culture (B=byssal thread; IG=inner gill; OG=outer gill). Bar=0.5 mm

Figure 63. Scanning electron micrograph of the inner gill filaments. Bar= 75 μm

Figure 64. Higher magnification of the proximal gill ciliation (F=frontal cilia; LF=laterofrontal cirri). Bar= 10 μm

Figure 65. Higher magnification of the distal (near the food groove) gill ciliation. Bar= 20 μm
Figures 66-70. Light and transmission electron micrographs of the histopathogenesis associated with infestations of *Lampsilis ventricosa* glochidia on the gills of largemouth bass (*Micropterus salmoides*).

Figure 66. Light micrograph showing fusion and hyperplasia of gill filaments surrounding encapsulated glochidium. Numerous eosinophils invaded affected tissues. Bar= 15 μm

Figure 67. Light micrograph showing proliferation of mucous cells (arrow) in host capsule. Bar= 20 μm

Figure 68. Transmission electron micrograph of host mucous cell. Bar= 3 μm

Figure 69. Transmission electron micrograph of host debris cell. Bar= 1 μm

Figure 70. Transmission electron micrograph of host chloride cell. Proliferation of chloride cells was also observed. Bar= 2 μm
SECTION 3.

EFFECTS OF CADMIUM ON LARVAL AND JUVENILE LAMPSILIS VENTRICOSA
(BIVALVIA: UNIONIDAE) FROM THE UPPER MISSISSIPPI RIVER
Effects of cadmium on larval and juvenile *Lampsilis ventricosa* (Bivalvia: Unionidae) from the Upper Mississippi River

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ABSTRACT

The declining health of mussel populations in the Upper Mississippi River suggests that habitat deterioration, including presence of metal contaminants, may be having a serious impact. There are few reports of metal toxicity in larval and juvenile unionid bivalves. Additionally, there are apparently no published reports of growth or histopathological effects from metal exposures. The acute toxicities (48-h) of cadmium in laboratory-reared larval and juvenile *L. ventricosa* were determined. Based on LC50's and EC50's (derived from stressed and dead juveniles), early juvenile stages (recently excysted and 7-d old) were more sensitive than older juveniles (14-d old). Glochidia were very tolerant to cadmium exposures; the LC50 was approximately three times greater than that for any of the juvenile stages. Growth and histopathological effects of cadmium on juvenile *L. ventricosa* were evaluated using a 7-d static renewal test. Morphometric analysis of external shell parameters revealed exposure to 10 μg Cd/L for 7-d significantly reduced anterior shell growth. Histopathological alterations (atrophy, increased vacuolization and tissue separations) appeared more severe for mantle, ganglia and digestive gland tissues as compared to foot and muscle tissues. Exposures to 30-100 μg Cd/L resulted in dissolution of the crystalline style in several juveniles. Histological examinations also indicated that juvenile feeding was reduced, lipid catabolism was altered and mucous hypersecretion may have occurred.
INTRODUCTION

Reductions in populations and species diversity of mussels in the Upper Mississippi River (UMR) suggest that habitat deterioration, including pollution, may be seriously impacting mussel populations (Thiel 1981; Duncan and Thiel 1983). The life cycles of unionid mussels are complex and usually include an obligate parasitic stage (the glochidium). Glochidia must encapsulate, with few exceptions, on an appropriate fish host in order to metamorphose (transform) into free-living juveniles. There is little information on the sensitivity of different life stages of unionid bivalves to chemical contaminants.

Adult mussels have been used in a wide variety of contaminant studies, including biomonitoring, bioaccumulation, lethality and histopathology studies (Anderson 1977; Heit et al. 1980; Robinson and Ryan 1986; Herwig et al. 1989; Lowe and Clarke 1989; Hemelraad et al. 1990a, 1990b, 1990c); whereas juveniles have been used almost exclusively in lethality studies (Harrison et al. 1984; Ringwood 1990; Keller and Zam 1991). Until recently, studies with young unionid freshwater juveniles were difficult to conduct because of their small size at metamorphosis (approximately 90-400 μm in length) and problems associated with maintaining and rearing juveniles in the laboratory. Advances in laboratory culture and the use of artificial media to transform glochidia have increased the availability of juvenile freshwater mussels for use in contaminant studies (Isom and Hudson 1982; Hudson and Isom 1984; Keller and Zam 1990; Wade 1989, 1990).

When acute toxicities are used as a measure of relative sensitivity, it has generally been found that embryonic and larval stages of marine bivalves are more sensitive to metal contaminants than either juvenile or adult stages. Ringwood (1990) reported embryo and larval stages of the marine bivalve * Isochrysis galbana* were more sensitive to cadmium than juveniles and adults the least sensitive. Unlike marine bivalves, freshwater unionids brood embryos to the glochidial stage within specialized gill chambers called marsupia. The juvenile represents the first free-living stage in the life cycle and, therefore, may be the stage most sensitive to metal contaminants.

Cadmium is one of the principle heavy metals found in industrial and municipal discharges into the Mississippi River (Rada et al. 1990). The relatively high toxicity of cadmium and its expanding use have heightened concern regarding its effect on aquatic
ecosystems. Based on the chemical's toxicity and exposure potential, cadmium was ranked second on a priority list of 17 chemicals targeted by the U. S. Environmental Protection Agency (EPA) as "toxic enemies" (Science News 1991).

The purpose of this study was to: (1) determine relative sensitivities of larval and juvenile *Lampsilis ventricosa* to cadmium and (2) determine growth, histopathogenic and ultrastructural effects of cadmium on transformed juveniles.
MATERIALS AND METHODS

Experimental Animals

Gravid female *Lampsilis ventricosa* were hand-collected from Pool 7 (River Mile 702.5 to 714.3) of the UMR near Dresbach, MN. Infective glochidia were flushed from the marsupium and used to infect smallmouth bass (*Micropterus dolomieui*) and largemouth bass (*M. salmoides*). Infected fish were held in flow-through aquaria at 20-25 °C. Beginning approximately 20-21 days post-infection, fish tanks were siphoned daily and contents filtered through a 150 µm mesh screen. A dissecting microscope was used to collect released juveniles from filtered contents. Juveniles were maintained in 100 ml static aerated culturing chambers and fed a daily diet of laboratory cultured phytoplankton at a concentration of approximately 300,000-500,000 cells/ml.

Acute Toxicity Tests

**Test design**

Larval and juvenile mussels, 0-d (0-12 hours post-drop off), 7-d and 14-d old, were exposed to 5 cadmium concentrations (administered as CdCl₂) and a control. Cadmium concentrations for each stage were selected based on preliminary range-finding tests. All treatments were conducted in triplicate, with ten juveniles per replicate, for 48 hours. The mussels were not fed during experiments and all experiments were conducted in covered and aerated 250 ml crystallizing dishes under total darkness.

**Test water**

Dissolved oxygen (YSI Model 51B dissolved oxygen meter coupled to YSI Model 5739 dissolved oxygen probe), temperature, pH (Altex pH meter), conductivity (Hanna Model H18033 conductivity meter), alkalinity (titrimetric method, APHA et al. 1990), and hardness (titrimetric EDTA method, APHA et al. 1990) were measured for each replicate daily. Physical and chemical properties of treatment solutions are presented in Table 1.

Water samples (50 ml) for cadmium analyses were taken at the start and end of each experiment. Samples were promptly acidified with 16 N HNO₃ (Ultrex®, J. T.
Table 1. Physical and chemical properties of test solutions for acute and subchronic cadmium toxicity tests

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Unit</th>
<th>48-h Toxicity Tests Mean (±SD)</th>
<th>7-d Toxicity Test Mean (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water temperature</td>
<td>°C</td>
<td>21.4 (±0.5)</td>
<td>21.7 (±0.4)</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td>8.4 (±0.1)</td>
<td>8.3 (±0.2)</td>
</tr>
<tr>
<td>Dissolved oxygen</td>
<td>mg/L</td>
<td>8.4 (±0.1)</td>
<td>8.3 (±0.1)</td>
</tr>
<tr>
<td>Total hardness</td>
<td>mg/L as CaCO₃</td>
<td>149 (±6.3)</td>
<td>145 (±4.0)</td>
</tr>
<tr>
<td>Total alkalinity</td>
<td>mg/L as CaCO₃</td>
<td>108 (±13.9)</td>
<td>109 (±3.0)</td>
</tr>
<tr>
<td>Conductivity</td>
<td>µmhos/cm</td>
<td>310 (±12.9)</td>
<td>304 (±12.2)</td>
</tr>
</tbody>
</table>

Baker Chemical Co.) to pH<2.0 and total cadmium was determined by flame atomization (direct aspiration) with an Instrument Laboratory Model 551 atomic absorption spectrophotometer.

Quality assurance

Equipment, sample containers and glassware were washed with Liquinox® detergent, rinsed in tap water, soaked for at least 12 hours in 8 N HNO₃ and rinsed in deionized water. The mean recovery of cadmium from eleven spiked water samples was 100% (range 91-107%). The relative standard deviation from analyses of triplicate water subsamples for cadmium averaged 1.29% (range 0.4-3.6%).

Test endpoints

At 48-h, juveniles and glochidia were removed from exposure dishes and examined with a compound microscope. Juvenile condition was assessed as: (1) alive = active and moving; (2) stressed = no foot movement, but ciliary action evident and (3) dead = no foot or ciliary movement, or empty valves. Glochidia condition was assessed as: (1) alive = exhibited shell closure when exposed to 1-5% NaCl and (2) dead = no shell
closure when exposed to 1-5% NaCl. Probit analysis (Spearman-Karber method, Hamilton et al. 1977) was used to calculate the 48-h LC50 and EC50 (for juveniles only) and their 95% confidence interval for each test. The EC50 concentration was based on the total number of dead and stressed juveniles. Because there was no appreciable loss of cadmium during the 48-h exposures, LC50's and EC50's were based on cadmium concentrations determined at the end of experiments. Nominal and measured cadmium concentrations (μg Cd/L) for each experiment are presented in Table 2.

**Subchronic Toxicity Test**

A 7-d static renewal toxicity test was conducted using 0-d old *L. ventricosa*. Juveniles were exposed to four nominal cadmium concentrations (10, 30, 50, and 100 μg/L) and a cadmium control. Treatments were conducted in duplicate with 50 juveniles

Table 2. Nominal and measured cadmium concentrations (mean of three replicates) used in 48-h toxicity tests with larval and juvenile *Lampsilla ventricosa*

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Control</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glochidia:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nominal</td>
<td>0</td>
<td>100.0</td>
<td>250.0</td>
<td>350.0</td>
<td>500.0</td>
<td>1000.0</td>
</tr>
<tr>
<td>Measured</td>
<td>0</td>
<td>97.8</td>
<td>245.5</td>
<td>351.4</td>
<td>515.6</td>
<td>1021.8</td>
</tr>
<tr>
<td>0-d Juvenile:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nominal</td>
<td>0</td>
<td>50.0</td>
<td>100.0</td>
<td>250.0</td>
<td>500.0</td>
<td>1000.0</td>
</tr>
<tr>
<td>Measured</td>
<td>0</td>
<td>48.5</td>
<td>92.4</td>
<td>237.5</td>
<td>482.5</td>
<td>960.2</td>
</tr>
<tr>
<td>7-d Juvenile:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nominal</td>
<td>0</td>
<td>100.0</td>
<td>250.0</td>
<td>350.0</td>
<td>500.0</td>
<td>1000.0</td>
</tr>
<tr>
<td>Measured</td>
<td>0</td>
<td>71.5</td>
<td>222.6</td>
<td>324.2</td>
<td>485.6</td>
<td>967.8</td>
</tr>
<tr>
<td>14-d Juvenile:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nominal</td>
<td>0</td>
<td>100.0</td>
<td>250.0</td>
<td>500.0</td>
<td>1000.0</td>
<td>1500.0</td>
</tr>
<tr>
<td>Measured</td>
<td>0</td>
<td>101.8</td>
<td>241.1</td>
<td>489.4</td>
<td>977.0</td>
<td>1482.2</td>
</tr>
</tbody>
</table>

*Treatments.*
per replicate. Every 24 hours, treatment solutions were replaced and juveniles fed a diet of laboratory cultured phytoplankton. Dissolved oxygen, temperature, pH, alkalinity, hardness and conductivity were measured (same methods as used for acute toxicity tests) for each replicate daily (Table 1). Juveniles were removed at 1, 4 and 7 days. At these intervals, the condition (same categories used for acute toxicity tests) of juveniles was assessed, and ten juveniles were measured (length and height) from each replicate using a calibrated ocular micrometer on a compound microscope. Representative juveniles (approximately 5 per replicate) were also prepared for light and electron microscopy at 1 and 4 days. All juveniles still alive at 7 days were processed for microscopy and morphometric analysis.

External morphological measurements of the shell of 7-d old juveniles were determined using an optical imaging system (Biosonics, Inc.), calibrated in micrometers and linked to an Olympus microscope. Specimens were prepared by fixation in 10% neutral buffered formalin (15 minutes) and stored in 70% ethanol. Because the juvenile shell is formed along the periphery of the larval shell, it was possible to make measurements of both the glochidial and juvenile (postmetamorphic) shells. Parameters measured included: juvenile shell length (maximum anterior to posterior distance) and height (maximum dorsal to ventral distance); glochidial shell length and height; anterior and posterior juvenile shell length; glochidial shell area and juvenile shell area. Morphometric data were statistically analyzed by one-way analysis of variance (ANOVA) (SAS Institute, Inc. 1985). Null hypotheses were rejected at P<0.05.

Juveniles prepared for light microscopy were relaxed in an approximate 1:2 solution of 10% magnesium sulfate and water, fixed in Karnovsky's fixative (2.5% glutaraldehyde, 2.0% paraformaldehyde in 0.067 M Sorenson's phosphate buffer, pH 7.2) overnight, rinsed in buffer and post-fixed for 2 hours in 1% osmium tetroxide. Specimens were dehydrated in a graded ethanol series and embedded in Spurr's resin. Embedded juveniles were serially sectioned at a thickness of 0.5 μm. Sections were stained with 1% toluidine blue in borax or azure II/methylene blue.

Approximately ten juveniles (7-d old) from each replicate were processed for scanning electron microscopy (SEM). Juveniles were fixed either in Karnovsky's fixative with 1% osmium tetroxide post-fixation or 10% neutral buffered formalin (15 minute fixation and stored in 70% ethanol). Specimens were dehydrated in a graded ethanol series, critical-point dried with CO₂, sputter-coated with gold-palladium and
examined with a JEOL JSM-35 scanning electron microscope operated at 15 kV and 80 μA beam current.

Tissue sections were examined with light microscopes using brightfield and phase optics. The objective was to qualitatively assess the relative condition of juvenile organs based on detection of tissues in deteriorated conditions. Vacuolization, atrophy, necrosis, intercellular spaces and tissue separations were used as indicators of tissue degradation. Target organs assessed included: mantle, foot, muscle, ganglia, digestive glands, gills and stomach (Fig. 8). The following grading scale was used: (3) no distinguishable atrophy or deterioration; (2) intermediate atrophy or deterioration and (1) severe atrophy or deterioration. These criteria are similar to those used to assess starvation in larval fish (Thellacker 1986; Kushuba and Matthews 1984). Other criteria evaluated included: (1) the relative amount of algal cells in the stomach (3=many; 2=moderate; 1=few and 0=none); (2) the relative numbers of lipid bodies present in the digestive glands (3=digestive glands at least one-half full of lipid bodies; 2=less than one-half; 1=few and 0=none); (3) amount of debris adhering to outer shell (same scale as used for algae) and (4) presence (+) or absence (-) of the crystalline style.
RESULTS

Acute Toxicity Tests

There was very low mortality (less than 4%) of control juveniles in 48-h cadmium toxicity tests. There was decreasing sensitivity to cadmium with increasing juvenile age. 48-h LC50 and EC50 values for 14-d old juveniles were more than two times greater than that of 0- and 7-d old *Lampsilis ventricosa* (Table 3). Glochidia were tolerant to cadmium; the LC50 was approximately three times greater than that for any of the juvenile stages (Table 3).

Table 3. Acute toxicity of cadmium to larval and juvenile *Lampsilis ventricosa*, 48-h LC50 and EC50, with 95% confidence intervals (CI)

<table>
<thead>
<tr>
<th>Cadmium Concentration (µg/L)</th>
<th>Life Stage</th>
<th>LC50</th>
<th>95% CI</th>
<th>EC50</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glochidia</td>
<td>&gt;1000</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Juvenile:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 day</td>
<td></td>
<td>141</td>
<td>NR&lt;sup&gt;a&lt;/sup&gt;</td>
<td>91</td>
<td>76-110</td>
</tr>
<tr>
<td>7 day</td>
<td></td>
<td>166</td>
<td>144-190</td>
<td>107</td>
<td>74-154</td>
</tr>
<tr>
<td>14 day</td>
<td></td>
<td>345</td>
<td>293-406</td>
<td>291</td>
<td>246-344</td>
</tr>
</tbody>
</table>

<sup>a</sup>Not reliable because 100 µg Cd/L treatment had very high juvenile survival and 250 µg Cd/L treatment had low juvenile survival.

Subchronic Toxicity Test

Toxicity

The LC50 for 0-d old *L. ventricosa* juveniles decreased from 141 µg Cd/L in 48-h exposures to 82 µg Cd/L at 4 days and 38 µg Cd/L at 7 days. Similarly, EC50 concentrations decreased to 72 µg Cd/L and 24 µg Cd/L at 4 and 7 days, respectively.
Juvenile survival during the 7-d exposure was high in controls (97%) and 10 μg Cd/L treatments (93%) (Fig. 1). Exposures to 30 and 50 μg Cd/L were stressful, with only 77% and 31%, respectively, of juveniles surviving to day 7 (Fig. 1). By day 4 only 32% of juveniles exposed to 100 μg/L cadmium were alive, and none survived to day 7 (Fig. 1).

Growth effects

Morphometric data revealed that cadmium impaired the growth of juvenile L. ventricosa (Fig. 1; Table 4). Mean length of control juveniles at day 7 was 315 μm, and 93% of the controls reached an overall length of 300 μm or more (Table 4). Mean lengths for juveniles exposed to 30 and 50 μg Cd/L were 302 μm and 270 μm, respectively. None of the juveniles from 50 μg Cd/L treatments achieved a length of 300 μm (Table 4). Growth of the postmetamorphic shell was greatest at the anterior end, and this measurement appeared to be a sensitive indicator of cadmium toxicity. Analysis of variance indicated a significant reduction in anterior shell length for juveniles exposed to 10 μg Cd/L as compared to that of controls (Table 4).

Examination of juveniles with SEM also revealed shell growth impairments for cadmium-exposed juvenile L. ventricosa (Figs. 2-7). Control juveniles exhibited extensive anterior shell growth (Fig. 2) and filled shell pores (Fig. 3). No ultrastructural variations of the shell were observed for juveniles exposed to 10 μg/L (Fig. 4). Cadmium concentrations of 30 and 50 μg/L greatly reduced growth of the juvenile shell but did not affect filling of the larval shell pores (Figs. 5-7). One individual from the 50 μg/L cadmium treatment exhibited a shell anomaly that would not have been apparent using light microscopes or image analysis (Fig. 6).

Histopathology

Juvenile mussel sections were difficult to interpret because of variations in the overall relaxation of juveniles when fixed, staining variability, variable specimen orientation for sectioning and the apparent normal vacuolization of the tissues. Results of qualitative histological assessments of juvenile L. ventricosa are summarized below.

4 day

Feeding. The presence of algal cells in the stomach was used as an indicator of filtering or feeding activity. The relative amount of algal cells in the stomach decreased
Table 4. Morphometric analysis of shell characteristics of *Lampsilis ventricosa* (7-d) controls and cadmium-exposed juveniles (expressed as sample means in micrometer or percents)

<table>
<thead>
<tr>
<th>Cadmium µg/L</th>
<th>Glochidia</th>
<th>Juvenile</th>
<th>Shell Length</th>
<th>Growth (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>(%) &gt; 300 µm&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length</td>
<td>Height</td>
<td>Anterior</td>
<td>Posterior</td>
<td>Length Area</td>
</tr>
<tr>
<td>Control</td>
<td>30</td>
<td>226</td>
<td>257</td>
<td>315</td>
<td>299</td>
</tr>
<tr>
<td>10</td>
<td>30</td>
<td>228</td>
<td>257</td>
<td>309</td>
<td>295</td>
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<td>30</td>
<td>30</td>
<td>228</td>
<td>261</td>
<td>302*</td>
<td>296</td>
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<tr>
<td>50</td>
<td>13</td>
<td>217*</td>
<td>250*</td>
<td>270***</td>
<td>278*</td>
</tr>
</tbody>
</table>

<sup>a</sup>Percent growth based on length (juvenile shell length-glochidial shell length X 100) and based on area (juvenile shell area-glochidial shell area X 100).

<sup>b</sup>Percent juveniles greater than 300 µm in length.

Superscript symbol indicates significance levels with respect to controls: (*)=p<0.05; (**)=p<0.01; (***)=p<0.001.
in juveniles exposed to cadmium (Table 5, Figs. 9-10). The mean amount of food in the stomach decreased from 2.2 for controls to 0.8 for juveniles exposed to 100 µg Cd/L.

**Lipid reserves.** Lipid bodies ranging from 2 to 5 µm in diameter (mean=3.7 µm) were observed in the digestive glands of juveniles. Although lipid content appeared somewhat variable (Table 5, Figs. 11-13), there was some indication that cadmium affected lipid catabolism. Fewer lipid bodies were found in juveniles exposed to 10 µg/L cadmium treatments (mean=0.8) (Fig. 12) as compared to controls (mean=1.4); whereas, juveniles from 100 µg Cd/L treatments contained large lipid reserves (mean level=2.5) (Table 5, Fig. 13).

**Debris.** The amount of debris and algal cells adhering to the outer shell may be an indirect measure of mucous production by juveniles. None of the control juveniles exhibited debris on the outer shell; whereas, all but one of the juveniles exposed to cadmium had at least some debris on the shell (Table 5, Fig. 14).

**Style.** The crystalline style is an acellular firm mucoprotein rod found in the stomach of most bivalves (Judd 1987) (Fig. 15). Cadmium concentrations of 30 µg/L and above appeared to inhibit formation of or cause dissolution of the crystalline style. The style was lacking in 9 of 15 juveniles examined from these treatments (Table 5, Fig. 16).

**Organs.** Juveniles exposed to 100 µg Cd/L treatments exhibited the most severe histopathological effects. Extreme vacuolar degeneration of most organs was observed (Table 6, Fig 17). Digestive glands appeared to be most affected by cadmium treatments of 30 and 50 µg/L. Necrotic areas were observed in the digestive gland tissue of several juveniles exposed to these cadmium treatments (Fig. 18).

**7 day**

**Feeding.** Fewer algal cells were observed in the stomachs of juveniles exposed to 30 and 50 µg/L cadmium as compared to control and 10 µg Cd/L exposed juveniles (Table 5).

**Lipid reserves.** By 7 days, lipids had been depleted by controls and juveniles exposed to 10 µg/L cadmium; whereas, a few lipid bodies remained in the digestive glands of 3 of the 9 juveniles sampled from 30 and 50 µg/L cadmium treatments (Table 5, Fig. 19).
Table 5. Histological assessments of juvenile *Lampsilis ventricosa* from 7-d cadmium toxicity test

<table>
<thead>
<tr>
<th>Cadmium µg/L</th>
<th>Food&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Lipids&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Debris&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Style&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean 3 2 1 0</td>
<td>Mean 3 2 1 0</td>
<td>Mean 3 2 1 0</td>
<td>+ -</td>
</tr>
<tr>
<td><strong>4 day:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.2 1 4 0 0</td>
<td>1.4 0 2 3 0</td>
<td>0 0 0 0 5</td>
<td>5 0</td>
</tr>
<tr>
<td>10</td>
<td>1.4 1 2 2 0</td>
<td>0.8 0 0 4 1</td>
<td>1.8 2 1 1 1</td>
<td>5&lt;sup&gt;e&lt;/sup&gt; 0</td>
</tr>
<tr>
<td>30</td>
<td>1.4 1 1 2 1</td>
<td>1.4 0 2 3 0</td>
<td>1.8 1 2 2 0</td>
<td>3 2</td>
</tr>
<tr>
<td>50</td>
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<td>1.6 1 2 3 0</td>
<td>2.0 2 2 2 0</td>
<td>2 4</td>
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<tr>
<td>100</td>
<td>0.8 0 0 3 1</td>
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<td>1 3</td>
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<td>1.0 1 0 2 2</td>
<td>5 0</td>
</tr>
<tr>
<td>10</td>
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<td>1.8 1 2 2 0</td>
<td>4 1</td>
</tr>
<tr>
<td>50</td>
<td>0.5 0 0 2 2</td>
<td>0.5 0 0 2 2</td>
<td>2.8 3 1 0 0</td>
<td>2 2</td>
</tr>
</tbody>
</table>

<sup>a</sup>Number of algal cells in the stomach (3 = many; 2 = moderate number; 1 = few; and 0 = none).

<sup>b</sup>Relative amount of lipids in the digestive glands (3 = digestive glands at least one-half full of lipid bodies; 2 = less than one-half; 1 = few lipid bodies; and 0 = none).

<sup>c</sup>Amount of debris adhering to the outer shell (3 = many; 2 = moderate; 1 = few; and 0 = none).

<sup>d</sup>Presence of crystalline style (+ = present; and - = absent).

<sup>e</sup>Style abnormal in one juvenile.
Table 6. Histological organ assessments of *Lampsilis ventricosa* juveniles from 7-d cadmium toxicity test (3 = no distinguishable atrophy or deterioration; 2 = intermediate atrophy or deterioration; and 1 = severe atrophy or deterioration)

<table>
<thead>
<tr>
<th>Cadmium μg/L</th>
<th>Mantle</th>
<th>Foot</th>
<th>Muscle</th>
<th>Ganglia</th>
<th>Dig. Gland</th>
<th>Gills</th>
<th>Stomach</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 Day:</td>
<td></td>
<td></td>
<td></td>
<td></td>
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Debris. Control juveniles exhibited less adherent debris on the outer shell than did juveniles exposed to 30 and 50 µg/L cadmium (Table 5, Fig. 20).

Style. The crystalline style was absent in 3 of 9 juveniles sampled from cadmium treatments of 30 and 50 µg/L (Table 5). An abnormal style was observed in one juvenile exposed to 10 µg/L cadmium and may be in the process of dissolution (Table 5, Fig. 21).

Organs. Qualitative histological assessments of controls and juveniles exposed to 10 µg/L cadmium were very similar (Table 6). Overall, foot and muscle tissue were less affected by cadmium than other organs assessed (Table 6). Cadmium treatments of 30 and 50 µg Cd/L produced the most severe effects on the mantle (Figs. 22-24), ganglia (Figs. 25-27) and digestive glands (Figs. 28-30). These organs were ranked 2 or below for all juveniles examined from 30 and 50 µg Cd/L treatments (Table 6). Vacuolization, necrosis and tissue separations were the most evident histopathological effects.

The mantle consisted of two simple epithelial layers: (1) a thin pallial epithelium and (2) an extrapallial epithelium which was one-cell layer thick (Fig. 28). These layers were joined by connective tissue. Epithelial cells of the mantle exhibited increased vacuolization following exposure to 30 and 50 µg/L cadmium (Figs. 23-24), and necrotic areas were observed in mantle tissues of juveniles exposed to 50 µg Cd/L (Fig. 24).

The pedal ganglion of controls consisted of neurons without intercellular spaces (Fig. 25). The pedal ganglion of juveniles exposed to 30 and 50 µg Cd/L exhibited apparent degeneration of the neurons, development of intercellular spaces and necrosis of epithelial cells (Figs. 26-27).

The paired digestive glands of young juveniles were undiverticulated, oblong and located laterally to the stomach. Digestive gland tissues were composed primarily of digestive and basophil cells. Digestive cells of controls contained large numbers of microvesicles and basophil cell nuclei were darkly stained (Fig. 28). Juveniles exposed to 30 µg Cd/L exhibited digestive cells that were devoid of contents and undergoing atrophy (Fig. 29). Digestive gland tissue of juveniles exposed to 50 µg Cd/L treatments was greatly vacuolated and exhibited severe degeneration (Fig. 30).
DISCUSSION

Cadmium Toxicity

Acute toxicity

Results of toxicity studies indicated that young juvenile stages of freshwater bivalves may be especially sensitive to metal contaminants. For *Lampsilis ventricosa*, juvenile stages appeared to be more sensitive to cadmium than either larval or adult stages. Additionally, tolerance of juveniles increased with age; an age difference of only one week, from 7- to 14-d old, more than doubled the LC50. Naimo (1990) conducted sublethal cadmium studies in which *L. ventricosa* adults were exposed to cadmium treatments of 30, 100 and 300 μg Cd/L for 28 days. The only mortality occurred at day 27 in the highest cadmium concentration. Young juveniles probably would not survive these cadmium exposures.

In order for there to be adequate protection of mussel populations, water quality criteria and management plans need to take into account sensitivities of all life stages of freshwater bivalves. Current water quality guidelines established by U. S. EPA (1986) recommend one-hour average concentrations of waterborne cadmium should not exceed 6.2 μg/L (at a water hardness of 150 mg/L as CaCO3) more than once every 3 years on the average. This one-hour average is lower than acute toxicities determined for 0- and 7-d old *L. ventricosa* juveniles in this study. Although, current EPA guidelines would probably adequately protect juveniles from acute waterborne cadmium exposures, the EPA guidelines do not address cadmium adsorbed to the sediments.

The lethal concentration of cadmium for *L. ventricosa* juveniles was lower than that reported for other freshwater invertebrates. Aquatic insect larvae are more tolerant of cadmium, with values ranging from 840 μg/L (96-h LC50) for ephemeropterans to 233,000 μg/L for odonates (Thorp and Lake 1974). *Lampsilis ventricosa* juveniles appeared to be slightly more tolerant to acute cadmium exposures than *Daphnia magna* (48-h LC50's= 58 and 65 μg/L) but similar in sensitivity to that of *D. pulex* (48-h LC50=115 μg/L) (as reported by Wong 1987).

Standardization of toxicity testing

Although standardized procedures exist for conducting toxicity tests with larvae of marine bivalves, there are no such procedures for freshwater glochidia or juveniles.
The present studies with *L. ventricosa*, and studies by Wade (1989, 1990) and Keller and Zam (1991) suggest young stages of juvenile freshwater bivalves can be suitable test organisms for use in standardized toxicity tests. Unlike adult mussels, young juveniles do not exhibit shell closures; therefore, chemical avoidance may not be a factor. Control mortalities in tests with *L. ventricosa* were low (0-3%) and juveniles did not appear to be sensitive to handling, rinsing and pipetting. Additionally, the transparency of the shell allowed for observation of ciliary action; therefore, stressed and morbid conditions could readily be determined. Shell morphology and growth can also be used as sensitive endpoints in toxicity tests. Most notably, the prominence of both larval and juvenile shells means that individual growth rates can be measured.

The sensitivity of *L. ventricosa* juveniles (48-h LC50=141 μg/L) was nearly identical to that reported by Keller and Zam (1991) for juveniles of *Anodonta imbecilis* exposed to cadmium in hard water (48-h LC50=137 μg/L). At this time it appears that *A. imbecilis* would be a suitable test species for use in standardized tests. To date, long-term survival (greater than 3 weeks) of juvenile mussels in laboratory culture is poor, with the exception of *A. imbecilis* juveniles. Hudson and Isom (1984) and Keller and Zam (1990) have developed successful protocols for transforming juveniles in vitro using artificial media, and they also have been successful in maintaining juvenile *A. imbecilis* for extended periods in the laboratory. In order for *A. imbecilis* to be used as the prototype in standardized freshwater juvenile toxicity tests, it would first be necessary to establish that its sensitivity to chemical contaminants is similar to that of other juvenile species (in addition to that of *L. ventricosa*). Most importantly, it would be necessary to establish that the sensitivity for any prototype be similar to that of endangered species or species of economic value.

Cadmium Growth Effects

There are few studies evaluating effects of metals on growth of juvenile freshwater bivalves. In this study, 7-d exposure to as little as 10 μg Cd/L resulted in significantly reduced anterior shell growth for juvenile *L. ventricosa*. Reductions in growth may be the direct result of contaminant exposures or a secondary effect produced by starvation or altered metabolism.

Further studies are required to determine if cadmium impaired biomineralization processes in *L. ventricosa* juveniles. Cadmium is reported to inhibit activity of alkaline
phosphatase, an enzyme that participates in bivalve biomineralization processes (Evtushenko et al. 1986). Cadmium can also stimulate demineralization of animal bones and shells (Miyahara et al. 1980; Hemelraad et al. 1990a). Hemelraad et al. (1990a) suggested that increases of \( \text{Ca}^{2+} \) in the hemolymph of *Anodonta cygnea* following prolonged cadmium exposures may be due to solubilization of calcium carbonate from the shell.

**Cadmium Histopathology**

**Cadmium uptake and bioaccumulation**

The chemistry of cadmium in freshwater systems is complex. Briefly, cadmium can exist in freshwaters in the ionic form (\( \text{Cd}^{2+} \)) or may bind to either soluble or insoluble ligands. The ionic form of cadmium is considered to be the most toxic. Cadmium speciation is influenced by the oxidation status and pH of the water, as well as by the concentrations of organic and inorganic anions and other metal cations (Brewers et al. 1987). Soluble cadmium forms can be removed from the water column by adsorption onto sediments and by aquatic biota. Studies suggest that metal uptake by mussels occurs via diffusion of the free ion across epithelial surfaces (Coombs 1979; Robinson and Ryan 1986) or by endocytotic processes of epithelial cells (free ion and loosely bound cadmium) (Marshall and Talbot 1979; Hemelraad and Herwig 1988).

Adult bivalves are well known for their ability to bioaccumulate high levels of trace metals in their tissues. For cadmium, uptake is reported to be greater in the digestive gland, kidney and gills, and lower in the digestive system, shell and muscle (George and Coombs 1977; Janssen and Scholz 1979; Adams et al. 1981; Evtushenko et al. 1986; Hemelraad et al. 1986; Robinson and Ryan 1986; Herwig et al. 1989). Histopathological alterations are expected to occur at sites of greater metal uptake. Although accumulation patterns were not evaluated in the present study, primary sites of histopathogenesis in *L. ventricosa* juveniles correlated to sites reported as being sites of major cadmium uptake for adults. Digestive gland tissues of juveniles exhibited the most severe histopathological alterations; whereas, muscle tissue, a site of low cadmium uptake, exhibited few tissue alterations.

**Digestive gland alterations**

Changes in the structure of digestive epithelium in response to contaminant exposures have been investigated for a number of marine bivalves (Sunila 1986;
Rasmussen 1982; Lowe and Clarke 1989; Pipe and Moore 1986). These studies indicate digestive gland tissues may be particularly sensitive to chemical toxicants. Rasmussen (1982) described effects of N-nitroso compounds on the marine mussel *Mytilus edulis*. Reported alterations in the digestive diverticula included atrophy of the digestive cells, infiltration of ducts with hemocytes and necrosis of epithelial linings of the ducts. Epithelial disruption and necrosis were also observed for *M. edulis* following cadmium and copper exposure (Yevitch and Yevitch 1985). The marine mussel *Venus verrucosa* exhibited both morphological (reduced cell volume or atrophy) and cytochemical (loss of lysosomal stability) changes following exposure to petroleum hydrocarbons (Axiak et al. 1988). Likewise, atrophy of digestive epithelial cells was observed for *M. edulis* exposed to petroleum derived hydrocarbons (Lowe and Clarke 1989). Hydrocarbon exposure also induces abnormal accumulations of lipids in the cytoplasm of digestive and basophil cells. It appears that atrophy of digestive cells is a general non-specific response to contaminant-induced stress. Juvenile *L. ventricosa* also exhibited this digestive gland alteration following short-term exposure to cadmium.

**Effects on lipid catabolism**

Many aquatic invertebrate larvae are dependent upon endogenous energy reserves, primarily lipids, during early post-hatching periods (Fraser 1989). Triacylglycerol (TAG) has been identified as the major storage lipid in animal cells. Holland and Spencer (1973) demonstrated that larval oysters (*Ostrea edulis*) utilize TAG to meet the high energy demands of metamorphosis. Nutritional and environmental stressors (i.e. contaminants) have been shown to alter lipid metabolism, primarily by the catabolization of TAG in an effort to meet increased energy demands (Gallager et al. 1986). Fraser (1989) reported that physiological condition indices based on quantification of TAG (more specifically the ratio of TAG to sterols) can be valuable tools for evaluating pollutant effects on fish and invertebrate larvae.

There are no studies evaluating the role of lipid reserves in energy metabolism for developing juvenile freshwater bivalves; however, it might be assumed that lipids are an essential energy reserve for metamorphosing juveniles. Cadmium appeared to affect lipid catabolism by juvenile *L. ventricosa*. Exposure to sublethal levels of cadmium (10 µg Cd/L) seemed to have a stimulatory effect on lipid metabolism, as shown by reduced levels of lipids in the digestive glands. There are many reports of sublethal levels of metals having a stimulatory effect on physiological processes (refer to Stebbing 1981).
Capuzzo et al. (1984) found reduced TAG levels in American lobster larvae, *Homarus americanus*, exposed to 0.25 ppm crude oil for 96-h. Adult lamellibranchs utilize carbohydrates rather than lipids as a major energy reserve (Holland and Spencer 1973). Hemelraad et al. (1990b) observed depletion of glycogen reserves by adult *Anodonta cygnea* following exposure to sublethal levels of cadmium.

Lethal levels of cadmium (30-100 µg/L) appeared to impede lipid catabolism by *L. ventricosa* juveniles. At 4-d, larger amounts of lipid bodies were observed in digestive glands of juveniles exposed to 100 µg Cd/L as compared to controls. By 7-d, a few lipid bodies remained in digestive glands of juveniles from 30 and 50 µg Cd/L treatments, whereas none were found in control and 10 µg Cd/L exposed juveniles. Lowe and Clarke (1989) suggested three possible mechanisms for abnormal accumulations of lipids in the digestive glands of *Mytilus edulis*: (1) disrupted neurosecretory control associated with nutrient storage; (2) increased lipid synthesis or (3) inability to catabolize lipids.

**Crystalline style dissolution**

Cadmium concentrations as low as 10 µg/L affected the normal morphology of the crystalline style of juvenile *L. ventricosa*, and treatments of 30 µg Cd/L and greater resulted in loss of the style. Hameed and Raj (1989) reported dissolution of the crystalline style for the freshwater bivalve *Lamellidens marginalis* (adults) following exposure to sublethal concentrations of cadmium (11 mg/L), copper (9.75 mg/L) and mercury (6.5 mg/L). Dissolution times ranged from 18 minutes for copper and cadmium to 24 minutes for mercury. The style persisted in control *L. marginalis* throughout metal exposures. Style reformation was also hindered by metal exposures. Bernard (1973), Dean (1980) and Hameed and Raj (1989) reported additional environmental stressors, such as exposure to air, anaerobic conditions and starvation, also can contribute to style dissolution. For *L. ventricosa* juveniles, style dissolution may be a primary effect of cadmium or a secondary effect of starvation or metabolic dysfunction.

**Future studies**

This study indicates that additional research is needed to evaluate metal contaminant effects on juvenile freshwater mussels. Some recommendations for future study include: (1) determining lipid biochemistry for freshwater juveniles with further evaluation of
effects of metal exposures on lipid reserves; (2) evaluating effects of metals on biomineralization processes (including effects on pore-filling); (3) conducting metal toxicity tests with additional freshwater bivalve species; (4) determining metal uptake patterns in juveniles; (5) identifying detoxification and sequestration mechanisms; (6) evaluating effects of metal exposure on larval encystment rates and metamorphosis and (7) continuing histological studies on effects of contaminants on growth and development. Histological examinations could be useful in assessing health of field-collected juveniles.


Figure 1. Survival (A) and growth (B) of *Lampsilis ventricosa* juveniles in 7-d cadmium toxicity test.
Figures 2-7. Scanning electron micrographs of 7-d old juvenile *Lampsilis ventricosa* from 7-d cadmium toxicity test

Figure 2. External shell surface of control juvenile showing larval shell (l) and postmetamorphic juvenile shell growth (a=anterior juvenile shell; p=posterior juvenile shell). Bar= 75 μm

Figure 3. Higher magnification of control juvenile showing larval shell surface with filled pores (arrowheads) and larval and juvenile shell commissure. Bar= 20 μm

Figure 4. Juvenile exposed to 10 μg Cd/L treatment. Bar= 75 μm

Figure 5. Juvenile exposed to 30 μg Cd/L treatment showing reduced anterior growth of the juvenile shell. Bar= 75 μm

Figure 6. Juvenile exposed to 50 μg Cd/L treatment showing an apparent shell anomaly (arrowhead) of the postmetamorphic shell (compare with control above). Bar= 75 μm

Figure 7. Juvenile exposed to 50 μg Cd/L treatment showing extreme reduction in growth of the juvenile shell. Bar= 75 μm
Figures 8-16. Light micrographs of 4-d old juvenile *Lampsilis ventricosa* from 7-d cadmium toxicity test

Figure 8. Control juvenile (a=anterior adductor muscle; pa=posterior adductor muscle; e=esophagus; f=foot; p=pedal ganglion; g=gills; c=crystalline style; s=stomach; m=mantle). Bar= 60 μm

Figures 9-10. Stomach of juveniles showing algal cells (arrowheads) (h=hinge ligament). Fig. 9. Control juvenile, graded 3. Fig. 10. Juvenile exposed to 30 μg Cd/L, graded 1. Bars= 20 μm

Figures 11-13. Lipid bodies (I) in the digestive gland of juveniles. Fig. 11. Control juvenile, graded 2. Fig. 12. Juvenile exposed to 10 μg Cd/L, graded 1. Fig. 13. Juvenile exposed to 100 μg Cd/L, graded 3. Bars= 15 μm

Figure 14. Algal cells and other debris adhering (arrowhead) to the outer shell (sh) of a juvenile exposed to 50 μg Cd/L, graded 3. Bar= 15 μm

Figure 15. Stomach of control juvenile showing the crystalline style (c) (p=pedal ganglion; g=gastric shield). Bar= 40 μm

Figure 16. The stomach of a juvenile exposed to 100 μg Cd/L. The crystalline style is absent (g=gastric shield). Bar= 30 μm
Figures 17-18. Light micrographs of 4-d old juvenile *Lampsilis ventricosa* from 7-d cadmium toxicity test

Figure 17. Juvenile exposed to 100 μg Cd/L. Vacuolar degeneration of tissues is evident (a=anterior adductor muscle; d=debris; dg=digestive gland; s=stomach; g=gills; m=mantle; sh=shell). Bar= 60 μm

Figure 18. Digestive gland tissue of juvenile from 30 μg Cd/L treatment showing large necrotic areas (arrowheads). Bar= 15 μm

Figures 19-24. Light micrographs of 7-d old juvenile *Lampsilis ventricosa* from 7-d cadmium toxicity test

Figure 19. Digestive gland of juvenile exposed to 30 μg Cd/L showing lipid bodies (arrowhead)

Figure 20. Juvenile exposed to 50 μg Cd/L with debris adhering to outer shell, graded 3. Bar= 60 μm

Figure 21. Abnormal crystalline style (arrowhead) of juvenile exposed to 10 μg Cd/L treatment. Bar= 20 μm

Figures 22-24. Mantle of juveniles. Fig. 22. Juvenile exposed to 10 μg Cd/L treatment, graded 3. Fig. 23. Juvenile exposed to 30 μg Cd/L treatment, graded 2. Fig. 24. Juvenile exposed to 50 μg Cd/L treatment, graded 1. Arrowhead indicates necrotic area. Bars= 15 μm
Figures 25-30. Light micrographs of 7-d old juvenile *Lampsilis ventricosa* from 7-d cadmium toxicity test

Figures 25-27. Pedal ganglion of juveniles (n=neurons; f=foot). Fig. 25. Control juvenile, graded 3. Fig. 26. Juvenile exposed to 30 μg Cd/L treatment, graded 2. Fig. 27. Juvenile exposed to 50 μg Cd/L treatment, graded 1. Arrowheads indicate large intercellular spaces. Bars= 15 nm

Figures 28-30. Digestive gland of juveniles (n=neurons; f=foot). Fig. 28. Control juvenile, graded 3. Fig. 29. Juvenile exposed to 30 μg Cd/L treatment, graded 2 (arrowhead indicates atrophied digestive cells). Fig. 30. Juvenile exposed to 50 μg Cd/L treatment, graded 1 (note extreme vacuolization and degeneration of tissues). Bars= 15 μm
GENERAL SUMMARY

Specific reasons for recent declines in mussel populations in the Upper Mississippi River (UMR) are currently unknown. Overharvesting, impoundments, dredging, siltation and pollution have been implicated as contributing factors for the declining mussel populations. Water quality has improved in the UMR in recent years; however, sediments remain sufficiently contaminated with metal pollutants at levels that potentially could be a threat to aquatic biota. Benthic filter-feeding bivalves may be especially vulnerable. Very little is known about the effects of habitat alterations on the life stages of freshwater unionids.

There is little information on early growth, development or habitat requirements of juvenile unionid mussels. New information is critically needed in order to develop recovery plans that will adequately protect threatened and endangered mussel species, as well as commercially and ecologically important species. The early shell development of juvenile *Lampsilis ventricosa* and *Pleurobema cordatum*, reared in the laboratory, was described using scanning electron microscopy. There was limited shell growth during encystment. The glochidial (larval) shell exhibited numerous pores which extended through the calcareous shell layer but did not penetrate the outer shell cuticle. The postmetamorphic (juvenile) shell was added to the peripheral edges of the larval shell. Shell deposition into larval shell pores coincided with early formation of the juvenile shell. This pore-filling had not previously been observed. Growth of the juvenile shell was rapid for *L. ventricosa*, and by 21 days, concentric growth rings were evident.

Light microscopy and electron microscopy were used to describe post-larval development of internal organs of *L. ventricosa* reared in the laboratory for up to 56 days. Glochidia underwent metamorphosis while encysted on the gills of fish hosts. Prior to drop off, presumptive digestive organs, three pairs of rudimentary gill filaments, mantle, a protractile foot and a well developed nervous system were present. Digestive glands contained large numbers of lipid bodies. At the time of release, juveniles exhibited ontogenetic changes required to ingest and process phytoplankton. Most notably, the stomach had differentiated into an anterior gastric portion and a posterior style sac. The style sac contained a translucent crystalline style and dense short cilia. Subsequent developments in the digestive system consisted of elongation of the intestine and further elaboration of ciliary sorting areas and linings.
The postmetamorphic alignment of the alimentary system was not altered until 56 days, when the style sac shifted ventrally. By 2 weeks, lipid bodies were absent from digestive glands. This loss of lipids correlated to increases in mortality of laboratory-reared juveniles. At 3 weeks, digestive glands had become diverticulated, and by 8 weeks, numerous diverticula surrounded the stomach and projected ventrally into the foot. Growth of gill filaments, by papillary outgrowths, began approximately 3 weeks following drop off. Frontal gill surfaces were covered with distinct lateral, frontal and laterofrontal ciliary tracts, which are characteristic for gills of adult lamellibranchs. The juvenile foot was well developed at release, and surfaces were morphologically distinct. Lateral dorsal surfaces were covered with a microvillar epithelium; whereas, ventral surfaces were covered with long dense cilia. By 8 weeks, the byssus complex had formed. Byssus glands were diverticulated, and a ciliated groove (the gland opening) had formed along the ventral length of the foot. A byssus thread was not elaborated by any of the juveniles examined.

When establishing acceptable metal exposure limits for unionid bivalves, the sensitivities of all life stages need to be taken into account. The juvenile stage represents the first free-living stage and, therefore, may be the stage most vulnerable to metal exposures. In the present study, cadmium was used to determine the relative sensitivities of larval and juvenile stages of *L. ventricosa* to metal exposure. Acute toxicity (48-h) tests were performed on *L. ventricosa* glochidia and juveniles aged 0- (0-12 hours post-drop off), 7- and 14-d old. Based on LC50's and EC50's (derived from number of stressed and dead juveniles), young juveniles (0- and 7-d old) were more sensitive to cadmium than 14-d old juveniles. Glochidia were very tolerant to cadmium; the LC50 was approximately three times greater than that of any of the juvenile stages.

The effects of metals on growth and development of juveniles apparently has not been evaluated. A 7 day static renewal test was used to determine growth and histopathological effects of cadmium on 0-d old juveniles. Morphometric analysis of external shell parameters revealed significant reductions in anterior shell length following exposure to 10 µg Cd/L. Histopathological alterations (atrophy, increased vacuolization and tissue separations) appeared more severe for the mantle, ganglia and digestive gland tissues than for foot and muscle tissues. Exposure to 30-100 µg Cd/L
resulted in dissolution of the crystalline style in several juveniles. Histological examinations also indicated that juvenile feeding was reduced, lipid catabolism was altered, and mucous hypersecretion may have occurred.
LITERATURE CITED


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