Studies on Lampsilis Mussels of the Upper Mississippi River

Diane Robinson Waller

Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/rtd

Part of the Zoology Commons

Recommended Citation
https://lib.dr.iastate.edu/rtd/9314

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
INFORMATION TO USERS

The most advanced technology has been used to photograph and reproduce this manuscript from the microfilm master. UMI films the original text directly from the copy submitted. Thus, some dissertation copies are in typewriter face, while others may be from a computer printer.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyrighted material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each oversize page is available as one exposure on a standard 35 mm slide or as a 17" × 23" black and white photographic print for an additional charge.

Photographs included in the original manuscript have been reproduced xerographically in this copy. 35 mm slides or 6" × 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.
Studies on *Lampsilis* mussels of the upper Mississippi River

Waller, Diane Robinson, Ph.D.

Iowa State University, 1987
PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark ✓.

1. Glossy photographs or pages ✓
2. Colored illustrations, paper or print
3. Photographs with dark background ✓
4. Illustrations are poor copy
5. Pages with black marks, not original copy ✓
6. Print shows through as there is text on both sides of page
7. Indistinct, broken or small print on several pages ✓
8. Print exceeds margin requirements
9. Tightly bound copy with print lost in spine
10. Computer printout pages with indistinct print
11. Page(s) lackling when material received, and not available from school or author.
12. Page(s) seem to be missing in numbering only as text follows.
13. Two pages numbered. Text follows.
14. Curling and wrinkled pages
15. Dissertation contains pages with print at a slant, filmed as received
16. Other

-----------------------------------------
-----------------------------------------
-----------------------------------------

UMI
Studies on *Lampsilis* Mussels of the Upper Mississippi River

by

Diane Robinson Waller

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

DOCTOR OF PHILOSOPHY

Major: Zoology

Approved: Members of the Committee

In Charge of Major Work

For the Major Department

For the Graduate College

Iowa State University

Ames, Iowa

1987
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>iv</td>
</tr>
<tr>
<td>GENERAL INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>EXPLANATION OF DISSERTATION FORMAT</td>
<td>20</td>
</tr>
<tr>
<td><strong>SECTION I. MORPHOLOGY OF THE GLOCHIDIA OF</strong></td>
<td>21</td>
</tr>
<tr>
<td><strong>LAMPSILIS HIGGINSI</strong> COMPARED</td>
<td></td>
</tr>
<tr>
<td>WITH OTHER UPPER MISSISSIPPI RIVER MUSSEL SPECIES</td>
<td></td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>22</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>25</td>
</tr>
<tr>
<td>RESULTS</td>
<td>27</td>
</tr>
<tr>
<td>CONCLUSIONS AND DISCUSSION</td>
<td>59</td>
</tr>
<tr>
<td><strong>SECTION II. DETERMINATION OF SUITABLE FISH</strong></td>
<td>61</td>
</tr>
<tr>
<td>HOSTS FOR THE GLOCHIDIA OF <strong>LAMPSILIS HIGGINSI</strong></td>
<td></td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>62</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>64</td>
</tr>
<tr>
<td>RESULTS</td>
<td>68</td>
</tr>
<tr>
<td>CONCLUSIONS AND DISCUSSION</td>
<td>73</td>
</tr>
</tbody>
</table>
ABSTRACT

The glochidial stage of the endangered freshwater mussel, *Lampsilis higginsi*, and several related species was studied to provide information on their early life histories. The glochidia of *L. higginsi* and the three species, *L. radiata siliquoidea*, *L. ventricosa*, and *Ligumia recta*, were compared using morphometrics and scanning electron microscopy (SEM). The glochidia of *L. higginsi* were morphometrically similar to those of the related species; however, they could be distinguished using SEM by the position of the hinge ligament and the dorsal ridge width. Fifteen species of fishes were tested for their suitability as hosts for the glochidia of *L. higginsi*. The following were found to produce at least one juvenile mussel: northern pike (*Esox lucius*), brook stickleback (*Culea inconstans*), bluegill (*Lepomis macrochirus*), green sunfish (*L. cyanellus*), largemouth bass (*Micropterus salmoides*), smallmouth bass (*M. dolomieui*), yellow perch (*Perca flavescens*), and walleye (*Sitzostedion vitreum vitreum*). A test of host quality using three members of the Family Centrarchidae ranked smallmouth bass highest with a transformation of 7.68%, followed by green sunfish (2.43%) and bluegill (0.00038%). Two propagation methods for juvenile *Lampsilis* mussels were tested. *In vitro* culture averaged 1.28 juveniles/plate, with a transformation of 1.05%. Transformation averaged 15-24 juveniles/fish on infected host
fish. The pathogenesis associated with *L. radiata siliquoides* on a suitable (walleye) and an unsuitable host (common carp) was compared using light and transmission electron microscopy. Encapsulation of glochidia on walleye gills was completed by 4-6 hr at 21°C. At 24-48 hr, the capsule was thin and compact. Fibrous tissue appeared in the capsule at 48 hr and increased in quantity to the end of the infection. Excystment occurred by thinning of the capsule aided by movement of the juvenile. Most of the glochidia attached to the common carp gills did not encapsulate. Partial capsular growth was evident in some, but the portions of the capsule distal to the bite consisted of necrotic cells and debris. A few complete capsules were found at 12-48 hr; however, all glochidia were sloughed by 60 hr. There was no evidence of leucocytosis; however, the number of heterophil type cells was greater in the capsular tissue of the common carp than in walleye.
GENERAL INTRODUCTION

The Upper Mississippi River (UMR) contains a unique and rich fauna of freshwater unionid mussels. Historically about 50 species occurred in the UMR, but today the number has dropped to about 30 (Fuller 1978) and many of the remaining species are threatened by extirpation. Information on the early life history of many mussels, in particular the endangered species *Lampsilis higginsi*, is scarce. The glochidia cannot be positively identified using the descriptions provided by early researchers, although the identification of fish hosts for a mussel species were often based on examinations of natural infections of gills. The mechanism responsible for host specificity of a mussel species has not been determined, although the *in vitro* requirements for transformation of the glochidium to the juvenile stage were determined by Isom and Hudson (1982). In the following studies, the glochidial stage of several *Lampsilis* species was examined to provide specific information on the early life history of *L. higginsi*, and to provide general information on the parasitic stage of *Lampsilis* mussels.

Reproduction

In 1695 Leuwenhoek studied the reproductive processes of freshwater mussels and learned that the eggs pass from the
ovaries to the gill passages where embryonic development is completed. His work was lost to science for about 150 years and the general belief that the tiny organisms found in mussel gills were parasites led to glochidia being named *Glochidium parasiticum*. Carus, in 1832, suggested that these tiny organisms were bivalve larvae, but he was unable to bring about continued development after separation from the parent mussel (Jones 1950). In 1866 Leydig discovered that the glochidia of freshwater mussels are parasitic on the gills of fishes.

Preliminary information on the reproduction of freshwater mussels and the sudden boom in commercial harvest of freshwater mussels initiated a large scale effort in naiad research in the early 1900s, particularly by the U. S. Bureau of Fisheries in the Upper Mississippi River. Lefevre and Curtis (1910b) first described the early life history of commercially valuable mussels in the Mississippi River, and Surber (1913) used observations of natural infections to identify the fish hosts for several of these species. Howard (1913, 1914a) reported narrow ranges of host specificity in experimental infections with commercially important mussel species in the Upper Mississippi River. Exceptional cases of metamorphosis on a *Necturus* and without a fish were also reported (Howard 1915; Lefevre and Curtis 1911). Coker et al. (1921) studied various aspects of the natural history of
mussels including habitat and reproduction. Various phases of the parasitic period were reviewed by Howard and Anson (1922). Life history data on the mollusca of Wisconsin, including species in the UMR, were compiled by Baker (1928). The decline of the pearl button industry and the cessation of studies at the Fairport Biological Station, Fairport, Iowa, brought a near halt to research on freshwater mussels after the 1930s. These early mussel studies did, however, provide the groundwork for more recent research on naiads.

Early studies demonstrated that freshwater mussels could be subdivided into three subfamilies on the basis of 1) hooked or hookless glochidia, 2) long or short term breeding, and 3) use of all or a portion of the gills as marsupia. The life cycle was shown to begin with release of sperm by the male into the water. Sperm are received by the female through the incumbent siphon. Both fertilized and unfertilized eggs pass into the gill chambers of the marsupium, a modified portion of the females' gill, where the glochidia develop. The mature glochidia are released from the marsupia and attach to the gills or fin of an appropriate fish host. They encapsulate and undergo organogenesis while attached to the fish and then fall off and become benthic-living juveniles (Lefevre and Curtis 1910a).

In recent years the focus of mussel research has been on the reproductive cycle and determination of host-parasite
relationships. Reproductive cycles of several species have been studied by histological examination of gonadal tissue and/or by identifying periods of glochidial release. Zale (1980) and Zale and Neves (1982a) collected glochidia in stream drift and examined the gonadal tissue of 4 species of lampsilines in Virginia to study the reproductive cycle of each. Similar studies have been conducted on other unionids including *Amblema plicata* (Stein 1973, Kammer 1986), *L. ventricosa* and *Proptera alata* (Kammer 1986), *Elliptio complanatus* (Matteson 1948), *Pleurobema cordatum* (Yokley 1972), *Pleurobema oviforme* (Weaver 1981), and *Anodonta* species (Heard 1975; Trdan and Hoeh 1982; Dudgeon and Merton 1983; Parker et al. 1984). Host-parasite relationships have been studied by observations of natural infections; Tedla and Fernando (1969a,b; 1970) examined the parasite fauna of the gills of yellow perch (*Perca flavescens*) and found it to be a host for *Lampsilis radiata*. Murphy (1942) examined infections of salmonids of the Truckee River to determine those species susceptible to infection by glochidia of *Margaritifera margaritifera*. Natural infections of the rock bass (*Ambloplites rupestris*) and pumpkinseed (*Lepomis gibbosus*) were studied by Havek and Fernando (1978a,b,c). Other researchers have also examined the gills of fish from various areas for evidence of glochidial infections (Hare and Frantsi 1974; Wiles 1975; Gruninger et al. 1977; Atkins 1979;
Artificial infection experiments, often used jointly with examinations of natural infections, have been the second major tool for studying host-parasite relations of freshwater mussels (Seshaija 1941; Matteson 1948; Tedla and Fernando 1969a,b; Tompa 1979; Trdan and Hoeh 1982; Weaver 1981; Neves et al. 1985). Zale (1980) and Zale and Neves (1982b) artificially infected various fish species with the glochidia of four mussel species from Virginia. Subsequently, they examined the gills of field collected fish to confirm the use of laboratory hosts in nature. Sylvester et al. (1984) artificially infected species of fish with glochidia of Lampsilis ventricosa. No juvenile mussels were produced by any fish species, but it was suspected walleye and largemouth bass were hosts based on prolonged attachments at 15-16°C. A comprehensive list of the mussel-host data has been compiled by Fuller (1974).

Research on the juvenile stage of the life cycle is very sparse. Isely (1911) presented preliminary work on the ecology of some stream species and Lefevre and Curtis (1910a), Howard (1914b), Tucker (1927), and Matteson (1955) presented general descriptions of anatomy and behavior of a few species. However, the juveniles of most species, including L. higginsi, have not been described and they are seldom reported in surveys (Matteson 1955). The habitat and
food requirements of juveniles are virtually unknown, with the exception of artificially propagated individuals (Howard 1914b) such as L. ovata and Ligumia recta (Hudson and Isom 1984).

Research on the life history of Lampsilis higginsi is especially limited. Lefevre and Curtis (1910a,b) collected gravid females in May and September. The glochidia were described and measured by Surber (1913), and Grier (1921) detailed the mantle flap of the female. Two species of fishes have been reported as hosts for the glochidia: sauger (Stizostedion canadense) (Wilson 1916; Coker et al. 1921; Surber 1913) and freshwater drum (Aplodinotus grunniens) (Wilson 1916; Coker et al. 1921). The gametogenic cycle of L. higginsi has not been studied, and its endangered status prevents histological examination of the gonadal tissue. However, L. ventricosa is a closely related species, and Kammer (1986) studied the life history of UMR populations of this species. Gravid females of L. ventricosa were collected from April through October, and release of glochidia was estimated to begin at about 19°C. Syngamy was exhibited in late July and early August, corresponding to peak ambient temperatures in the river.
Habitat

As benthic, filter feeding organisms, freshwater mussels are highly dependent on environmental cues. The typical dense, diverse mussel bed occurs along channel borders, in substrates of mixed particle size with well oxygenated water; however each mussel species within and between beds may have different habitat requirements. Early research of general habitats and biotic associates of mussels was reviewed by Coker et al. (1921). Specific rivers and river areas have been studied, including some Wisconsin rivers (Stern 1978), the St. Croix River (Stern 1983), Pool 10 of the Mississippi River (Duncan and Thiel 1983), the Illinois River (Starrett 1971), and the UMR (Fuller 1978, 1980) to assess the mussel population and associated habitat types.

*Lampsilis higginisi* is reportedly a low density species (Pratt 1876, Witter 1883), found in deep water in large rivers (Baker 1928). Information on its habitat requirements has come mainly from surveys reporting the habitat type and substrate on which individuals were found. Coker et al. (1921), in a review of the habitat literature to date, reported finding *L. higginisi* in sand, sand/gravel, gravel and clay/sand, in areas of fair to good current. Mathiak (1979) found *L. higginisi* on substrates of sand/gravel/boulder. Kindschi (1980) summarized survey findings of *L. higginisi* in the Mississippi River from 1966 to 1980. Individuals of this
species were usually found along main channel borders and side channel habitats in substrate combinations of mud, sand, and gravel, and at depths of 8-30 feet. Most recently Duncan and Thiel (1983) surveyed Pool 10 of the UMR and found _L. higginisi_ primarily in main channel border habitats; 53% of the individuals were collected in the east and west channels of the river at Prairie du Chien, Wisconsin. The habitat requirements of _L. higginisi_ adults may reflect their ability to burrow in various substrate types. Marking and Bills (1977) found _L. higginisi_ adults were unable to burrow into rocky substrates, but were able to burrow into silt, clay, sand, and/or pebble/gravel. The shortest burrowing time occurred in silt while the longest was in pebble-gravel.

Several surveys have found faunal associations between _L. higginisi_ and other mussel species (Duncan and Theil 1983; Havlik 1981). Specifically, _Obovaria olivaria_ and _Quadrula metanevra_ have been found to cluster with _L. higginisi_. Whether this association is due to common requirements for substrate, current, fish hosts or some other environmental factor is not known.

**Factors suspected to cause the decline of populations**

The decline of freshwater mussel populations began in the early 1900s due to a number of factors, most notably the commercial harvest of shells by the button industry.
Commercial clamming began about 1889 (Carlander 1954) and rapidly depleted beds in the UMR between Muscatine, Iowa and Red Wing, Minnesota. Clamming was constant even during the mussel spawning season and all sizes of mussels were taken. Carlander (1954) reported that from 1914 to 1915, 3,000-4,000 tons of shells were taken from Lake Pepin, but by 1919 only about 150 tons were harvested. In addition, changes in the river system and water quality were suspected to contribute to declines in populations. The construction of the lock and dam system in the 1930s produced physical alterations of the river including increased siltation and changes in the current regime and in the fish fauna. In the pool systems, lentophilous mussel species such as Anodonta species and three-ridge (Amblema plicata) prospered while lotophilous species, such as buckhorn (Tritogonia verrucosa) and muckets (Actinonaias and Lampsilis species) declined (van der Schalie 1938). Greater inputs of domestic and industrial wastes and agricultural inputs of sediment also contributed to degradation of mussel habitat. Ellis (1931) reported poor replacement of mussels, which he attributed to the effects of silt. Only two species of mussels, the maple leaf (Quadrula quadrula) and hickory nut (Obovaria olivaria), of the 15 principal commercial species, showed a replacement with young mussels four years or older. Ellis cited siltation effects such as the covering of beds, the covering of juveniles, the
poorer condition of glochidia, and the increase in oxygen demand on river bottoms as possible causes for poor mussel replacement.

In more recent years, similar factors are being named as suspected causes for further declines of many mussel species. Commercial harvest of mussels has been renewed for use in the Japanese pearl culture industry. Regulations on the species and size of shells taken have been implemented by most states to prevent the harvest of threatened and endangered species and to prevent the extirpation of the commercially valuable species. However, to harvest suitable species, commercial clammers must disturb the beds and habitats of non-commercial species. Mussels are non-selectively harvested by brail bars and then thrown back if they do not meet specifications. Clammers that harvest by diving may clear entire areas of mussel habitat and remove all harvestable species present. Imlay (1972) demonstrated that mussels displaced by artificial means have a lower survival than those displaced naturally suggesting that even species not harvested by commercial clammers are still affected by clamming activities.

Channel maintenance activities by the U. S. Army Corps of Engineers and dredging by private individuals may affect the mussel fauna in several ways, including physical removal of mussels from the river, an increase of the turbidity of
the water, resuspension of contaminants, and erosion of dredge material onto mussel beds. Havlik and Marking (1980) found 33 \textit{L. higginsi} shells from 10 sample sites in material dredged from the Mississippi's east channel at Prairie du Chien, Wisconsin, and Fuller (1980) found one juvenile \textit{L. higginsi} in dredge material at Brownsville, Minnesota. Rosenberg and Henschen (1986) demonstrated that heavy metals adsorbed to sediment particles which had gotten trapped in the pallial spaces caused staining of the nacre in \textit{Amblema plicata}. In addition, they suggested that the release of heavy metals from the particles may disturb calcium metabolism in the mussel by competing with calcium binding sites. Marking and Bills (1977) exposed \textit{Lampsilis radiata luteola} and \textit{Fusconaia flava} to overlays of sand or silt in the laboratory and found overlays of 18 cm or more were required to prevent emergence of \textit{L. radiata} and 10 cm prevented emergence of 50\% of the \textit{F. flava}.

A number of other factors have been implicated in the decline of the UMR mussel populations including the input of waste from industry, municipalities, and agriculture. Havlik and Marking (1987) reviewed reports on the effect of toxins. Contaminants were found to be detrimental to mussel populations by direct toxic effects and indirect effects on essential food organisms and host fish. A number of contaminants were reported to be toxic, including cadmium,
ammonia, potassium, chromium, arsenic trioxide, copper and zinc. However, few specific effects of contaminants have been determined and related stresses responsible for mussel declines have not been identified and quantified. The silt load from tributaries, especially in agricultural areas, may have a detrimental effect on the mussel population. Dennis (1984) found survival of mussels was poorer in heavily silted areas than in unsilted areas.

Changes in the biotic community are suspected as the cause of decline in several mussel species. The ebony shell, *Fusconaia ebena*, disappeared from the upper reaches of the Mississippi River because its sole host, the skipjack herring (*Pomolobus chrysochloris*), could not migrate upstream after impoundment. The asiatic clam (*Corbicula*) is a growing threat to indigenous species of mussels. It proliferates in disturbed areas, does not require a host fish for reproduction, and may be able to outcompete and replace natural populations of other mussels (Fuller and Imlay 1976; Fuller 1980). Fuller and Richardson (1977) reported large numbers of dislodged mussels in the Savannah River and found *Corbicula* clams burrowing under the mussels. *Corbicula* has become much more widespread since 1924 (McMahon 1982). Fuller (1980) reported *Corbicula* from the Upper Mississippi River and from the St. Croix River.
Special remarks on the endangered mussel Lampsilis higgininsi

*Lampsilis higgininsi* is one of two federally endangered species of the UMR. It was never historically abundant in the river, but it became increasingly rare around the turn of the century (Coker, 1919), experiencing an estimated 53% reduction from its historical range (pre-1965) to its present range (1965-1980) (Havlik 1981). The decrease in range and the limited numbers of individuals reported prompted its consideration for endangered species status, which it was granted in 1976, Federal Register 41FR24064, June 14, 1976. The early life history of *L. higgininsi* is not well known. Effective recovery of the species requires basic life history information on its reproduction, life cycle, and habitat requirements. The United States Fish and Wildlife Service initiated study of *L. higgininsi* in 1984 as part of the Endangered Species Program. The early life history research was funded through a Cooperative Education Agreement between Iowa State University and the U. S. Fish and Wildlife Service and is presented in this dissertation.

Classification of Lampsilis higgininsi

The classification of *Lampsilis higgininsi* is as follows (Johnson 1980):
Superfamily Unionacea
Family Unionidae
Subfamily Ambleminae
Tribe Lampsilini

*L. higginsi* is member of the *orbiculata* complex.

Conspecifics, of this complex include:

- *L. pinguis*—Call (1885)
- *L. abruptus*—Ball (1922), Call (1885)

*L. higginsi* and *L. orbiculata* are similar in appearance and have been considered separate species, subspecies, and synonyms. Generally, *L. higginsi* is considered to be the Mississippi River counterpart of *L. orbiculata* of the Ohio River system but the question of synonymy has not been settled.

**Distribution of Lampsilis higginsi**

Historically, *Lampsilis higginsi* was found throughout a wide range of the Mississippi River, but was never reported to be abundant. Early reports of *L. higginsi* from the Mississippi River between McGregor and Davenport, Iowa, were relatively frequent. Tryon (1864) reported *L. higginsi* and *L. orbiculata* from Davenport, Iowa. At that time, *L.*
higginsi was known only from Iowa. Witter (1883) noted L. higginsi was common in the UMR but thought that the species was probably L. orbiculata. Pratt (1876) identified L. orbiculata from the Mississippi River at Davenport but did not mention L. higginsi. Shimek (1922) secured both L. orbiculata and L. higginsi from the river at McGregor, Iowa, although he reported both species as rare. Baker's "Catalogue of the Mollusca of Illinois" (1904), lists L. higginsi from the Mississippi River, although no specific locale is given. Utterback (1914) listed the range of L. higginsi as Iowa to Kansas and he collected dead shells of this species from the Mississippi River at Hannibal, and at Louisiana, Missouri. Coker (1919) agreed L. higginsi was uncommon but stated that a few could be found in any carload of shells from Ohio, Illinois, or from the middle Mississippi River.

Grier (1926) appraised the mussels in the Upper Mississippi River (1920 to 1925) and found that some commercial species, including L. higginsi, constituted a smaller proportion of the naiad fauna in some areas in 1925 than in 1920. L. higginsi was considered commercially important at the time, although it was comparatively rare. A total of 6 were found from Red Wing, Minnesota to Homer, Minnesota. Ellis (cited by van der Schalie and van der Schalie, 1950) surveyed the Mississippi River from Cairo,
Illinois, to Lake Pepin, Minnesota 5 years later, and found that *L. higginisi* was a wide ranging species that seldom occurred in large numbers.

The aforementioned surveys were conducted before impoundment of the Mississippi River, and few data were available to assess the impacts of impoundment until 1965. Since 1965, a number of mussel surveys have been conducted at various sites in the Upper Mississippi River. Finke (1966) surveyed Pools 4A, 5, 6, and 7 to assess the effects of clamming, which had resumed in 1964. He reported specimens of *L. higginisi* from Pool 7 at a frequency of 0.49% of the total specimens found. Davis and Cawley (1975), repeating the 1930 survey of Ellis, reported no *L. higginisi*, although Ellis had reported two at comparable sites. Coon et al. (1977) found no *L. higginisi* in pools 8, 9, and 10. Ecological Consultants (1977) collected one individual from Slyvan Slough at Rock Island, Illinois. Cawley (1978) found one female, approximately 10 years old, from Pool 12. Individuals were found in Pools 11, and 14 (4 sites) with a frequency of 8.1% by Perry (1979).

The river surveys mentioned so far all used brailing, sometimes supplemented by handpicking and raking. Later studies found that brailing is low in efficiency, collecting only 1% of the mussels present compared to 100% efficiency when SCUBA is used (Thiel 1981). As a result, it is
conceivable that *L. higginisi* may have been present at other survey sites but the inefficiency of the collection method and the low abundance of this mussel species resulted in low procurement. The Wisconsin Department of Natural Resources (Thiel 1981), using diving and brailing in the survey area of Grier (1922, 1926) and Ellis (van der Schalie and van der Schalie 1950), found *L. higginisi* to have the same abundance as in the previous surveys. Other recent findings of *L. higginisi* have included dead individuals from Pools 7 and 8 (Havlik 1983), and live individuals from Pools 9 and 10 (Fuller 1980; Duncan and Thiel 1983; Miller et al. 1985), 14, 16, and 17 (Ecological Analysts 1981a,b), and 18, and 19 (Cawley 1985).

*Lampsilis higginisi* was historically present in a number of tributaries of the Mississippi River. In Illinois it was reported from the Rock River (Baker 1926), the Illinois River (Danglade 1914, Kelly 1899), and the Sangamon River (Havlik 1981). Starrett (1971) reported that *L. higginisi* was recently eliminated from the Illinois River as a result of pollution and siltation. Dawley (1947) found *L. higginisi* in the Minnesota River. Wilson and Danglade (1914) reported none from Minnesota rivers, and no specimens have been found there in more recent surveys. In Wisconsin, *L. higginisi* was reported from the St. Croix River (Dawley 1947; Fuller 1980; Stern et al. 1982). *Lampsilis higginisi* was also reported
from the Iowa, Cedar, and Wapsipinicon Rivers in Iowa (Witter 1883; Shimek 1888; Gieser 1910), but it has not been reported from these rivers since. Utterback (1914) collected *L. higginsi* from Missouri lakes and from the Missouri River proper. Records exist of *L. higginsi* in the Elkhorn River, Nebraska (Havlik 1981) and in the Nemaha River, Nebraska, (Aughey 1877), but Havlik (1981) suggested these rivers were out of its range. Specimens have been recorded as *L. higginsi* from several southern rivers, including the White River in Arkansas and Arkansas River in Tennessee (Wheeler 1918; USFWS 1950). However, Havlik (1981) suggests these specimens are a form of the related *L. orbiculata*. Presently, *Lampsilis higginsi* reportedly exists in the UMR, in the St. Croix River near Hudson, Wisconsin, and perhaps in isolated areas in the Wisconsin and Illinois Rivers.

The "*Lampsilis higginsi* Recovery Plan" (Stern et al. 1982) listed seven "essential habitat" areas for *L. higginsi* that should be preserved. The locations were designated as essential based on (1) the historical and present distributional records, (2) the nature of the collection data, and (3) the nature of the associated fauna. The essential habitat areas included:

- St. Croix River opposite Hudson, WI
- Mississippi River, Pool 9, at Whiskey Rock, opposite Ferryville, WI
Mississippi River, Pool 10, at Harper’s Slough
Mississippi River, Pool 10, main and east channel at
Prairie du Chien, WI and Marquette, IA
Mississippi River, Pool 10, at McMillan Island
Mississippi River, Pool 14, Cordova, IL
Mississippi River, Pool 15, Sylvan Slough

Nine secondary habitats were also designated in the Mississippi River between Pool 11 and 17.

A survey of tributaries such as the Wapsipinicon, Rock, and Minnesota Rivers may also determine whether these historic habitats are suitable for re-establishing *L. higginsi* and associated species. These tributaries are generally less disturbed by impoundment, navigation, dredging, and mussel harvest than the Mississippi mainstream populations, and as a result may be possible relocation sites.
EXPLANATION OF DISSERTATION FORMAT

The dissertation is presented in four chapters, each of which is meant to stand alone. Each chapter contains its own introduction and discussion. A general summary of the dissertation follows chapter four and a single list of references serves all chapters.

Chapter 1 details efforts to differentiate the glochidia of *Lampsilis higginsi* from related species. This was a necessary first step in the study of this mussel’s early life history.

Chapter 2 deals with efforts to determine suitable fish hosts for glochidia of *Lampsilis higginsi* in the laboratory, following field efforts to differentiate the glochidia.

Chapter 3 details efforts to develop methods for culturing the glochidia and juveniles of three *Lampsilis* species, including *Lampilis higginsi*, in the laboratory for future research and conservation programs.

Chapter 4 examines the pathological response of susceptible and non-susceptible fish species to infection by glochidia of *Lampsilis radiata siliquoidea*. Transmission electron microscopy was used for the first time to examine the host cellular and tissue responses to glochidia.
SECTION I. MORPHOLOGY OF THE GLOCHIDIA OF LAMPSILIS HIGGINSI COMPARED WITH OTHER UPPER MISSISSIPPI RIVER MUSSEL SPECIES

Submitted for publication in Malacological Review
INTRODUCTION

The glochidia of unionid freshwater mussels are obligate parasites on the gills or fins of fishes. Glochidia discharged from the marsupial gills of females attach and encapsulate on fish and undergo organogenesis to the juvenile stage (Coker et al. 1921). Information on the life history and recruitment of mussel species can be readily developed by the collection and identification of glochidia. For example, Zale and Neves (1982a,b) using light microscopy, determined the timing of glochidial release, periods of infection, and the identity of fish hosts for four lampsiline mussels by collecting and identifying glochidia in stream drift and on fish gills.

Glochidia of the endangered mussel Lampsilis higginsi are morphologically similar to those of several other lampsiline species in the Upper Mississippi River (Surber 1912, 1915). Information about the reproductive cycle and host fishes could be readily determined if a method for operational/field identification of the glochidia of L. higginsi was available.

Several methods have been used to study glochidia. Lea (1860) first described the glochidia of 38 species of freshwater mussels, including L. radiata, L. ventricosa, and Ligumia recta. Using light microscopy, Lea provided drawings
and brief descriptions of each. Some of the features Lea used for differentiating species were the granulation patterns and diameter of the granules on the external surface, the shape and size of the single adductor muscle, and the color of the larval shells in the marsupium. Unfortunately, Lea’s drawings were not scaled to size and no measurements were provided for most species. Subsequent investigators have also described the shell shape and gross features using light microscopy (Lefevre and Curtis 1910a; Surber 1912, 1915; Utterback 1933; Inaba 1941); standard length and height measurements were made by Surber (1912, 1915) and Wiles (1975). Inaba (1941) added a length:height ratio, and Zale and Neves (1982a) included a measurement of hinge length.

Scanning electron microscopy (SEM) has been used by several researchers for description and differentiation of glochidial species. Rand and Wiles (1982) were able to distinguish Anodonta cataracta from A. implicata using SEM to examine the structure of shell valves and terminal plate structures. Clarke (1981, 1985) described anodontine glochidia of the Tribe Alasmidontine using SEM. L. radiata (Calloway and Turner 1978) and Actinonaias carinata (Heffelfinger 1969) have been the only lampsiline species studied with SEM to date. Although Surber (1912) provided camera lucida drawings and measurements of glochidial length
and width from samples of *L. higginsi*, he provided no definitive identification of the species. No further studies of *L. higginsi* glochidia have been reported.

Our objective was to ascertain simple techniques that could be used routinely in the field, including light microscope examination and measurements of shell dimensions, to differentiate the glochidia of *L. higginsi* from those of other in the Upper Mississippi River system. In addition, scanning electron microscopy was used to study the comparative ultrastructure of the shells of *L. higginsi* and the three most similar species of the UMR, *L. radiata silikuioidea*, *L. ventricosa*, and *Ligumia recta*. 
MATERIALS AND METHODS

Gravid females of 18 species of mussels were collected from the Upper Mississippi River (Navigation pools 7 and 10) by handpicking and brailing. The species collected included: *Lampsilis higginsi*, *L. radiata siliquoidea* (here termed *L. radiata*), *L. ventricosa*, *L. teres*, *Ligumia recta*, *Amblema plicata*, *Arcidens confragosus*, *Ellipsaria lineolata*, *Elliptio dilatata*, *Leptodea fragilis*, *Megalonaias gigantea*, *Obliquaria reflexa*, *Obovaria olivaria*, *Quadrula pustulosa*, *Q. quadrula*, *Strophitus undulatus*, *Truncilla donaciformes*, and *T. truncata*. Glochidia were extracted from females by flushing the marsupial portion of the gill with water by means of a hypodermic needle and syringe. Glochidia that were viable and therefore selected for examination responded by snapping their valves shut when placed in a 1.0% NaCl solution. Other glochidia came from females preserved in 10% formalin or 70% ethanol. For measuring length (maximum anterior-posterior), height (maximum dorsal-ventral), and hinge length, we examined 20 glochidia per female under a microscope (100x) fitted with an ocular micrometer. In addition, the glochidia of *L. ventricosa* were used to detect changes in shell size due to preservation. Twenty glochidia from six *L. ventricosa* females were measured fresh and 20 were measured after preservation in formalin or 70% ethanol. Comparisons of
length, width, and hinge length were made using analysis of variance.

Data analyses were conducted using the Statistical Analysis System (SAS Institute 1979) at Iowa State University, Ames. Statistical significance is defined as P>0.05.

Photographs were taken of representative specimens of each species for qualitative comparisons of general shell features. All aspects of the shell were photographed, including lateral views showing the shape of the shell and hinge, and the position, size, and shape of adductor muscle; a dorsal view showing the hinge and beak sculpture; and an anterior-posterior view showing the flange and shell gape.

Glochidia samples of L. higginsi, L. radiata, L. ventricosa, and Ligumia recta were fixed in 10% formalin and held in 70% ethanol for scanning electron microscopy. Samples were prepared by critical point drying and sputter coating with platinum palladium (Postek et al. 1980). Shells were studied at magnifications of 300x to 10,000x.
RESULTS

Light microscopy

Our general observations of the shells were similar to those of Lefevre and Curtis (1910a,b) for each shell type in shape of the shell, the double margin around the periphery of the shell, granulations on the lateral surface, the position and shape of the adductor muscle, and the presence of microprojections. The glochidia could initially be categorized as hooked or hookless and further separated into groups by their overall shape and relative size (Table 1):

A. Hookless

1. Lampsiline—elliptical or horse-shoe shape, length greater than width, hinge length approximately one-half width (Figures 1 and 2).

   a. Small sized—length less than 0.100 um.

      Quadrula quadrula (Figure 3)  
      Leptodea fragilis (Figure 4)  
      Truncilla donaciformes (Figure 5)  
      Truncilla truncata (Figure 6)

   b. Medium sized—length 0.230-0.270 um

      Lampsilis higginsi (Figure 7)  
      Lampsilis radiata (Figure 8)  
      Lampsilis ventricosa (Figure 9)  
      Ligumia recta (Figure 10)
Obovaria olivaria (Figure 13)
Lampsilis teres (Figure 14)
c. Large sized—length greater than 0.280 μm.
Quadrula pustulosa (Figure 15)
Megaloniais gigantea (Figure 16)
Ellipsaria lineolata (Figure 17)

2. Anodonte—nearly circular; length and width approximately equal, hinge length greater than one-half width.
Obliquaria reflexa (Figure 18)
Amblema plicata (Figures 19 and 20)
Elliptio dilatata (Figures 21 and 22)

B. Hooked—triangular valve with prominent ventral hook.
Arcidens confragosus (Figure 23)
Strophitus undulatus (Figure 24)

Lampsilis higginsi was most similar in shape and size to L. radiata, L. ventricosa, and Ligumia recta (Table 1). The shape and appearance of the shells of these four species were so similar that identification by observation with the light microscope was not possible (Figures 7-10). When profiles of the shells of each species were compared by overlaying transparencies of shells of the same size, no obvious differences in shape could be detected, although in
Figure 1. *Lampsilis* glochidia following removal from the marsupium are gaping and open to an angle of about 90°.

Figure 2. Conglutinate of *Lampsilis* glochidia which are not yet infective. Many glochidia are still surrounded by a thin membrane and do not close in a saline solution.
Figure 3. Glochidia of *Quadrula quadrula*

Figure 4. Glochidia of *Leptodea fragilis*
Figure 5. Glochidia of *Truncilla truncata*

Figure 6. Glochidia of *T. donaciformes*
Figure 7. Glochidia of *Lampsilis higginisi*

Figure 8. Glochidia of *L. radiata siliquoidea*

Figure 9. Glochidia of *L. ventricosa*

Figure 10. Glochidia of *Ligumia recta*

Figure 11. Gaping shell of a *L. higginisi*
   A pair of microprojections is evident in each valve.

Figure 12. Abnormally long glochidia of *Ligumia recta*
Figure 13. Glochidia of *Obovaria olivaria*

Figure 14. Glochidia of *L. teres*

Figure 15. Glochidia of *Q. pustulosa*

Figure 16. Glochidia of *Megalonaias gigantea*
Figure 17. Glochidia of *Ellipsaria lineolata*

Figure 18. Glochidia of *Obliquaria reflexa*
Figure 19. Glochidia of *Amblema plicata*

Figure 20. Anodontine glochidia have a shell gape of 180°.

Figure 21. Glochidia of *Elliptio dilatata* and an unfertilized egg (arrow) which was also present in the marsupium.

Figure 22. Gaping and closed glochidia of *Elliptio dilatata*.
Figure 23. Glochidia of *Strophitus undulatus*

Figure 24. Glochidia of *Arcidens confragosus*
one female *Ligumia recta* about 4% of the glochidia were much higher than most glochidia of this species (height $x=296 \mu m$) (Figure 12). The relative position of the hinge ligament could be discerned in some glochidia of each species at 400x. The hinge ligament in *Ligumia recta* was centrally located whereas that in the three *Lampsilis* species was more posterior. The adductor muscle is lost soon after a glochidium attaches to a fish and as a result, it is not a reliable feature for use in glochidial identification. Other features of the glochidium were not adequately resolved by light microscopy to be useful for species identification.

**Statistical analyses**

One-way analysis of variance revealed that overall significant differences existed among *L. higginisi*, *L. radiata*, *L. ventricosa*, and *Ligumia recta* in the three morphometric characteristics measured. However, the source of the difference was not due to *Lampsilis higginisi*, but to *L. radiata*, which was significantly greater in length, height, and hinge length than the other three species (which did not differ significantly from one another, Table 1). Ranges of parameters also overlapped, with those of *L. higginisi*, in *Obovaria olivaria* and *L. teres*. However, both were statistically smaller in all dimensions and the shape of
Table 1. Measurements of three shell parameters: \(N=\)number of glochidia measured and \(f(N)=\)number of females sampled

<table>
<thead>
<tr>
<th>Species</th>
<th>N</th>
<th>f(N)</th>
<th>Mean height ±S.D. (µm)</th>
<th>Range</th>
<th>Mean length ±S.D. (µm)</th>
<th>Range</th>
<th>Mean hinge ±S.D. (µm)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lampsilines</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leptodea fragilis</td>
<td>120</td>
<td>6</td>
<td>88.1 ± 3.55</td>
<td>79-95</td>
<td>75.6 ± 3.39</td>
<td>68-84</td>
<td>37.4 ± 2.11</td>
<td>31-42</td>
</tr>
<tr>
<td>Quadrula quadrula</td>
<td>100</td>
<td>5</td>
<td>92.9 ± 3.10</td>
<td>83-100</td>
<td>83.6 ± 3.22</td>
<td>78-93</td>
<td>37.8 ± 3.27</td>
<td>31-47</td>
</tr>
<tr>
<td>Truncilla truncata</td>
<td>60</td>
<td>3</td>
<td>68.3 ± 2.95</td>
<td>58-74</td>
<td>54.7 ± 2.79</td>
<td>52-63</td>
<td>30.9 ± 1.58</td>
<td>26-36</td>
</tr>
<tr>
<td>T. donaciformes</td>
<td>20</td>
<td>1</td>
<td>62.9 ± 0.59</td>
<td>60-63</td>
<td>49.4 ± 2.63</td>
<td>47-53</td>
<td>27.3 ± 2.15</td>
<td>26-32</td>
</tr>
<tr>
<td>L. higginisi</td>
<td>76</td>
<td>3</td>
<td>259.8 ± 8.24</td>
<td>243-274</td>
<td>215.9 ± 5.93</td>
<td>207-232</td>
<td>110.3 ± 4.28</td>
<td>98-119</td>
</tr>
<tr>
<td>L. radiata</td>
<td>220</td>
<td>11</td>
<td>270.1 ± 11.67</td>
<td>231-305</td>
<td>227.7 ± 8.12</td>
<td>205-252</td>
<td>119.5 ± 5.47</td>
<td>105-137</td>
</tr>
<tr>
<td>L. ventricosa</td>
<td>556</td>
<td>19</td>
<td>257.1 ± 9.18</td>
<td>223-283</td>
<td>216.1 ± 7.53</td>
<td>199-238</td>
<td>107.4 ± 4.78</td>
<td>95-124</td>
</tr>
<tr>
<td>L. teres</td>
<td>20</td>
<td>1</td>
<td>232.6 ± 4.59</td>
<td>228-243</td>
<td>186.3 ± 4.75</td>
<td>176-197</td>
<td>99.4 ± 3.97</td>
<td>93-104</td>
</tr>
<tr>
<td>Ligumia recta</td>
<td>180</td>
<td>9</td>
<td>258.5 ± 9.61</td>
<td>236-284</td>
<td>213.0 ± 7.42</td>
<td>199-231</td>
<td>107.1 ± 4.86</td>
<td>89-116</td>
</tr>
<tr>
<td>Species</td>
<td>N</td>
<td>DC</td>
<td>Mean L</td>
<td>Min-L Max-L</td>
<td>Mean W</td>
<td>Min-W Max-W</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------------</td>
<td>----</td>
<td>----</td>
<td>--------</td>
<td>-------------</td>
<td>--------</td>
<td>-------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Obovaria olivaria</strong></td>
<td>60</td>
<td>3</td>
<td>240.6</td>
<td>228-254</td>
<td>199.2</td>
<td>186-212</td>
<td>101.3</td>
<td>93-109</td>
</tr>
<tr>
<td><strong>M. gigantea</strong></td>
<td>20</td>
<td>1</td>
<td>335.3</td>
<td>321-347</td>
<td>261.6</td>
<td>248-280</td>
<td>152.7</td>
<td>140-166</td>
</tr>
<tr>
<td><strong>Quadrula pustulosa</strong></td>
<td>20</td>
<td>1</td>
<td>285.1</td>
<td>278-294</td>
<td>228.1</td>
<td>226-231</td>
<td>87.2</td>
<td>79-95</td>
</tr>
<tr>
<td><strong>E. lineolata</strong></td>
<td>20</td>
<td>1</td>
<td>363.8</td>
<td>347-378</td>
<td>270.4</td>
<td>252-284</td>
<td>109.5</td>
<td>105-116</td>
</tr>
<tr>
<td><strong>Anodontines</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Amblema plicata</strong></td>
<td>50</td>
<td>3</td>
<td>209.9</td>
<td>189-226</td>
<td>202.9</td>
<td>189-226</td>
<td>130.3</td>
<td>116-152</td>
</tr>
<tr>
<td><strong>Elliptio dilatata</strong></td>
<td>20</td>
<td>1</td>
<td>219.9</td>
<td>212-228</td>
<td>203.6</td>
<td>197-212</td>
<td>143.9</td>
<td>135-150</td>
</tr>
<tr>
<td><strong>Obliquaria reflexa</strong></td>
<td>86</td>
<td>5</td>
<td>232.3</td>
<td>210-243</td>
<td>220.8</td>
<td>210-233</td>
<td>121.1</td>
<td>105-140</td>
</tr>
<tr>
<td><strong>Hooked species</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>A. confraaosus</strong></td>
<td>20</td>
<td>1</td>
<td>371.7</td>
<td>357-389</td>
<td>361.2</td>
<td>347-378</td>
<td>239.7</td>
<td>221-252</td>
</tr>
<tr>
<td><strong>S. undulatus</strong></td>
<td>20</td>
<td>1</td>
<td>299.3</td>
<td>284-326</td>
<td>359.1</td>
<td>315-389</td>
<td>268.3</td>
<td>242-284</td>
</tr>
</tbody>
</table>
Obovaria olivaria was more rounded than that of *L. higginsi* (Figures 7 and 13).

Multivariate (principal component) analysis also did not separate *L. higginsi* from the other species (Figure 25). The first principal component had similar loadings for all three characteristics (height=0.59, length=0.60, hinge length=0.54) and accounted for 77% of the total variance in the correlation matrix; component 2 (hinge length=0.83, height=0.47, length=0.29) accounted for 16% of the variance. Again, *L. radiata* could be separated from the other three species by its larger size, but *L. higginsi* did not differ significantly from *L. ventricosa* and *Ligumia recta*.

Glochidia of *L. higginsi* were correctly classified in 39% of the observations by discriminant analysis, but 55% were misclassified as either *L. ventricosa* or *Ligumia recta* (Table 2). Correct classifications were 50% for *L. ventricosa* and 48% for *Ligumia recta*. Discriminant function analysis was the most accurate for glochidia of *L. radiata*, correctly classifying 83% of the glochidia; 10% were misclassified as *L. higginsi*. 
Figure 25. Principal component analysis of three shell parameters of the glochidia of four lampsiline mussels
Plot of Principal 1 vs. Principal 2

- L. higginsi
- L. ventricosa
- L. radiata
- Ligumia recta
Table 2. Discriminant analysis classification summary

<table>
<thead>
<tr>
<th>Species</th>
<th>L. higginsi</th>
<th>L. radiata</th>
<th>L. ventricosa</th>
<th>Ligumia recta</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. higginsi</td>
<td>39</td>
<td>6</td>
<td>22</td>
<td>33</td>
<td>96</td>
</tr>
<tr>
<td>L. radiata</td>
<td>10</td>
<td>83</td>
<td>5</td>
<td>2</td>
<td>220</td>
</tr>
<tr>
<td>L. ventricosa</td>
<td>19</td>
<td>8</td>
<td>50</td>
<td>24</td>
<td>556</td>
</tr>
<tr>
<td>Ligumia recta</td>
<td>20</td>
<td>5</td>
<td>27</td>
<td>48</td>
<td>180</td>
</tr>
</tbody>
</table>

There was no significant difference between measures of live and preserved samples (Table 3). It was assumed that no appreciable shrinkage occurred in the formalin and alcohol fixative and therefore differences in shell dimensions were not attributable to fixation.
Table 3. Mean measurements of live and preserved glochidia of *Lampsilis ventricosa*. Means for each measured characteristic are not significantly different from each other ($P > 0.05$)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Number of valves</th>
<th>Length (µm)</th>
<th>Width (µm)</th>
<th>Hinge length (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live</td>
<td>100</td>
<td>263.5</td>
<td>220.4</td>
<td>111.2</td>
</tr>
<tr>
<td>Preserved</td>
<td>120</td>
<td>259.4</td>
<td>221.0</td>
<td>109.7</td>
</tr>
</tbody>
</table>

**Scanning electron microscopy**

Scanning electron microscopy showed that all four species have similar surface features (Figure 26). A series of semi-circular ridges on the lateral surface become wrinkled near the hinge (Figure 27). In addition, each valve has many pits on both the internal and external surface, which have sometimes been interpreted as pores (Figures 27, 28 and 29) (Arey, 1924; Zs.-Nagy and Labos, 1969; Calloway
and Turner, 1978; Rand and Wiles, 1982). When viewed from the lateral external surface, the shell does not appear to be porous, but in cross-sectional and internal examinations of the valves, the pits appeared to be continuous. This apparent discrepancy may have been explained by Calloway and Turner (1978), who noted that the external surface appeared perforated only at accelerating voltages of 20 kilovolts and greater. They concluded that the periostracum was not perforated and that the appearance of pores on the outer surface was an artifact of the scanning electron microscope. Perhaps electrons penetrate the periostracum at 20 kilovolts, making it appear transparent and the pits appear as pores. Posterior and anterior edges of each valve are flattened near the dorsal aspect, forming a smooth surface, about 65 μm long (dorso-ventral) and 25-40 μm wide (medial-lateral), here referred to as dorsal ridges (Figure 26). The peripheral edges of the valves are turned inward to form a continuous shelf around the inner margin. Ventrally, the shelf forms a flange or lip believed to be analogous to the hook of anodontine mussels described by Lefevre and Curtis (1910a,b). The flange is about 13-19 μm wide and extends the width of the ventral shell margin (Figure 28, 29). Fine, tooth-like projections, previously described as microstyles (Clarke, 1985), cover all except the proximal one-third of the flange (Figure 28). The microstyles decrease in length to
micropoints on the inner edge of the flange. In all four species, the microstyles are arranged in irregular vertical rows, and about 14-17 rows cover the flange from the inner to the outer edge. The inner shell margin provides an attachment site for the mantle, a thin sheet of epithelium covering the inner valve surface except in the region of the adductor muscle. The single adductor muscle was also seen internally near the dorsal margin. A pair of cylindrical microprojections, about 24-26 μm long, previously described as sensory hairs (Lefevre and Curtis 1910a), is near the ventral margin of the valve (Figure 11 and 30). At the dorsal edge of the valve, the shelf folds inward forming an articulating surface for junction of the valves. The larval ligament connects the valves at this hinge line (Figure 31).

We concentrated on three features of the shell in our efforts to distinguish among the species: (1) position of the hinge ligament, (2) flattened dorsal ridge width, and (3) sculpturing on the lateral shell surface. The first two features proved to be the most useful for separating L. higginsi. Hinge ligaments were central in glochidia of Ligumia recta, whereas they were slightly more posterior in L. higginsi, L. ventricosa, and L. radiata (Figure 31). The dorsal ridges of each valve, measured at their greatest width, differed among species (Table 4). The ridge width was narrower in L. higginsi and Ligumia recta (250-300 μm) than
in *L. ventricosa* and *L. radiata* (280–400 μm).

The shell sculpture showed no discernable differences among the species, though there was some subtle variation. We attempted to identify photographs of each species on the basis of shell sculpture alone, but could not consistently detect a representative pattern on each shell.
Figure 26. Anterior view of a glochidium of *L. higginsi*
Scanning micrograph, d=dorsal ridge

Figure 27. Lateral view of a typical *Lampsilis* shell
The shell sculpturing becomes more rugose near the dorsal shell margin. Scanning micrograph

Figure 28. Ventral flange (f) showing microstyles and internal pits (arrow) Scanning micrograph

Figure 29. Ventral flange (f) and a portion of the lateral shelf Scanning micrograph

Figure 30. Gaping shell of *L. radiata*
The thin sheet of mantle tissue covers the inner surface of both valves. Microprojections and the adductor muscle are also evident. Scanning micrograph

Figure 31. Internal view of the dorsal shell margin in *L. radiata*
The hinge ligament (L) is posteriorly located in this species. Scanning micrograph
Table 4. Comparison of the mean width of the dorsal ridge of four lampsiline mussels. Means with the same superscript are not significantly different from each other ($P > 0.05$)

<table>
<thead>
<tr>
<th>Species</th>
<th>Number</th>
<th>Mean width (μm)</th>
<th>S.D. (μm)</th>
<th>Range (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. higginsi</em></td>
<td>9</td>
<td>27.20$^a$</td>
<td>1.75</td>
<td>25.00-29.20</td>
</tr>
<tr>
<td><em>L. radiata</em></td>
<td>9</td>
<td>33.48$^b$</td>
<td>3.13</td>
<td>28.00-37.90</td>
</tr>
<tr>
<td><em>L. ventricosa</em></td>
<td>10</td>
<td>34.70$^b$</td>
<td>3.06</td>
<td>30.00-40.00</td>
</tr>
<tr>
<td><em>Ligumia recta</em></td>
<td>8</td>
<td>28.66$^a$</td>
<td>1.46</td>
<td>25.80-30.00</td>
</tr>
</tbody>
</table>
CONCLUSIONS AND DISCUSSION

The objective of this study was to find an operational/field method for routine identification of *Lampsilis higginsi* glochidia. Light microscopy and statistical analyses of shell dimensions were found to be inadequate for species differentiation. Scanning electron microscopy can be used to differentiate glochidia of *L. higginsi* from the other three species on the basis of the position of the hinge ligament and the width of the dorsal ridges, but the technique is expensive and impractical for identification of small samples of glochidia collected in the field. The technique may be of use when there is justification for a significant investment of time and expense in identification of glochidia. Hoggarth and Cummings (1986) used scanning electron microscopy to identify glochidia of *Anodonta grandis grandis* on fish in field collections and suggested this technique was more labor efficient than artificial infection experiments for determining host fishes. On the contrary, we have found that artificial infection requires much less equipment, training, and expense than scanning electron microscopy and is more practical for routine use.

Laboratory culture of glochidia and juveniles (Isom and Hudson 1982; Hudson and Isom 1984) may be another route for
developing early life histories. Investigators may follow the growth of a mussel and document developmental stages at which *L. higginsi* can be positively differentiated from related species by light microscopy. One could then verify fish hosts by holding field-collected fish in the laboratory until juvenile mussels have dropped off and developed into an identifiable stage.
SECTION II. DETERMINATION OF SUITABLE FISH HOSTS FOR THE GLOCHIDIA OF
LAMPSILIS HIGGINSI

Accepted for publication in Malacological Review
INTRODUCTION

The life cycle of freshwater unionid mussels includes a parasitic larval stage on the fins or gills of fishes. Larvae (glochidia), released from the marsupial gills of female mussels, must attach and become encapsulated in the gills or fins of appropriate fish species before they can develop to the juvenile stage (Coker et al. 1921). Information on this critical component of the life cycle of *Lampsilis higginsi*, an endangered mussel of the Upper Mississippi River, is scarce. Early investigators reported that the sauger, *Stizostedion canadense*, (Coker et al. 1921; Surber 1913; Wilson 1916), and freshwater drum, *Aplodinotus grunniens* (Coker et al. 1921; Wilson 1916), were fish hosts, based on observations of natural infections. However, the validity of these field determinations is questionable because the glochidia of *L. higginsi* closely resemble those of other species. Sylvester et al. (1984) suspected that the walleye (*Stizostedion vitreum vitreum*) and largemouth bass (*Micropterus salmoides*) might also be hosts for *L. higginsi*, based on prolonged attachments of the glochidia on artificially infected fish of these species. However, no transformed juveniles were recovered. Walleye and largemouth were demonstrated to be suitable hosts for the closely related species *L. ventricosa* (Waller et al. 1986). We
describe the results of laboratory tests of the suitability of 16 species of fishes as hosts for glochidia of L. higginsi. In addition, we ranked the quality of three species of the Family Centrarchidae as hosts for the glochidia of L. higginsi to determine if glochidia demonstrated a specificity for host fishes within a family.
MATERIALS AND METHODS

Glochidia were obtained from three different female Lampsilis higginisi. Mussels were collected in June 1985 and 1986 from the Mississippi River near Prairie du Chien, Wisconsin, and the glochidia of each female were used in separate trials. Glochidia were flushed from the marsupia with distilled water, using a hypodermic syringe and needle. The viability of glochidia was determined by adding a few grains of sodium chloride to a 1-2 ml suspension of glochidia; larvae capable of attachment to fish responded by snapping shut (Zale and Neves 1982b).

Fifteen species of fishes were exposed to glochidia of L. higginisi in four separate trials. Fish were exposed to glochidia by one of two methods; to minimize handling, smaller fish were infected by placing individuals in a fingerbowl (15 cm in diameter) containing a suspension of glochidia for 30 sec (Lefevre and Curtis 1910a,b). Larger fish were exposed by pipetting a drop (200-1000 individuals) of the glochidial suspension into one branchial cavity. All fish were young-of-the-year and all were hatchery reared except northern pike (Esox lucius), sand darter (Ammocrypta clara), log perch (Percina caprodes), Johnny darter (Etheostoma nigrum), white bass (Morone chrysops), and long nose gar (Lepisosteus osseus). The latter were collected
from the Upper Mississippi River in areas where populations of mussels were small or absent. Field-collected specimens were checked for natural glochidial attachments before the tests were begun. The number of individuals of each species tested varied, depending on availability at the time of testing (Figure 1).

Six species of fishes were exposed by the bowl method in the first series: fathead minnow (*Pimephales promelas*), common carp (*Cyprinus carpio*), bluegill (*Lepomis macrochirus*), largemouth bass, smallmouth bass (*M. dolomieu*) and yellow perch (*Perca flavescens*). Water temperatures fluctuated between $15^\circ C$ and $28^\circ C$ due to flow rate changes in the water system, and mortality was high in the largemouth bass, smallmouth bass, and yellow perch. A second series of tests was performed on largemouth bass and smallmouth bass, but yellow perch were not available for retesting. Bluegills were also retested in trial 2 because one juvenile mussel was recovered in the first test. Three additional species were used in the second trial, green sunfish (*Lepomis cyanellus*), northern pike, and walleye. All fishes except walleye were infected by the pipette method in the second trial.

In trials 3 and 4, glochidia from an individual *L. higginsi* were used for infections. Four groups of fishes were infected by the pipette method in trial 3: brook stickleback (*Culea inconstans*), white bass, emerald shiner
(Notropis atherinoides), and sand darter. In trial 4, brook sticklebacks were retested; they maintained attachments for the duration of trial 3 but only 12 juveniles were recovered. Sand darters were retested since they were field collected and a question existed about possible immune development. In addition, longnose gar, northern pike, log perch, and Johnny darter, were examined. Fish were infected in trial 4 by the pipette method.

Fishes were inspected under a dissecting microscope for attached glochidia 1 hr after exposure to assess initial infestation rates; thereafter half of the fish in each tank were examined at about 3 day intervals. Fish were held in 38 l flow-through aquaria at temperatures averaging $21.6^\circ \pm 5^\circ$, $21.4^\circ \pm 5^\circ$, $22.2^\circ \pm 3^\circ$, and $21.9^\circ \pm 3^\circ$, respectively. A polyurethane hose, 1.5 cm in diameter, and a 150 \(\mu\)m nylon mesh screen were used to siphon the bottom of the tanks daily to check for transformed juveniles. Siphoned material was examined under a dissecting microscope. Metamorphosis was considered to have occurred if juvenile features were observed (i.e., movement of the foot, ciliary action, opening and closing of the shell, and separation of two adductor muscles).

The tests of host quality were run on three species of the Family Centrarchidae, largemouth bass, bluegill, and green sunfish, based on the results of earlier infection
trials. Ten fish of each species were infected by pipetting one drop of glochidia into the right branchial cavity. Fish were then held separately in 3.4 l jars in a static system at 20-21°C. The total number of sloughed glochidia and metamorphosed juveniles produced per fish was determined by pouring the contents of each jar through a 150 µm mesh screen every other day and examining the contents under a dissecting microscope. The gills of each fish were also checked every 2-3 days to count the number of glochidia attached. The percent transformation for each fish was determined by the following:

\[
\frac{\text{number of juveniles recovered}}{\text{total number of glochidia sloughed and attached}} \times 100
\]
RESULTS

Glochidia of *L. higginisi* encapsulated on and produced at least one metamorphosed juvenile on each of nine species: northern pike, brook stickleback, bluegill, green sunfish, largemouth bass, smallmouth bass, white bass, yellow perch, and walleye. The trials indicated that largemouth bass, smallmouth bass, walleyes, yellow perch, brook stickleback, and white bass were suitable hosts for glochidia of *L. higginisi*. Juveniles were found in the tanks containing these species beginning 14 to 20 days post infection. Green sunfish produced 41 juvenile mussels, but some fish lost their infections beginning on the fifth day of infection. One juvenile mussel was produced by bluegills and northern pike. The number of infected bluegills, northern pike, and Johnny darters declined over the course of the experiment. Common carp, fathead minnow, emerald shiner, sand darter, log perch, and longnose gar lost all glochidia in the first 48 hr of infection. The rate of development of *L. higginisi* on the nine suitable host species was similar; the recovery of juveniles peaked 18-24 days post-infection.

The test of host quality among the three Centrarchidae species, largemouth bass, green sunfish and bluegill, ranked largemouth bass highest by virtue of the number of juveniles produced per fish (7.68%). Transformation was lowest on
Figure 1. Artificial infection of fishes with glochidia of *Lampsilis higginsi* in four separate trials

\( n=\)number of fish, \( j=\)number of juveniles produced, 
CAP=common carp, BLG=bluegill, FHM=fathead, 
LMB=largemouth bass, SMB=smallmouth bass, 
YEP=yellow perch, NOP=northern pike, GSF=green sunfish, WAE=walleye, ES=emerald shiner, SD=sand darter, Stk=brook stickleback JD=Johnny darter, 
Gar=longnose gar, LP=logperch  Shaded areas indicate periods during which juveniles were recovered. Arrows indicate the peak time of juvenile recovery, if one existed
Figure 1 (continued)

DAYS POST INITIAL INFECTION
bluegills (0.00038%) with green sunfish showing an intermediate condition (2.43%). Analysis of variance showed a significant difference in percent transformation between that found for largemouth bass and values for the other two species (P>0.005). However, no significant differences existed between bluegill and green sunfish. The greatest sloughing of glochidia occurred in the first three days of the test. Consequently, the number of glochidia still attached to the gills after day three appeared to be a better indicator of the number of glochidia that attached and encapsulated than counts done earlier. Percentages of juveniles produced per fish were higher when the initial 3 day drop-off was excluded but did not change the ranking order of the species.

Juveniles were recovered from day 18 to 25 in jars containing largemouth bass. Partially transformed juveniles appeared from day 11 to 15 in the green sunfish jars, indicating sloughing before complete transformation. Juveniles were found on days 16 to 18. One partially transformed juvenile (day 15) and one completely transformed individual (day 18) were recovered from a single bluegill.
CONCLUSIONS AND DISCUSSION

The variation in the rate of development of *L. higginisi* juveniles between the four trials may have resulted from differences in water temperature. Higher water temperatures have been shown to increase the rate of metamorphosis (Howard and Anson 1922; Zale and Neves 1982b). The variation in the number of juveniles that developed on host fish species may have been related to differences in the number of glochidia that initially became attached. These differences may have resulted from different infection methods and variation in the surface areas of the gills or the viability of glochidia from different female mussels. However, a portion of the variation in numbers of juveniles produced by each species also appeared to be due to differences between species as shown by results of the host quality test.

Sylvester et al. (1984) suspected that walleye and largemouth bass were hosts for *L. higginisi*, based on observed glochidial attachments of 35-36 days. The present results support this conclusion. Conversely, Sylvester et al. (1984) reported that green sunfish sloughed all glochidia in 1-3 days. In the present study, juveniles were produced by green sunfish. This difference may due to differences in temperatures during the studies: 12° C (Sylvester et al. 1984) vs 19-22° C (this study); or in the sample size (10
fish vs 36 in the present study). A range in host suitability was apparent in the present study. Largemouth bass, smallmouth bass, walleye, yellow perch, brook stickleback and white bass produced relatively large numbers of juveniles. Although transformations on green sunfish were successful, many glochidia dropped off before complete metamorphosis. Bluegills and northern pike were only marginal hosts, each species producing only one juvenile. The possibility of immunity in the field collected species was considered. However, the prolonged attachment time on some species (northern pike and Johnny darter) suggests that they were not immune; sloughing of glochidia reportedly occurs within 1-3 days in immune fish (Arey 1932b). In addition, the development of immunity generally requires more than one exposure to glochidia (Arey 1932b; Reuling 1919; personal observation) and since these were young-of-the-year fish, it is unlikely that they had been infected more than once in the season. Lastly, reports of metamorphosis on darters are rare (Fuller 1974) so these results are not unusual.

In general, fishes of the Families Cyprinidae, Ictaluridae, and Catostomidae, tested by Sylvester et al. (1984) and in the present study were unsuitable hosts for the glochidia of L. higginii, whereas members of the highly derived families (e.g., Percidae, Centrarchidae,
Gasterosteidae, and Percichthyidae) were suitable. Adult *L. higginisi* live in the main channel and field observations indicate that it is a long-term (bradytactic) breeder that releases glochidia during June and July. Fishes typically sharing the habitat of *L. higginisi* during these months include Percidae species, such as walleyes, saugers, and yellow perch. Although centrarchids primarily inhabit backwater areas where they would be less likely to encounter *L. higginisi* females during glochidial release, they also commonly occur in the main channel.

Zale and Neves (1982b) used field examinations of fishes to determine which species were suitable hosts for four mussel species. Unfortunately, the glochidia of *L. higginisi* are so similar to those of other *Lampsilis* forms that they cannot be readily differentiated from related species (Section I. Morphology). Field verifications of host use are therefore not possible with light microscopy.

Susceptibility to infection with the glochidia of *L. higginisi* varied in the three Centrarchidae species tested. A similar effect appeared in members of the Percidae. Though host quality tests were not conducted on these species, the following gradation was observed: 1) an immediate sloughing of glochidia (sand darter and logperch), 2) a lengthy shedding period with no or little juvenile development (bluegill and Johnny darter) 3) sloughing of some glochidia
but also with production of juveniles (green sunfish and yellow perch) and 4) production of many juveniles (largemouth bass and walleyes). *Lampsilis higginisi* is a member of the Subfamily Ambleminae (Johnson, 1980), which is considered to be a specialized group of freshwater mussels because of parasitic modifications. The apparent selectivity of fishes of highly derived families as hosts by *L. higginisi* may reflect coevolution of this mussel species and its fish hosts. Similarly, the greater susceptibility of certain fishes within a family to infection may reflect the phylogenetic relationships and physiological or biochemical similarities of species within the family.
SECTION III. ARTIFICIAL PROPAGATION OF THE GLOCHIDIA OF LAMPSILIS MUSSELS
INTRODUCTION

The cultivation of freshwater unionid mussels has recently become important to agencies working to preserve the numerous species listed as endangered and threatened. Large scale culture of mussels would be invaluable to the study and preservation of threatened and endangered mussels. It would allow ongoing experimental study without the depletion of natural populations. In addition, the establishment of new populations or the renewal of existing populations might be facilitated by introducing laboratory reared juveniles into suitable habitats. Isom and Hudson (1982) generated considerable interest in artificial cultivation when they reported high rates of success transforming large numbers of glochidia of 12 different mussel species in an artificial medium.

In the early 1900s the U. S. Bureau of Fisheries first considered cultivation when mussel populations were drastically declining due to overharvest by commercial fishermen. Lefevre and Curtis (1910a,b) developed a method for artificially infecting fish to determine host specificity. In 1912, their method was adopted for practical operations. Host fish were artificially infected to produce juvenile mussels. Crews working in the Upper Mississippi River routinely infected fish with glochidia before returning
fish to a river (Ellis, Westfall, and Ellis undated). By 1914, approximately 150 million glochidia of an unknown number of mussel species had been planted in the Upper Mississippi River, the Wabash River, Indiana, and the Black and White Rivers in Arkansas (Coker 1914).

The U. S. Bureau of Fisheries continued to be interested in obtaining juvenile mussels via fish infection for many years (Rich 1924, 1925; Higgins 1933, 1934, 1936; Ellis, Westfall, and Ellis undated); however, cultivation of mussels without a fish host also became an important part of the Bureau's large scale mussel propagation program (Higgins 1927, 1928, 1929). Lefevre and Curtis (1910a) tried various substrates, including fish blood, frog and Necturus blood, fish tissue extracts and bouillon, as culture media all with negative results. Following a series of experiments, Ellis and Ellis (1926, 1927) successfully transformed glochidia of Lampsilis fallaciosa in a solution containing salts, sugar, and an amino acid mixture. However, Ellis and Ellis never published their methods, and details of the media were not made available. In vitro mussel culture was largely abandoned until Isom and Hudson (1982) adopted what was known about Ellis and Ellis' techniques and developed a medium that supports transformation of Ligumia recta, Lampsilis ovata, and several other mussel species. The medium, which was initially designed to support fish cell
growth, included inorganic salts, amino acids, vitamins, glucose, fish blood sera, antibiotics, and antimycotics. Plasma from a variety of fish species, including non-host fishes, was found to support transformation of the glochidia of 12 mussel species (Isom and Hudson 1984).

*Lampsilis higginisi*, a federally endangered species of the Upper Mississippi River, is one of the mussel species identified for propagation by the U.S. Fish and Wildlife Service. We have attempted to propagate juveniles from the glochidia of *Lampsilis higginisi* and two related species, *L. ventricosa* and *L. radiata siliquoidea*, in the artificial medium developed by Isom and Hudson (1982) and via infection of host fish in order to compare the success rate and efficiency of each method for large scale mussel cultures.
MATERIALS AND METHODS

Gravid female mussels of *Lampsilis higginisi*, *L. ventricosa* and *L. radiata siliquoidea* were collected from the Upper Mississippi River and held in aquaria in the laboratory. Glochidia were extracted from the marsupia with a sterile pipette and scalpel and rinsed 5-6 times in a sterile petri plate with sterile deionized water. The glochidia were tested for viability by placing a subsample in a 1% NaCl solution. Viable glochidia responded by snapping shut. Fish blood plasma was collected from rainbow trout (*Salmo gairdneri*), largemouth bass (*Micropterus salmoides*), yellow perch (*Perca flavescens*), and common carp (*Cyprinus carpio*). Blood was prepared by centrifuging for 10 minutes at 1,000 rpm and at 5,000 rpm, removing the plasma, and then filtering the plasma through a 0.20 μm or 0.45 μm pressure filter.

The medium was prepared following the techniques of Isom and Hudson (1982). It consisted of Unionid ringer's solution (NaCl, MgCl₂, NaHCO₃, KCl, and CaCl₂), Eagle's essential and non-essential MEM amino acids (Gibco), MEM (Gibco) vitamins, and 20%w/v dextrose. Culture dishes were prepared with 2 ml artificial medium, 1 ml fish plasma, three antibiotics (Achromycin, Geopen, and Garamycin), and one antimycotic (Amphotericin B). The pH of the media was brought to 7.2-7.4
using 1 M NaOH. One drop of water containing glochidia (200-1000) was added and then each plate was placed in a CO₂ and air incubator at 24°C. Culture in the artificial medium was attempted in six separate trials with 68 replicate plates (x=10.3 plates per trial). An average of 570 glochidia were placed in each plate. To alleviate problems with contamination several different modifications were attempted, including weekly additions of antibiotic and antimycotics at a concentration of 100 μg/ml or increasing the initial dose of the drugs to 200 or 1000 μg/ml. In the last three trials, the plates were also placed under an ultraviolet light for 15 min-24 hr prior to the addition of glochidia. The success rate of transformation was defined as the percentage of juveniles produced per total number of viable glochidia inoculated.

The second method of propagation involved artificial infection of known host fishes. Suitable host fishes for *L. higginisi* and *L. ventricosa* were determined in previous studies (Waller et al., 1986; II. Host Determination). The hosts for *L. radiata siliquoides* have been previously documented (Fuller, 1974). Young-of-the-year, hatchery-reared fishes, including 120 walleye (*Stizostedion vitreum vitreum*), 60 largemouth bass, and 30 yellow perch, were obtained for artificial infections. Fishes were infected by placing one drop of the glochidial suspension (200-1000) into
one branchial cavity (yellow perch) or by placing fish into a bowl containing glochidia for 30-60 seconds (walleyes and largemouth bass). Fishes were held separately in flow-through aquaria at 20-22°C and maintained on a diet of frozen brine shrimp; the aquaria bottoms were siphoned daily beginning approximately 12 days post-infection. Material was filtered through a 150 μm mesh screen and examined under a dissecting microscope for the presence of juveniles.
RESULTS

Glochidia of *Lampsilis higginsi* were successfully transformed in two trials, and juveniles of *L. ventricosa* were produced in one trial, producing a total of 414 juveniles in 13 separate plates. The percentage of transformation averaged 1.67% (x=6.0 juveniles/plate, S.D.=7.78%, range of 0.0%-55.27%). Seventy-six plates failed to produce transformed juveniles (Table 1). Transformations occurred in cultures containing common carp and rainbow trout plasma and were complete about 20-25 days after inoculation. Metamorphosis of glochidia was evidenced by movement of the foot, separation of the two adductor muscles, and opening and closing of the valves.

There was no clear trend in the success and failure of different culture trials. Metamorphosis was found in plates receiving 100 µg/ml and 200 µg/ml, but not in plates receiving 1000 µg/ml. Transformation occurred in both plates that were treated with U. V. light and those that were not.

Artificial infection of fish produced juveniles of all three species 14-20 days post-infection (Table 2). The rate of transformation was not calculated for the individual fish in this study (see II. Host determination). The number of juveniles produced per fish was based on the total number of juveniles produced per total number of fish infected.
Table 1. **In vitro** culture of *Lampsilis* glochidia

<table>
<thead>
<tr>
<th>Trial</th>
<th>No. Plates</th>
<th>% Transformed per plate</th>
<th>Blood source</th>
<th>Dosage (μg/ml)</th>
<th>UV trt</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>L. higginisi</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>1.56</td>
<td>c.carp</td>
<td>100</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.35</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.47</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>0.31</td>
<td>c.carp</td>
<td>100</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2.30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.77</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>0.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8</td>
<td>0.0</td>
<td>c.carp</td>
<td>200</td>
<td>yes</td>
</tr>
<tr>
<td><strong>L. ventricosa</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>0.0</td>
<td>perch, lm.bass</td>
<td>200</td>
<td>no</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>0.0</td>
<td>c.carp</td>
<td>1000</td>
<td>yes</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>1.0</td>
<td>20% carp</td>
<td>200</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.77</td>
<td>80% rainbow</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.94</td>
<td>trout</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>24.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>55.20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Low viability of glochidia (31.5%).
Host mortality occurred within the first 48 hours of infection and was attributed to over-infection of fishes. When mortality was taken into account, the average number of juveniles produced per fish in each tank, was substantially smaller (3.6-4.2), except in the yellow perch, which remained the same since they experienced 0% mortality.

Table 2. Results of cultivation of glochidia of *L. higginisi* *L. radiata*, and *L. ventricosa* via fish infections

<table>
<thead>
<tr>
<th>Fish</th>
<th>Mussel</th>
<th>Juveniles</th>
<th>Fish w/ mortality</th>
<th>Fish wo/ mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>walleye</td>
<td><em>L. higginisi</em></td>
<td>252</td>
<td>75%</td>
<td>4.2</td>
</tr>
<tr>
<td>walleye</td>
<td><em>L. ventricosa</em></td>
<td>225</td>
<td>75%</td>
<td>3.7</td>
</tr>
<tr>
<td>yellow perch</td>
<td><em>L. radiata</em></td>
<td>545</td>
<td>0%</td>
<td>18.2</td>
</tr>
<tr>
<td>lm. bass</td>
<td><em>L. ventricosa</em></td>
<td>285</td>
<td>80%</td>
<td>3.6</td>
</tr>
</tbody>
</table>

no. juveniles/fish
CONCLUSIONS AND DISCUSSION

Isom and Hudson (1982) reported successful transformation of 12 species of mussels in the artificial medium. A transformation rate of 40-60% was common for Lampsilis species (August, 1985, B. G. Isom, Tennessee Valley Authority, Muscle Shoals, AL). In the present study, this success rate was achieved only once in 87 replicates. The remaining rates were much lower (0.0%-24.2%). The major problem in the cultures seemed to be contamination of media and subsequent mortality of the glochidia. In plates that did not yield juveniles, the lack of success was attributed to bacterial and fungal growth. Treatment of the medium with U. V. light prior to addition of the glochidia did help alleviate the problem of bacterial and fungal growth. However, contamination still occurred in these plates, indicating the glochidia themselves were a source of contamination. Low doses of U. V. light to the glochidia may be attempted to lower bacterial and fungal growth caused by the introduction of non-sterile glochidia.

Transformations were successful in plates containing fish blood plasma from common carp and rainbow trout. However, the source of the plasma was not considered to be a contributing factor in the success of transformation of glochidia in this study. These same plates received the
U. V. treatment; in addition, common carp and rainbow trout are not suitable hosts for the glochidia of these three Lampsilis mussels, whereas, largemouth bass and yellow perch are hosts. Transformation would be less likely in the plasma of the common carp and rainbow trout if it were affecting the success of the cultures.

Artificial infection of fishes produced juveniles more consistently than the \textit{in vitro} method. Juveniles were produced in every experimental infection trial, whereas, only 50% of the \textit{in vitro} culture trials yielded juveniles. The transformation rate on each fish was not determined in this study; however, in earlier studies (II. Host Determination), glochidia of \textit{L. ventricosa} averaged 7.68% transformation on smallmouth bass (\textit{Micropterus dolomieu}) compared to 1.67% in the media. Mortality of walleyes and largemouth bass greatly decreased the average number of juveniles produced via artificial infection, reaching values similar to that produced in the medium. Walleyes and largemouth bass were infected by the bowl methods and their high mortality rates may have resulted from prolonged exposure times and overinfection. Mortality from overinfection can be decreased by infecting only one branchial arch by the pipette method.

The rate of transformation is a measure of the success of the culture method, but other factors must be weighed when
considering a culture system. There are advantages and disadvantages to \textit{in vitro} and host propagation of juveniles. Success rates for \textit{in vitro} culture may be low, particularly in early cultures and this method of propagation may require considerable start-up time. Contamination is a constant problem in a medium, even when great care is used in preparation and antibiotics/antimycotics are used. In artificial infection, disease may be a problem only if unhealthy fish are used or water quality is poor. In addition, obtaining fish plasma for use in the medium can be difficult especially in large enough quantities for large scale culture. On the other hand, juveniles obtained via \textit{in vitro} culture may be "cleaner" and thus better suited for experimental purposes. Juveniles can also be examined at different stages of metamorphosis and the effect of various factors on development can be monitored.

Infection of fishes also has its disadvantages. The host fish for the mussel species to be cultured must be known and must be available while any species of fish may serve as a source of fish plasma for \textit{in vitro} culture. In addition, mortality of fish can range from 0-100% if the dose of glochidia given to each fish is not controlled. The collection of juveniles from aquaria may be tedious and time consuming, and juveniles collected in such a manner may introduce other organisms into a culture system.
In conclusion, both of the tested propagation techniques can be of use for production of juvenile mussels. The best choice of culture technique depends on the availability of equipment for either, on information concerning the host specificity of the mussel species of interest, and the intended use of the propagated individuals.
SECTION IV. COMPARISON OF THE PATHOGENESIS ASSOCIATED WITH LAMPSILIS GLOCHIDIOSIS IN A SUSCEPTIBLE AND NON-SUSCEPTIBLE HOST SPECIES USING LIGHT AND ELECTRON MICROSCOPY
INTRODUCTION

An important phase in the development of freshwater mussels is the obligatory parasitic period spent on an appropriate fish host. The larvae (glochidia) infest and become encapsulated on the gills or fins of a fish and undergo organogenesis to the juvenile stage. There are two general types of glochidia. A type with hooks are typical of Anodonta species and generally are considered to be mainly parasites of fish fins. The glochidia of Lampsilis species are hookless and are primarily gill parasites. Lampsilis glochidia show no appreciable increase in size during parasitism; they undergo only internal development while on the fish.

Host specificity

The specificity of a mussel species for certain host fishes became apparent to early investigators who discovered that the glochidia of some mussel species were sloughed soon after infesting unnatural hosts, while those on suitable hosts were retained to the juvenile stage. Several mechanisms have been offered to explain host specificity: glochidia may simply attach only to selected hosts; in some cases, glochidia may reject a fish because it lacks specific chemical cues; the glochidia may attach, but not remain on a
host because of a lack of certain vital nutrients; and glochidia may be rejected by the fishes' immune system. Selective attachment of glochidia has been tested by studying the glochidial response to various stimuli. Lefevre and Curtis (1910a) described wild "snapping" of Lampsilis glochidia in the presence of fish blood and several inorganic salts. Young (1911) described the same response in Margaritifera margaritifera exposed to fish blood, fish mucus, and to fin and gill tissue. Arey (1921) exposed glochidia of four Lampsilis species to various concentrations of acids (hydrochloric acid, acetic acid, and picric acid), a base (KOH), alcohols (ethyl and methyl), sugars, (saccharose and dextrose), oils, (wintergreen and cloves), and salts (KCl, KBr, KI, NaCl, LiC, MgCl₂, and CaCl₂). Arey found that increased concentrations of all the substances (within physiological limits) elicited increasingly stronger closing responses.

Lefevre and Curtis (1910a) found that Lampsilis glochidia responded only slightly to tactile stimulation and concluded that the chemicals of a fish elicited the closing response. However, Arey (1921) and Young (1911) found that the tactile response was much stronger than the response to chemical in Lampsilis glochidia. They surmised that tactile stimulation alone was sufficient for closure and that chemicals may enhance closure after initial attachment.
Davenport and Warmuth (1965) showed that the glochidia of *Anodonta implicata* showed no discrimination in initial host attachment. Glochidia attached equally well to the excised fins of 3 non-host species and to their host, the alewife (*Alosa pseudoharengus*). Meyers et al. (1980) found that glochidia of *M. margaritifera* had a three-fold higher attachment rate for the gills of chinook salmon (*Oncorhynchus tshawytscha*), than for the gills of coho salmon (*O. kisutch*). Glochidia closed on the gills of coho salmon but within minutes opened slowly and detached.

Once attached to the host, glochidia rapidly become covered by a capsule of host tissue comprised of epithelial and connective tissue stroma (Arey 1932a). It has been suggested that the parasite may induce this tissue proliferation (Young 1911; Reuling 1919). Schierholz (1888), Faussek (1895, 1901), Harms (1907), Young (1911), and Lefevre and Curtis (1910a) describe encapsulation as a process of cell proliferation resulting from rapid mitotic division in cells around the glochidial shell. However, Arey (1932a,c) reported no significantly higher numbers of mitotic figures in cells of infected gill tissue during early encapsulation. He suggested that the process of encapsulation was a reparative mechanism accomplished by cell migration into the wound site and not a response to the glochidia per se. He demonstrated the same phenomenon with an aluminum clip
fastened to a gill filament of a fish.

Host immunity to glochidial infestation has been suggested to explain the sloughing of glochidia before metamorphosis is complete. Such sloughing occurs within the first 48 hours of infestation. Lefevre and Curtis (1910a) suggested several mechanical parameters, including the configuration of the mouth parts, texture of the gills, size of the gill opening and rapidity of fin movement, may be factors in the resistance of largemouth bass (Micropterus salmoides) for glochidia of Lampsilis teres. These factors would be more important in preventing solid attachment of the glochidia to the gills rather than causing sloughing of partially encapsulated glochidia.

Arey (1932b) reported that the capsules of "immune" fish were overgrown and thicker in the early part of infection compared to capsules on natural hosts. In some instances, cells of encapsulated glochidia became necrotic presumably due to some cytolysin from the fish; the glochidium would subsequently gape and slough off. In other cases the entire capsule and glochidium were sloughed. Fustish and Millemann (1978) reported that chinook salmon infected with glochidia of M. margaritifera developed smaller diameter capsules and less hyperplasia than coho salmon, which are reportedly less susceptible than chinook salmon to infection. Glochidia of M. laevis were destroyed by host tissue proliferation in
yearling Oncorhynchus muaou, and Salvelinus leucomaenis and in 1-2 year old Salmo gairdneri (Awakura 1968). Eosinophils have been found in the capsule area (Arey 1932b; Fushish and Millemann 1978) as well as eosinophilic plastids and basophilic spherules (extruded chromatin) (Arey 1932b), implying a cytological response by the fish. Reuling (1919) postulated that the mucus of certain fishes may be incompatible with various glochidia or that differing amounts of fibrous tissue in the gills of fishes may play a role in host specificity; however, he found no significant difference in the amount of mucous cells or in the amount of fibrous tissue between susceptible and non-susceptible fishes. Subsequently he tested the blood of non-susceptible fishes for the presence of a cytolysin and demonstrated lysis of glochidia in situ placed in blood from an immune bass starting at 2 hours post-exposure; Lampsilis glochidia placed in plasma from susceptible fishes remained normal for 48 hours. Meyers et al. (1980) found that blood samples taken from a salmonid species resistant to infection (coho salmon) and from a salmonid species susceptible to infection (chinook salmon) by glochidia of M. margaritifera, showed significant increases in hematocrit, hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin, and leukocyte numbers compared with control fish. In salmonids susceptible to infection only the hematocrit, erythrocyte
numbers, and mean corpuscular volume were increased in response to the glochidial challenge while the mean corpuscular hemoglobin decreased. Antibodies to glochidia were demonstrated in the blood plasma and mucus of both species of salmonids 8 to 12 weeks post-infection. Glochidial extracts incubated with mucus and plasma from fishes 8 to 12 weeks post-infection showed the formation of a precipitate 48 hours after incubation. In addition, the in vitro survival time of parasites in mucus and plasma from resistant salmonids was less than in the fluids of susceptible species. In one hour, 60% of the glochidia had died in coho salmon mucus while mortality was 33% in mucus from chinook salmon; in three hours, 41% of the glochidia died in the plasma from coho salmon compared to only 26% in plasma from chinook salmon. Surprisingly, in light of these observations, Isom and Hudson (1984) reported successful transformation of glochidia from 12 mussels species in a medium containing plasma from different fishes including that of reportedly immune species.

Pathogenesis

Glochidiosis has a range of effects on hosts and non-hosts apparently related to the dose of infection and size of the fish. Following infection doses of 600-1200 M. margaritifera glochidia/fish, Murphy (1942) reported 52%
mortality in salmonids due to glochidia blocking blood vessels of the filaments. At 250-700/fish no mortality occurred until fully transformed juveniles began dropping off. The majority of these deaths were attributed to secondary infection of the lesions by aquatic fungi and bacteria. No mortality was reported in fish receiving 30-50 glochidia. Mortality at high infection doses was generally attributed to impaired respiratory and circulatory function, while mortality at lower doses and later in the course of the infection was attributed to secondary infection of the lesion by fungi and bacteria. Moles (1983) also reported a decrease in growth and a decreased fat content in coho salmon fry infected with glochidia of *Anodonta oregonensis*.

**Study objectives**

The ultrastructure of the host capsule and its association with the glochidium have not been examined. In addition the ultrastructure of the capsule in susceptible hosts has not been compared with that in resistant fish. The main objective of this study was to describe the process of encapsulation, at both the tissue and cellular levels, of glochidia by known host fishes. In addition, to determine if cytological responses play a role in resistance to glochidial infestation, the early cellular response of a suitable host species was compared with that of a resistant species.
MATERIALS AND METHODS

Females of *Lampsilis radiata siliquoidea* were obtained from the Upper Mississippi River at La Crosse, Wisconsin. Glochidia were removed from the marsupium by flushing it with water injected via a hypodermic needle and syringe. Walleye (*Stizostedion vitreum vitreum*), averaging 70 mm total length and common carp (*Cyprinus carpio*), averaging 40 mm total length, were obtained as young-of-the-year from the National Fishery Research Center, La Crosse, Wisconsin. Four separate infection trials, two per species, were run in this study. Fishes were individually infected with glochidia by pipetting a one drop suspension (200-1000 glochidia) into the right branchial cavity. Each species was held separately in a 38 l flow-through aquarium at 20-22°C and fed twice daily a diet of frozen brine shrimp and commercial trout food. Control fishes were held separately under the same water, feeding and temperature regimes as the experimental fishes. Fishes were sacrificed and samples of the right gills were taken according to the following schedule:

- **Carp:** 3 fish at 0, 2, 4, 6, 12, 24, and 48 h post-infection
- **Control:** 3 fish at 12 hr in trial 1, 3 fish at 6 hr in trial 2
Walleye: 2 fish at 0, 2, 4, 6, 12, 24, 48 h, 5, 8, 11, 14, 17, 20 and 23 d post-infection

Control: 3 fish at 20 d in trial 1, 3 fish at 6 hr in trial 2

For comparison, observations were also made of uninfected gill tissue on the experimental fishes.

Material for paraffin sectioning was fixed in Bouin's fluid overnight, washed in 70% ethanol and dehydrated through a graded ethanol series, cleared in xylene and embedded in paraffin. Sections of 6-7 µm were stained with hematoxylin and eosin, phosphotungstic acid hematoxylin (PTAH) or periodic acid Schiff's (PAS) reagent.

The glochidial shell is composed partly of calcium crystals and even at this early stage in development, it is hard and crystalline. Fixation methods for ultrastructural study were chosen on the basis of their use in eliminating the problems associated with the shell, including infiltration of the fixative and resin into the shell and softening of the shell for thin sectioning. Three different fixation methods were used for ultrastructure study samples: 1) freeze substitution, 2) Karnovsky's fixative with post-fixation in osmium tetraoxide with decalcification in EDTA, and 3) Karnovsky's fixative, post-fixation in osmium tetraoxide without decalcification. Freeze substitution samples were infiltrated with a graded series of glycerol in
cacodylate buffer at 0° C. Samples were then submersed in 2-methylbutane at -160° C and then placed in 100% ethanol with 1% osmium tetraoxide at -75° C for three days. Tissue was infiltrated and brought to room temperature through a graded ethanol-resin series. Samples for Karnovsky’s fixation were fixed in cold 2.5% gluteraldehyde and 2% paraformaldehyde solution overnight followed by washing for 1 hr in 0.1 M sodium cacodylate buffer, (pH 7.2, 2mM calcium chloride, 5.5% sucrose). One set of samples was then decalcified in 10% EDTA for 30 min. Material was postfixed for 1 hr in 1% osmium tetraoxide in sodium cacodylate buffer, washed in buffer for 1 hr and dehydrated in a graded acetone series. Material was embedded in Medcasts epoxy resin and sectioned on an LKB ultramicrotome with a diamond knife. Semi-thin sections (0.5 μm) were stained with warm 1% toluidine in borax for light microscopy. Ultrathin sections (500 Å) were mounted on formvar coated grids, stained with uranyl acetate and lead citrate and examined in a Hitachi HU11E transmission electron microscope at 50 kV.

Common carp were also examined for evidence of a cellular immune response. Peripheral blood smears were made from 10 fish per sample period. Slides were stained with Giemsa stain and a total leucocyte count was made for each sample.

Identification and description of normal cells of the
gills were obtained from Newstead (1967) and Laurent and Dunel (1980). Leucocytes were identified based on information provided by Weinreb (1963), Ferguson (1976), Lester and Desser (1975) and Cannon et al. (1980). Tissue from all fishes at each sampling period was examined using light microscopy and included complete serial sectioning of two branchial arches from each of the fishes. Ultrastructural examinations at each sampling periods were as follows:

<table>
<thead>
<tr>
<th>Species</th>
<th>Sample time</th>
<th>No. Capsules Examined</th>
<th>No. Fish Examined</th>
</tr>
</thead>
<tbody>
<tr>
<td>Walleye</td>
<td>0-2 hr</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>4-6 hr</td>
<td>14</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>12-48 hr</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>5 day</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>8-11 day</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>14 day</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Carp</td>
<td>0-2 hr</td>
<td>13</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>4-6 hr</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>12 hr</td>
<td>6</td>
<td>3</td>
</tr>
</tbody>
</table>
Measurements of tissue and cell structures were taken from micrographs, and are provided as a general reference of relative sizes of structures. Sample sizes ranged from 2-5 per measurement.
RESULTS

Fixation of cells and organelles was optimal in the Karnovsky's fixative followed by post-fixation in osmium tetraoxide. The EDTA was added in one set of samples to decalcify the glochidial shell and thus make thin sectioning of the tissue easier. However, the EDTA did not make a significant difference in the hardness of the shell and as a result it was eliminated from the procedure. Some tissue samples prepared by the freeze substitution method showed poor fixation as evidenced by extensive intercellular and intracellular vacuolation and poor definition of cell organelles and double membranes. These features were not seen in the samples fixed in the Karnovsky's fixative.

Normal Walleye Gill Tissue

The structure of uninfected walleye gill tissue was similar to that of other species of teleosts as described by Newstead (1967). The width of the most distal lamellae on the filament ranged from approximately 6.7-8.7 μm with a length of about 10-16 μm. The interlamellar space was about 3.0 mm (Figures 1 and 2). Each lamella consisted of support and epithelial cells; pillar cells formed the structural support for the primary blood space (Figures 3 and 4).
Figure 1. Uninfected walleye gill tissue
hematoxylin-eosin stain (Scale bar=50 \( \mu m \))

Figure 2. Uninfected walleye gill tissue
toluidine blue stain (Scale bar=25 \( \mu m \))

Figure 3. Lamella tip of uninfected walleye gill, electron micrograph (Scale bar=1 \( \mu m \))

Figure 4. Middle of lamella of uninfected walleye gill, electron micrograph (Scale bar=1\( \mu m \))

B=basal lamina, Bs=primary blood space of lamella, E=epithelia, En=nucleus of epithelial cell, Pn=pillar cell nucleus, T=thrombocyte
Pillar cells each had four flanges; two flanges on each side extending to those from an adjacent pillar cell formed the blood space. The pillar cell body was approximately 2.5-3.5 \( \mu m \) in diameter and contained most of the cell organelles and nucleus. The nucleus was oval or rectangular and contained dense patches of chromatin, especially around its periphery. Immediately external to the pillar cell was a basal lamina approximately 0.12 \( \mu m \) thick. The lamina covered the pillar cells along the entire length of the column. External to the basal lamina was a double layer, an inner basal layer and an outer mucosal layer. The epithelial cells, most of which were pavement cells, were elongate (11.0-14.0 \( \mu m \)) and flattened, and the mucosal layer had an extensive endoplasmic reticulum and Golgi apparatus. The junction of the cells showed interdigitating membranes and tight junctions. Epithelial cell nuclei were also elongate (6.3 \( \mu m \)) with peripherally dispersed chromatin. The epithelial layer had a width range of 0.5-2.5 \( \mu m \) and the outer surface had microridges of 0.11-0.45 \( \mu m \) in length covering it. A secondary blood space (up to 1.6 \( \mu m \) wide) between the two epithelial layers contributed to a greater lamellar width in some areas. This space contained various small cells and myelin bodies.

The filament of the gill was composed of several cell types. Mucous cells, approximately 6.0 \( \mu m \) in diameter, were
located on the interlamellar region of the filament (Figure 5). They were characterized by large membrane-bounded vesicles, about 0.6-0.8 \( \mu \text{m} \) in diameter, which appeared to originate from a prominent Golgi apparatus at the base of the cell. Nuclei of mucous cells were basally located. Squamous pavement cells, similar to those on the lamellae, lined the external boundary of filaments. They had an extensive Golgi apparatus and endoplasmic reticulum and a more extensive series of microridges on the external surface than pavement cells on the lamella. Cells containing dark granules were found in the second or third cell layer of the filament and occasionally in the secondary blood space. These cells were about 8.0 \( \mu \text{m} \times 3.0 \mu \text{m} \) and the granules were 0.45 \( \mu \text{m} \) in diameter. Dark granule cells contained a moderate amount of mitochondria and rough endoplasmic reticulum; the nucleus occupied about one-fourth of the cell and chromatin was aggregated in patches around the periphery. These cells were thought to be heterophils. Chloride cells contained a large number of elongate mitochondria (2.0 \( \mu \text{m} \times 0.5 \mu \text{m} \)) associated with a densely branched network of tubules of the endoplasmic reticulum (Figure 6). The chloride cells were relatively large (7.0\( \mu \text{m} \times 9.5 \mu \text{m} \)) and contained a large oval nucleus with sparse, homogeneous chromatin. These cells were mainly on the interlamellar region of the filament and rarely on the
proximal end of the lamella, and were more numerous than the mucous cells.

Other cell types that were found in more proximal layers of the filament included pigment cells, neuroepithelial cells, chondrocytes, erythrocytes, and thrombocytes. Pigment cells contained dense concentrations of dark pigment-like material. The neuroepithelial cells were characterized by myelin-bound vesicles and large myelin bodies, and a prominent rough endoplasmic reticulum. Erythrocytes were only found in the primary blood spaces and were about 10.0 μm x 2.5 μm in size. Thrombocytes were relatively abundant (one to six per lamella) and were also found in the primary blood space (Figure 4). These cells were approximately 7.0 μm x 4.0 μm; each had a lobed or crescent shaped nucleus which contained very dense areas of aggregated chromatin. The cytoplasm contained many small vesicles filled with myelin material and a moderate number of small mitochondria (0.26 μm-0.66 μm).

Infested gill tissue: 0-2 hr

Glochidia were found attached at several sites on the gills; 9.4% were found on the gill rakers and 90.6%, of a total of 66 glochidia, were attached to filament tips, along
Figure 5. Interlamellar region of uninfected walleye gill
Mucous gland cells contain electron lucent granules.
The cell containing electron dense granules may be a heterophil.
Electron micrograph (Scale bar=1 μm)

Figure 6. Interlamellar region of uninfected walleye gill
Chloride cells are characterized by a prominent mitochondria and an extensive endoplasmic reticula.
Electron micrograph (Scale bar=1 μm)

Figure 7. Infected walleye gill tissue at 0-30 min post-infection
A glochidium is attached on the middle of the filament and the surrounding filaments appear relatively normal.
Hematoxylin-eosin stain (Scale bar=100 μm)
CC=chloride cell, E=epithelial cell, He=heterophil, m=mitochondria, mc=glochidial mantle cells, MG=mucous gland cell, n=nucleus, p=periostracum of glochidium, PC=pigment cell
the length of the filament and between two filaments (Figure 7). Closure of the shell on the gill tissue compressed and distorted the structure of the filament and occluded the blood space, blocking circulation into the tissue held by the glochidium (Figure 8). At some infestation sites a mass of cells, primarily erythrocytes and necrotic cells, surrounded the glochidium. This may have been the result of hemorrhage when the glochidium initially attached. At other sites, the mass of cells was absent but cells of the lamellae immediately adjacent to the glochidium were necrotic.

At 0-30 min post-infection, hyperplasia was seen on one to nine lamellae distal to the glochidium (Figures 9 and 10). The most proximal lamellae were hyperplastic in all but a few cases. Two cell types accounted for most of the hyperplasia: epithelial cells were aggregated on the lamella and in interlamellar spaces; chloride cells were found on the lamella (Figure 11) in 2-3x greater numbers than in normal gill tissue.

Ultrastructural examination at 0-30 min post-infection showed that the epithelial cells in the immediate vicinity of the glochidial bite were abnormally shaped. Many were elongate and some appeared to be in the process of forming the capsule around the glochidium. All the cells in the area of the bite had pseudopodia-like extensions, emanating
Figure 8. Infected walleye gill tissue 0-30 min post-infection
Attachment of the glochidium caused compression and occlusion of the blood space. The outer epithelia assumed a ruffled appearance.
Toluidine blue stain (Scale bar=10 μm)

Figure 9. The 3 lamellae adjacent to the glochidium show a slight degree of hyperplasia at 0-30 min post-infection in walleye
Hematoxylin-eosin stain (Scale bar=25 μm)

Figure 10. Hyperplasia was evident on infected filaments (F*) in the walleye, while adjacent, uninfected filaments appeared relatively normal (F)
Hematoxylin-eosin stain (Scale bar=25 μm)

Figure 11. Chloride cells were found on the outer lamellae of infected gill tissue at 0-30 min post-infection (arrows) in walleye
Toluidine blue stain (Scale bar=5 μm)

C=capsule, CC=chloride cell,
F=uninfected filament, F*=infected filament,
G=glochidium, H=host tissue, * indicates shell crystals, arrows indicate some areas of abnormally high numbers of chloride cells
from the main cell body. The cellular extensions often made contact with other cells and lacked well-defined membranous cell organelles. Intercellular spaces, 1.2-3.5 \( \mu \text{m} \) in diameter, were extensive and the interdigitating junction between epithelial cells was widened so membranes were not interlocked. Some cell contacts were maintained only at tight cell junctions.

Lamellae near the distal, lateral portion of the capsule showed several cell changes; the outer epithelial cells often had bulges or stalks rather than the regular border of microridges as seen in controls (Figure 12). The epithelial layers were more widely separated (2.9 \( \mu \text{m} \)-4.4 \( \mu \text{m} \)), creating an enlarged secondary blood space. The cell layer overlying the basement membrane of the pillar cells was pulled away in these lamellae (Figures 13 and 14). The lamellar blood space created by pillar cells was occluded or decreased, apparently because of compression by the shell.

The most striking intracellular change was the foamy appearance of many organelle-rich cells, here referred to as transformed cells (Figure 15). In these cells, the intramembranous spaces of the mitochondria, rough endoplasmic reticulum, and Golgi apparatuses were widened and vesicular, and assumed much of the volume of the cell. Many of these cells appeared to be transformed chloride cells.
Figure 12. Middle of a lamella at 0-2 hr post-infection in walleye
The outer epithelia is irregularly stalked
Electron micrograph (Scale bar=1 μm)

Figure 13. Lamella at 0-2 hr post-infection in walleye
The outer epithelia has pulled away from the inner epithelia and basal lamina, creating an enlarged secondary blood space.
Electron micrograph (Scale bar=1 μm)

Figure 14. Lamella at 0-2 hr post-infection in walleye
The outer epithelia has maintained relatively normal microridges but cells have pulled away from the inner epithelial layer.
Electron micrograph (Scale bar=1 μm)

Figure 15. Transformed cells in the filament tissue adjacent to the glochidium in walleye
The mitochondria and endoplasmic reticula are vesicular and swollen.
Electron micrograph (Scale bar=1 μm)
B=basal lamina, E=epithelia, Er=erythrocyte, m=mitochondria, n=nucleus, Pn=pillar cell nucleus, TC=transformed cell
Figure 16. Glochidium and surrounding capsule at 2 hr post-infection in walleye
The capsule is very irregular in shape, thickness, and cell composition. Many of the outer cells of the capsule appear necrotic.
Hematoxylin-eosin stain (Scale bar=50 \( \mu m \))

Figure 17. Glochidium and surrounding capsule at 2 hr post-infection in walleye
The capsule is thinner and symmetrical relative to Figure 16.
Hematoxylin-eosin stain (Scale bar=50 \( \mu m \))

Figure 18. Cells of the capsule at 2 hr post-infection in walleye
Cells were elongate and contained pseudopodia-like extensions. An inner layer was often associated with the periostracum.
Electron micrograph (Scale bar=1 \( \mu m \))

Figure 19. Cells of the capsule at 2 hr post-infection in walleye, electron micrograph (Scale bar=1 \( \mu m \))
\( a= \) adductor muscle of glochidium, \( C= \) capsule tissue, \( E^*= \) epithelioid cells, \( G= \) glochidium, \( H= \) host tissue, \( mc= \) mantle cells of glochidium, \( P= \) periostracum, \( pe= \) pseudopodia-like extensions
At 2 hr post-infection, glochidia were contained in a capsule 1-4 cell layers thick (1.0 μm-18.0 μm) and very irregular in thickness, shape, and cell composition (Figure 16 and 17). The innermost cell layer of the capsule was commonly found closely associated with the periostracum (Figure 18). Outer capsule layers were generally less compact particularly in areas where the capsule was thicker (4.5-18.0 μm). The capsule was composed of different cell types and virtually all of the cells had pseudopodia-like extensions (Figure 19). Normal chloride cells were found in the capsule in only a few instances. These cells were usually found in areas near the bite and in the outer areas of the capsule. The most common cell types in the capsule were epithelioid cells, transformed cells, and necrotic cells (Figure 20). Elongate epithelioid cells composed most of the innermost layer of the capsule and were found in other layers of the capsule as well. Epithelioid cells contained few membranous cell organelles and their nucleoplasm and cytoplasm were very homogeneous compared to any cells in the uninfected gill tissue. The pseudopodia-like extensions of these cells were often very slender and long. Transformed cells contained extensive endoplasmic reticulum and their mitochondria were often so vesicular and swollen that they could not be individually distinguished. The nuclei contained relatively homogeneous nucleoplasm with
Figure 20. Cells of the capsule at 2 hr post-infection in walleye

The most abundant cells in the capsule were epithelioid cells, transformed cells, and necrotic cells.

Electron micrograph (Scale bar=1 \( \mu \text{m} \))

Figure 21. Lamella incorporated into the capsule at 2 hr post-infection in walleye

The pillar cell bodies and flanges are distorted and compressed, and the epithelia has pulled away from the basal lamina.

Electron micrograph (Scale bar=1 \( \mu \text{m} \))

Figure 22. Capsule and glochidium at 4-6 hr post-infection in walleye

This capsule appears relatively thin and there is evidence of compaction of cells in the inner capsule layer.

Hematoxylin-eosin stain (Scale bar=25 \( \mu \text{m} \))

B=basal lamina, Bs=primary blood space, C=capsule, E=epithelia, F=filament, H=host tissue, m=mitochondria, mc=mantle cells of glochidium, n=nucleus, NC=necrotic cell, Pn=nucleus of pillar cell, TC=transformed cell
few dense areas of chromatin. These cells were highly variable in shape; some were elongate with slender pseudopodia-like extensions while others were very large (up to 8.0 μm in diameter) and globular with short extensions. Necrotic cells were distinguished by their scanty cytoplasm and nucleoplasm, thickened mitochondrial membranes (approximately 1.5x), and in some cases, partial or complete loss of the cell membrane. Pillar cells and the associated basal lamina were incorporated into the capsule but the outer bilayer of epithelia was usually not intact (Figure 21). The pillar cell flanges were distorted and the entire pillar column and basal lamina had areas of infolding. The cell body often contained intracellular spaces and myelin bodies and the nucleus contained homogeneous nucleoplasm.

Hyperplasia was evident 3-4 lamellae from the bite to the entire length of the infected filament, and the structure of the lamellae and cells of the filament became increasingly abnormal more proximal to the glochidium. Lamellae within 4-5 lamellae from the glochidium had 3-4x more chloride cells in the outer cell layer than uninfected gill tissue. These chloride cells were found in the secondary blood space or in the epithelial layer and increased the width of the lamellae by about a factor of about 0.5x. Microridges and detached vesicles were found on the external surface of the chloride cells, indicating secretory activity. The lamellae adjacent
to the shell were abnormal in several respects. Transformed cells were located in areas of the lamellae corresponding to the location of chloride cells on uninfected lamellae. The epithelium was distinguishable but was pulled away from the pillar cells and had many pseudopodia-like extensions. The pillar cells were compressed and their cell bodies contained small intracellular vacuoles. Myelin bodies (0.05-.15 μm in diameter) were seen in some pillar cells. The lamellar width was as great as 11.35 μm in some areas. The lamellae 1-4 proximal to the glochidium exhibited a few transformed cells, were stalked, and had surface bulges. Distortion of the pillar cells and associated epithelium was not as great as in lamellae adjacent to the shell.

An inflammatory response was not evident in the 0-2 hr samples. Small lymphocytes were found in the primary and secondary blood spaces in tissue adjacent to the capsule and between cells of the capsule, and leucocytes were seen in the primary blood space; but, the number of white blood cells was not different than that seen in uninfected tissue.

4-6 hr post-infection

The capsule was complete around all the glochidia sampled by 6 hours post-infection (Figure 22). By this time, the capsule was irregular in outline and thickness but the innermost layer showed evidence of compacting. The
cells of the innermost layer were elongate and thin and stained darker than those of the outer layers. Generally, in areas of relatively thick capsule, the outer capsule layers were less compact and often contained aggregates of cell debris and leucocytes. The outermost layer was commonly composed of large, light staining cells with swollen nuclei, and appeared necrotic (Figures 23 and 24).

Tissue distal to the capsule showed more extensive hyperplasia and lamellar fusion than seen in the 0-2 hr samples (Figure 25). In some instances the lamellae of an entire filament were fused. Glochidia were sometimes attached to the lamellae of 2 adjacent filaments and both filaments subsequently contributed to the capsule tissue (Figure 26). Epithelial lifting from the outer lamella was also seen on some filaments adjacent to the glochidium. Tissue changes were not seen on filaments beyond 2 filaments from a glochidium.

Electron microscopy showed that the cell changes seen at two hours were more pronounced and more extensive at 4-6 hr. Epithelial stalking and the formation of intercellular spaces between cell layers was extensive (Figure 27). The capsule consisted of 1-7 cell layers and was 2.0-17.0 \( \mu \text{m} \) thick. Compact and thinner areas of the capsule were
Figure 23. Glochidia and capsules at 4-6 hr post-infection in walleye
Capsule at the lower left appears symmetrical and compact compared to the irregular, thick appearance of the capsule to the right.
Hematoxylin-eosin stain (Scale bar=50 μm)

Figure 24. Glochidium and capsule at 4-6 hr post-infection in walleye
This capsule contains thin areas of capsule growth as well as thick, irregular areas.
Hematoxylin-eosin stain (Scale bar=50 μm)

Figure 25. Infected walleye filament at 4-6 hr
The interlamellar spaces are filled with epithelial cells.
Hematoxylin-eosin stain (Scale bar=50 μm)

Figure 26. Glochidium and capsule at 4-6 hr post-infection in walleye
The capsule is composed of cells from the infected filament and the adjacent filament
Hematoxylin-eosin stain (Scale bar=25 μm)
a=adductor muscle of glochidium, Bs=blood space, C=capsule, G=glochidium, H=host tissue, L=lamella, mc=mantle cells of glochidium, n=necrotic cells
composed of elongate, thin cells with electron dense and homogeneous cytoplasm and nucleoplasm. Few membranous organelles were found in these cells and much of each cell consisted of pseudopodia-like extensions. In some areas, the inner layers were composed solely of layered cell extensions. The capsule was thickest and least compact adjacent to the bite and the greatest diversity of cell types was usually found in this area (Figures 28 and 29). Many areas of the outermost layer of the capsule had microridges similar to those on normal epithelium. Intercellular spaces, common between layers of the capsule, contained isolated cells of various types, including small lymphocytes, heterophils, and debris-filled cells. In most cases, contact between cells of the capsule consisted of interdigitating membranes, although a few tight cell junctions were also seen.

By 6 hr post-infection, the most common cell types of the capsule and proximal areas of the filament were again the epithelioid cells, transformed cells, and necrotic cells (Figure 30). The number of transformed cells was greater than in the 0-2 hr samples and cells containing debris and myelin bodies appeared the 4-6 hr samples. Transformed cells composed up to one-half the cells in some areas and the degree of mitochondrial swelling and endoplasmic reticular distension was greater in many cells than at 0-2
Figure 27. Lamella of infected walleye filament at 4-6 hr post-infection
A chloride cell is present between the epithelial layers, the intercellular spaces are extensive and the outer epithelia is irregular.
Electron micrograph (Scale bar=2 μm)

Figure 28. Cells of the capsule at 4-6 hr post-infection in walleye
Transformed cells were abundant in this portion of the capsule.
Electron micrograph (Scale bar=2 μm)

Figure 29. Outer capsule layers at 4-6 hr post-infection in walleye
Epithelioid cells with exaggerated microridges composed the outer capsule layer.
Electron micrograph (Scale bar=2 μm)

Figure 30. Necrotic and transformed cells of the capsule at 4-6 hr post-infection in walleye
Electron micrograph (Scale bar=2 μm)
Bs=primary blood space of lamella, CC=chloride cell, DC=debris cell, E*=epithelioid cell, Er=erythrocyte, m=mitochondria, n=nucleus, NC=necrotic cell, TC=transformed cell
hr (Figure 31).

Cells, here referred to as debris cells, were variable in shape and size and contained myelin bodies, karyotic and pyknotic cells, or cell debris and dark circular granules (0.7 μm). The dark granules often contained dense circular granules (probably nuclear debris) within them (Figure 32). The cytoplasm of the debris cells was usually light staining with an extensive rough endoplasmic reticulum. The nucleus was also electron lucent, did not assume a regular shape, and the chromatin was aggregated around the periphery. Debris cells may have phagocytosed some of the necrotic or transformed cells. With the exception of the transformed cells, debris cells, and leucocytes, the vast majority of cells in the capsule at 6 hr assumed a uniform appearance, with moderately dense, homogeneous cytoplasm; in some cells the nucleus showed a rim of aggregated chromatin.

A slight inflammatory response was evident at this time. Small lymphocytes, monocytes/macrophages, and a few heterophils were found in many areas, including in the primary and secondary blood spaces and between layers of the capsule, of the infected gill (Figures 33 and 34).

12-48 hr

At 12-48 hr the capsule appeared more "mature." The cells of the capsule and surrounding tissue showed cell
Figure 31. Transformed cell at 4-6 hr post-infection
Electron micrograph (Scale bar=1 μm)

Figure 32. Debris-filled cell between layers of the capsule
at 4-6 hr post-infection in walleye
The debris cells contains dark granules and
myelin material. Pillar cells and the
associated basal laminae compose the surrounding
layers of the capsule.
Electron micrograph (Scale bar=2 μm)

Figure 33. Capsular tissue in walleye 6 hr post-infection
Epithelioid cells are prominent in this area.
Several blood cells are also evident, including
a small lymphocyte, thrombocyte, and an
erythrocyte, but they are not contained in the
primary blood space.
Electron micrograph (Scale bar=2 μm)

Figure 34. Capsular cells in walleye at 6 hr post-infection
A heterophil type cell is present in the blood
space of the infected filament.
Electron micrograph (Scale bar=1 μm)

B=basal lamina, DC=debris cell, E*=epithelioid
cell, En=nucleus of epithelioid cell,
He=heterophil, L=lymphocyte, m=mitochondria,
P=pillar cell, Pn=pillar cell nucleus,
T=thrombocyte, TC=transformed cell
connections and many cells had apparently dedifferentiated. The capsule was uniform in thickness and cell composition by 48 hours. Around each glochidium, the capsule thickness was uniform and the inner layers were denser staining and more compact than at 6 hr (Figures 35, 36, and 37). Intercellular spaces, formed between pseudopodia-like extensions of cells, remained in the middle and outer layers of the capsule. The PTAH stain showed no signs of connective tissue formation in the capsule at 24 hr, but by 48 hr, fibrous material was evident. The capsule also contained aggregates of necrotic cells, erythrocytes, and heterophils.

Complete fusion of lamellae was found on areas adjacent to the infection, and commonly, lamellae along the entire length of an infected filament were fused (Figure 38). The pillar columns were distinguishable and maintained their structure, but the interlamellar spaces were completely filled with cells. As a result the outer border of the infected filaments had a smooth profile, often with a dense, thin, outer, layer. Lamellae on neighboring filaments usually had ruffled borders consisting of large, pale staining cells (probably chloride cells) that appeared to be necrotic. Hyperplasia was also seen on many lamellae.

The thickness of capsules ranged from 4.0 μm and three cells thick to 22.6 μm where pillar columns had been
Figure 35. Glochidium and capsule at 12-48 hr post-infection in walleye
The capsule is very uniform in thickness and the inner layers are compacted.
Hematoxylin-eosin (Scale bar=25 μm)

Figure 36. Glochidia and capsules at 48 hr post-infection in walleye
These capsules are thin and PTAH staining showed evidence of connective tissue cells of the capsule. PTAH stain (Scale bar=50 μm)

Figure 37. Glochidia and thinning capsules at 48 hr post-infection in walleye
The outer capsule walls were extremely thin and were composed of connective tissue.
PTAH stain (Scale bar=50 μm)

Figure 38. Infected walleye filaments 12-48 hr
Complete fusion of the lamella has occurred on the right filament (F*), while the adjacent filament (F) still has some distinguishable lamella distal to the site of infection.
Hematoxylin-eosin (Scale bar=50 μm)
a=adductor muscle of glochidium, C=capsule, E=erythrocytes, F=filament, G=glochidium, H=host tissue, mc=mantle cells of glochidium
incorporated into the capsule. The composition of the capsule was different from 12 to 48 hr, and micrographs indicated that a gradation of changes occurred during this time period. The number of debris and myelin-filled cells was greater than at any earlier times, whereas the number of transformed cells was smaller. By 48 hr the capsule contained few transformed cells, but debris cells were common between layers of the capsule (Figure 39). With the exception of the debris cells and the leucocytes, the cell population of the capsule continued to become increasingly uniform. It was dominated by epithelioid cells which were pseudopodate and often had a prominent nucleolus. These cells were characterized by well-developed rough endoplasmic reticulum and few other organelles. By 48 hr tight cell junctions were found between epithelioid cells, particularly between pseudopodia-like extensions and interdigitating membranes. Tracts of fibrous material were also found in these cells at 48 hr (Figure 40). Necrosis of cells in the capsule was evident but was not as common as in earlier samples. Necrotic cells at this time were most often in the outer layer of the capsule.

An inflammatory response was advanced in comparison to early samples. Leucocytes were present in relatively greater numbers in the filament blood space adjacent to the shell and in intercellular spaces of the capsule (Figures 39
Figure 39. Cells of the capsule at 12-48 hr post-infection in walleye

Elongate epithelioid cells are the main structural cells of the capsule. Between the layers of epithelioid cells other cell types are evident, including small lymphocytes and debris-filled cells.

Electron micrograph (Scale bar=1 μm)

D=debris filled-cell, E*=epithelioid cell, G=glochidium, L=small lymphocyte, P=periostracum, arrow indicates a tight cell junction between epithelioid cells
and 40). Cells including small lymphocytes, heterophils, monocytes and thrombocytes, were found adhering to the perimeter of the blood space, apparently undergoing diapedesis (Figure 41).

The lamellae adjacent to the capsule were varied at this time. One to two were incorporated into the capsule without occlusion of the primary blood space or substantial loss of epithelium (Figures 42 and 43). Lamellar fusion involved extensions and hyperplasia of epithelia of adjacent lamellae. Other capsules contained distorted lamella in which the epithelial bilayer was absent, pillar cells were compressed and their flanges were indistinguishable. The occluded blood space contained cell debris and the pillar cell body of some cells also contained debris, myelin and intracellular vacuoles.

5 day

The capsule was uniform in shape and cell composition (Figure 44). The cellular response appeared to be more localized and discrete at this time. Surrounding tissue of the infected filament and adjacent filaments did not show substantial change from the 0-48 hr samples; in some samples the lamellae of adjacent filaments showed less hyperplasia than in all earlier samples and there were no necrotic cells on the outer borders. The PTAH stain showed cells of the
Figure 40. Cells of the capsule at 12-48 hr post-infection in walleye
Small lymphocytes and heterophil-type cells were found between layers of the capsule. Epithelioid cells were joined by interdigitating membranes and a few tight cell junctions (arrow).
Electron micrograph (Scale bar=1 μm)

Figure 41. Filament blood space of infected walleye gill at 12-48 hr post-infection
Leucocytes in the blood space appear to be undergoing diapedesis.
Electron micrograph (Scale bar=1 μm)

Figure 42. Lamellae incorporated into the capsule at 12-48 hr post-infection in walleye
The lamellae compose the outer layers of the capsule and still retain normal pillar cell structure.
Electron micrograph (Scale bar=1 μm)

Figure 43. Glochidium and infected filament at 48 hr post-infection in walleye
The adjacent lamellae have been incorporated into the capsule. Many have are distorted and have lost the outer epithelia, while the primary blood space has not been occluded.
Toluidine blue stain (Scale bar=50 μm)
Bs=blood space, E*=epithelioid cell, En=nucleus of epithelial cell, Er=erythrocyte, G=glochidia, He=heterophil, L=lymphocyte (Figures 40 and 41), L=lamella (Fig 43), Le=leucocyte, Pn=pillar cell nucleus, arrow indicates tight cell junction
capsule and those in the fused interlamellar areas 2-3 lamellae distant from encapsulated glochidia contained fibrous connective tissue. Blood flow through pillar cells in the fused areas of the capsule and surrounding tissue appeared to be uninterrupted, and erythrocytes were seen in the pillar blood spaces (Figure 45).

The most pronounced feature in most cells in the capsule was the appearance of a substantial amount of fibrous material (Figures 46 and 47). The fibrous material was found in light staining bundles about 0.20-0.30 μm wide which appeared to run in all directions in the cells (Figure 48). The cells containing fibrous material also had areas of electron-dense cytoplasm, containing ribosomes. No other cell organelles were evident. The nuclei were typical of the epithelioid cells in the capsule at 12-48 hr; many also showed a prominent nucleolus. Tight cell junctions were very common between these cells. The fiber-filled cells appeared to form the main framework of the capsule at this time. Isolated small lymphocytes, heterophils, and debris cells filled the intercellular spaces of the capsule (Figure 49).

8-17 days

No significant changes were evident in the capsule from 8 d to 11 d (Figure 50). A mild degree of cellular
Figure 44. Glochidium and capsule at 5 d post-infection in walleye
The inner layers of the capsule are more compact than seen earlier, and the formation of the gut is evident in the glochidium.
Hematoxylin-eosin stain (Scale bar=25 μm)

Figure 45. Infected filament at 5 d post-infection
Blood flow continued in the fused lamellae of the infected filament although the interlamellar spaces were indistinguishable.
Hematoxylin-eosin stain (Scale bar=20 μm)

Figure 46. Capsular cells in walleye at 5 d post-infection
Tracts of fibrous material are present in the electron-dense cells of the capsule. Tight cell junctions were also more numerous at this time.
Electron micrographs (Scale bar=1 μm)

Figure 47. Cells of the capsule at 5 d post-infection in walleye
Fiber-filled cells and debris cells are evident in this area.
Electron micrograph (Scale bar=1 μm)
Bs=blood space, C=capsule, FC=fiber-filled cell, G=glochidium, g=developing gut of glochidium
separation was evident between cells in the capsule in some samples, usually in those with a larger amount of tissue associated with the capsule. The amount of fibrous connective tissue also appeared greater than in all earlier samples, as evidenced by the PTAH stain and TEM examination (Figures 51 and 52). The cells of the capsule at 11 d and 14 d were virtually filled with fibrous material and contained a number of ribosomes. At 11 d some capsules showed small areas of thinning, and by 14 d all the capsules showed extreme thinning of at least one wall (Figure 53). The capsule was broken open in some cases and the juveniles were partially excysted (Figure 54). In one gill sample, three empty capsules were seen in which the cavities were filled with cellular exudate (Figure 55).

Fully transformed juvenile mussels were recovered 14-17 d post-infection. Liberation of mature juveniles appeared to be a combination of extreme capsule thinning and mussel movement. The thin fibrous walls of the capsule may be disrupted by the mussel movements. The juveniles found partially excysted were usually slightly agape and the foot was protruded in some cases. Juveniles collected from the bottom of aquaria were active and fully metamorphosed as evidenced by the separation of the two adductor muscles and movement of the foot and valves. Histological sections of encapsulated juveniles at 14 d post-infection also showed
Figure 48. Fiber-filled cells of the capsule at 5 d post-infection in walleye
The fiber-filled cells contained an abundance of ribosomes, and were often joined by tight cell junctions. Electron micrograph (Scale bar=0.5 μm)

Figure 49. Capsular cells in walleye at 5 d post-infection
A debris-filled cell is found between layers of the capsule. Electron micrograph (Scale bar=1 μm)

Figure 50. Capsule and developing juvenile at 11 d post-infection in walleye
The capsule showed little evidence of change between 5 and 11 days post-infection, but organs of the developing juvenile were evident. Hematoxylin-eosin stain (Scale bar=25 μm)

Figure 51. Capsule and developing juvenile at 11 d post-infection in walleye
The amount of fibrous material increased in the capsule compared to 5 d post-infection as evidenced by the PTAH stain. (Scale bar=25 μm)

a=adductor muscle of juvenile, C=capsule, DC=debris cell, dg=digestive gland, dm=definitive mantle cells, f=fibrous tracts, ft=foot of juvenile, g=gut, gl=rudimentary gill, lm=larval mantle cells
Figure 52. Capsule and juvenile at 14 d post-infection in walleye
Cells of the capsule appear to be extremely thin and fibrous. The juvenile mussel is fully formed.
Hematoxylin-eosin stain (Scale bar=50 μm)

Figure 53. Capsule and juvenile at 14 d post-infection in walleye
The capsule walls were extremely thin in areas.
Hematoxylin-eosin stain (Scale bar=25 μm)

Figure 54. Juvenile in the process of excystment a 14 d post-infection
Hematoxylin-eosin stain (Scale bar=50 μm)

Figure 55. Capsules and juveniles at 14 d post-infection in walleye
Juveniles have apparently excysted from two of the capsule. The empty capsules contain non-cellular material.
Hematoxylin-eosin stain (Scale bar=100 μm)
C=capsule, dg=digestive gland, dm=definitive mantle cells, EC=empty capsules, f=foot of juvenile, g=gut, J=juvenile mussel
evidence of metamorphosis. The larval mantle was replaced by adult mantle cells, and the foot, gastrointestinal tract, digestive gland and rudimentary gills were also formed.

**Common carp studies**

Glochidia began to slough immediately from the gills of the infected common carp. All glochidia were sloughed by 60 h post-infection in the first trial and by 12 h post-infection in the second trial.

**Normal gill tissue**

The structure of the common carp gill is similar to that described for walleye. Lamellae at the distal end of the filament were about 15.0 μm long, 5.0 μm wide, with an interlamellar separation of 4.0 μm (Figure 56). Epithelium, basal lamina, pillar cells, mucous gland cells and chloride cells of the gill tissue were present and appeared very similar to those seen in the walleye (Figures 57, 58, and 59). In addition, cells that appeared to be eosinophilic heterophils, described by Weinreb (1963), were found in the normal common carp tissue. The eosinophilic cells contained granules (about 0.047 μm in diameter) with electron dense, slender rods. A second type of heterophil cell was similar to the tissue eosinophils described by Lester and Desser (1975). It contained small spherical granules (about 0.043 μm in diameter) within large granules (about 0.60 μm in
Figure 56. Uninfected common carp gill tissue
   Hematoxylin-eosin stain (Scale bar=25 μm)

Figure 57. Uninfected common carp gill tissue
   The structure of the lamella is similar to that in the walleye gill.
   Electron micrograph (Scale bar=1 μm)

Figure 58. Uninfected common carp gill tissue
   Chloride cells are abundant in the interlamellar region.
   Electron micrograph (Scale bar=2 μm)

Figure 59. Chloride cell on the uninfected common carp gill
   Electron micrograph (Scale bar=1 μm)

CC=chloride cell, E=epithelial cell, Er=erythrocyte, m=mitochondria, P=pillar cell
Pf=pillar cell flange, Pn=pillar cell nucleus
diameter). The exact nature of these cells is not known, but herein they are referred to as heterophil type 1 and heterophil type 2, respectively.

**Infested gill tissue: 0-2 hr**

The glochidia were located on the filaments, filament tips, and gill rakers, however, the distribution of glochidia among these sites differed from that of the walleye. Six glochidia were located on the filaments while four were found on the gill rakers at 0 hr. At 2 hr, the distribution was 6 glochidia on each. The tissue reaction at this time was similar to that seen in the walleye; a thin layer, 2-4 cells thick, of erythrocytes was found around some shells but no appreciable capsule growth had begun (Figures 60 and 61). Hyperplasia of lamellae was similar to that seen in the walleye.

Electron microscope samples also resembled those of the walleye at this time (Figures 62, 63, and 64). The majority of the cells had pseudopodia-like extensions, creating abnormal intercellular spaces. The nuclei of many cells, especially the epithelial cells, were homogeneous. Transformed cells were also found but were not numerous at this time. The membranous organelles of cells appeared swollen and vesicular. The endoplasmic reticulum was extensive, and intracellular vacuoles (about 0.03-0.1 \( \mu \)m in
Figure 60. Glochidium and infected gill tissue at 0-2 hr post-infection in common carp
There was little evidence of capsule growth around the glochidium at this time.
Hematoxylin-eosin stain (Scale bar=25 μm)

Figure 61. Infected common carp gill tissue at 0-2 hr
The glochidium is attached at the tip of the filament and no capsule growth has begun. The area of exudate to the right may be where a glochidium was attached and subsequently sloughed.
Hematoxylin-eosin stain (Scale bar=25 μm)

Figure 62. Infected gill of common carp at 0-2 hr post-infection Many cells have pseudopodia-like extensions and have pulled away from adjacent cells. Electron micrograph (Scale bar=1 μm)

Figure 63. Capsular tissue in common carp, 0-2 hr post-infection The pillar cells are distorted and the primary blood space is occluded. The outer epithelia is pulled away from the basal lamina. Necrotic cells are evident. Electron micrograph (Scale bar=1 μm) a=adductor muscle, B=basal lamina, E=epithelia, E*=epithelioid cell, F=filament, H=host tissue, He=heterophil type II, mc=mantle cells, n=nucleus, N=necrotic cells, P=periostracum, Pf=pillar cell flanges
Figure 64. Infected gill tissue at 0-2 hr post-infection in common carp

Many of the cell changes seen in the walleye at this time are evident in the common carp tissue, including separation of the outer epithelia from the basal lamina, formation of intercellular spaces, and pseudopodia-like extensions. Intracellular spaces are also evident in the common carp tissue.

Electron micrograph (Scale bar= 1 μm)

Bs=blood space, E*=epithelioid cell, N=necrotic cell, Pn=pillar cell nucleus
diameter) were present in some cells. The lamellae adjacent to the shell, but not within the glochidial bite, also appeared similar to those in the walleye. The outer epithelium of the lamellae and filaments had an irregular stalked or bulged profile and sometimes contained large necrotic cells. Blood smears and the sectioned tissues showed no increase in the number of leucocytes compared to the uninfected control tissue.

4-6 hr post-infection

The distribution of glochidia was 4 out of 6 on the rakers at 4 hr and 12 out of 15 on the rakers at 6 hr. Infected filaments and adjacent filaments showed hyperplasia and lamellar fusion similar to that seen in the walleye at this time. The extent of hyperplasia on infected and 3-4 filaments adjacent to the infection was more extensive than in the walleyes. The shells were not fully encapsulated at 6 hr. Areas of the capsule most distal to the bite consisted of necrotic cells, whereas areas proximal to the bite contained non-necrotic, elongate capsule cells (Figures 65, 66 and 67). Electron microscopy showed that the distal and inner portions of the capsule contained three to four layers of necrotic cells and cellular debris (Figures 68 and 69). The capsule did not have an inner layer closely associated with the periostracum in most common
Figure 65. Infected gill tissue and glochidium at 4-6 hr post-infection in common carp
Very little capsule growth has occurred at this time compared to that in the walleye (Figures 22-24). Hematoxylin-eosin stain (Scale bar=10 μm)

Figure 66. Infected gill tissue and glochidium at 4-6 hr post-infection in common carp
Some capsule growth is evident but much of it consists of necrotic cells.
Hematoxylin-eosin stain (Scale bar=50 μm)

Figure 67. Necrotic and normal areas of the capsule at 4-6 hr post-infection in common carp
Electron micrograph (Scale bar=25 μm)

Figure 68. Cells of the capsule at 4-6 hr post-infection in common carp
Many of the outer cells of the capsule and those most distal to the glochidial bite were necrotic.
Electron micrograph (Scale bar=1 μm)
C=capsule, ct=cartilage, H=host tissue, mc=mantle cells of the glochidium, N=necrotic cells
carp samples as it did in the walleye. Instead, there was often a large space (3.0-5.0 μm) between cells of the capsule and the periostracum; this space contained necrotic cells and/or cellular debris. Areas adjacent to the bite contained cells similar to those seen in the walleye (Figure 70); epithelioid cells were the most common types and these were dark staining, elongate with pseudopodia-like extensions, and contained a prominent nucleolus. The only cell organelles distinguished in these cells were mitochondria. There were gaps between filaments which appeared to be created by glochidia which had been sloughed (Figure 71). Tissue change was evident and the interlamellar and interfilamental spaces were filled with non-cellular material that appeared to be exudate. Cells in the area surrounding the gaps were necrotic and adjacent lamellae exhibited extensive hyperplasia.

Other cell and tissue changes at this time were similar to those in the walleye (Figures 72, 73 and 74); the lamellae were abnormally compressed and the epithelia were pulled away from the basal laminae, cells had pseudopodia-like extensions, and a prominent nucleolus. Transformed cells were also present but were not as numerous as in walleye at this time. Heterophil type 1 and 2 were found in the capsule in 10 samples at a density of 1-8 cells/20,000 μm² (Figure 75).
Figure 69. Necrotic cells of the capsule at 4-6 hr post-infection in common carp
Electron micrograph (Scale bar=1 μm)

Figure 70. Gill tissue adjacent to the glochidial bite at 4-6 hr post infection in common carp
Elongate epithelioid cells are prominent in the infected gill tissue.
Electron micrograph (Scale bar=1 μm)

Figure 71. Infected gill tissue at 4-6 hr post-infection in common carp
The pocket of non-cellular exudate may be an area where a glochidium was previously attached.
Hematoxylin-eosin stain (Scale bar=50 μm)

Figure 72. Infected gill tissue at 4-6 hr post-infection in common carp
Intracellular spaces and pseudopodia-like extensions were prominent in many cells of the infected filament.
Electron micrograph (Scale bar=1 μm)
E* = epithelioid cell, G = gap created by glochidium, m = mitochondria, N = necrotic cell, P = periostracum
12-48 hr

Glochidia were again found to be distributed mainly on the gill rakers, 7 out of 11 glochidia, and 3 out of 3 found there at 12 hr and 24 hr respectively. At 48 hr, 2 fish in Trial 1 and 0 fish in Trial 2 remained infected and the remaining glochidia were found only on the filaments. The capsules were highly variable and some were still not complete. Three capsules were found on one common carp at 48 hr that were essentially identical in shape, thickness and cell composition to those of the walleye at this time (Figure 76); however, the surrounding tissue appeared to be slightly more hyperplastic than the walleye. The other glochidia remaining attached at these times were not fully encapsulated, but were partially surrounded by exudate and necrotic material (Figures 77, 78, 79, and 80).

Blood smears taken at 12-48 hr showed no significant increases in white blood cells compared to the control fishes. The capsular tissue of 12 hr samples contained heterophil type 1 and 2 cells at a density similar to that at 6 hr.
Figure 73. Infected gill tissue at 4-6 hr post-infection in common carp
Many of the cell changes are similar to those seen in the walleye gill tissue including the epithelioid cells and pseudopodia-like extensions.
Electron micrograph (Scale bar=1 μm)

Figure 74. Infected gill tissue at 4-6 hr post-infection in common carp
Epithelioid cells are dominant in this area of the capsule. Electron micrograph (Scale bar=1 μm)

Figure 75. Cell material in the capsule at 4-6 hr post-infection in common carp
A space was often present between cells of the capsule and the periostracum. Cellular debris and heterophil type I and II cells were found in the capsular tissue.
Electron micrograph (Scale bar=1 μm)

Figure 76. Glochidia and capsules at 12-48 hr post-infection in common carp
These capsules and glochidia are similar to those found in the walleye at this time.
Hematoxylin-eosin stain (Scale bar=25 μm)
C=capsule, CS=space between capsule and periostracum, E*=epithelioid cell, G=glochidium, He=heterophil, L=small lymphocyte
Figure 77. Glochidia and sloughing capsular tissue at 12-48 hr post-infection in common carp
The capsule has advanced around the glochidia but is thin and appears to be sloughing.
Hematoxylin-eosin stain (Scale bar=50 μm)

Figure 78. Sloughed glochidium and encapsulated glochidium at 12-48 hr post-infection in common carp
The glochidium at the top of the picture has been sloughed off and some necrotic capsular cells are evident around it. The glochidium at the bottom of the picture appears to have encapsulated and is still intact.
Hematoxylin-eosin stain (Scale bar=50 μm)

Figure 79. Capsular tissue in infected gill tissue at 12-48 hr post-infection in common carp
Cellular debris and necrotic cells were abundant around the glochidium compared to the capsular cells in the walleye (Figures 39, 40, 46 and 47)
Electron micrograph (Scale bar=2 μm)

Figure 80. Capsular tissue in infected common carp at 12-48 hr post-infection
Electron micrograph (Scale bar=2 μm)
a=adductor muscle of glochidium,
C=capsule, G=glochidium, H=host tissue,
N=necrotic cells, TC=transformed cell
Encapsulation of glochidia of *Lampsilis radiata siliquoideae* in walleye tissue was similar to that reported by earlier researchers using light microscopy. Encapsulation was complete in the present study in 4-6 hr; Arey (1932a) reported complete encapsulation in 2.5-3.5 hr, and Young (1911) reported completion in 3-4 hr. However, early researchers did not report the temperature at which the infected fish were held, and the process has been shown to be temperature dependent (Howard and Anson 1922), becoming more rapid as the temperature increases. The rate of encapsulation also depends on the species of fish and mussel glochidia and on the site of infection. Larger glochidia and infections at the filament tips require longer encapsulation periods (Arey 1932a).

*Lampsilis* glochidia do not appear to cause severe bleeding from the gill tissue upon initial attachment. There was some evidence of hemorrhaging at the site of infection but it varied among glochidia and the tissue area bitten. Young (1911) reported extensive bleeding, and found a number of erythrocytes and leucocytes around the glochidial shell. Arey (1932a), on the other hand, found relatively little hemorrhaging in largemouth bass infected with glochidia of
Lampsilis sp. and reported the bite made by the glochidium did not severely cut the blood vessels. Hemorrhaging during the initial attachment of the glochidium is probably minimal due to the rapid clotting of fish blood and to the compression of the vessels by the glochidial valves.

The mechanism of capsule formation cannot be clearly defined by light microscopy. Several early researchers (Young 1911; Lefevre and Curtis 1910a; Faussek 1895, 1901; and Harms 1907) reported that the mechanism of capsule formation was a rapid proliferation of epithelium and some mesenchyme at the site of compression. New cells were then thought to be pushed up over the capsule. Arey (1932a,c) reported no significant increase in the number of mitotic figures in the cells at the site of infection, and he suggested that capsule formation occurs by a process of cell migration rather than cell proliferation. Arey (1932c) also cited evidence from other studies on epithelial cell cultivation which suggested that cell migration occurs initially, followed by compensatory mitosis. In this study, capsule formation appeared to begin by extension of cells surrounding the glochidium rather than by cell movement. Ultrastructural examination of the capsule showed the presence of extensive pseudopodia-like extensions on cells near glochidia and on cells composing the capsule; these cells were abnormally elongate and slender, indicating they
had "stretched". The subsequent thickening of the capsule and hyperplasia of adjacent lamellae may have resulted from cell migration and cell proliferation.

The capsule formed in the walleye gill tissue progressed from an asymmetrical, thick, structure at 0-6 hr to a more compact and uniform structure by 48 hr. The same progression in appearance of the capsule was seen by early researchers examining *Lampsilis* sp. infections (Arey 1932a; Young 1911; Lefevre and Curtis 1910a). Observations of the composition of the capsule at the light microscope level were similar to those of Arey (1932a). Cells of the mesenchyme were generally found in the inner layers of the capsule and epithelial cells covered the capsule and were found in the inner layers. Arey reported glochidia of *Lampsilis* sp. were embedded in the connective tissue stroma with a covering of epithelium, although Karna and Millemann (1978) found glochidia of *M. margaritifera* on salmonids were encapsulated only by epithelium. Discrepancies among these studies concerning the composition of the capsule are probably due to size differences in the glochidial species studied. Glochidia of *M. margaritifera* are smaller than those of *L. radiata siliquoidea* and do not bite as much tissue or bite as deeply. As a result, the connective tissue stroma is not incorporated into the capsule. Young (1911) also reported numerous mucous gland cells in the outer capsule layer but
these cells were rarely found in this study.

Ultrastructural examination of the capsule provided detail on the capsule composition and changes in cell types not evident by light microscopy. Initially, the capsule contained epithelioid, transformed, and necrotic cells; at 48 hr, debris-filled cells were greater in abundance than the transformed and necrotic cells. This may have resulted from removal and degradation of non-functional cells, such as the transformed and necrotic cells. The granules in the debris-filled cells may have contained lysosomal enzymes or cells may have been degraded by myelin-body formation.

The capsule, between the first days of infestation and shedding of the transformed glochidia, becomes increasingly fibrous. The PTAH stain showed the capsule contained fibrous material by 48 hr, and ultrastructural examination showed an increasing presence of fibrous tracts in cells from 48 hr to 14 d post-infection. Early researchers (Arey 1932a; Young 1911) using light microscopy, reported few changes in the capsule between the first day of infestation and shedding of the transformed glochidia. During the final week of infestation, Young (1911) found lymph accumulations which formed large intercellular spaces in the outer layers of the capsule, while the inner layers of the capsule remained compact. She believed this weakened the capsule, causing pieces to break off and enabling the juvenile to excyst.
Arey (1932a) reported cell separation in some capsules but did not consider it a regular occurrence. In this study, there was evidence of cell separation in the thicker capsules; however, the formation of large intercellular spaces was less evident than the occurrence of extremely thin areas of the capsules.

Excystment of the juveniles appeared to be a combination of capsule thinning and glochidial movement. Thinning of the capsule may have resulted from further compaction and thinning of cells, especially the fibrous-epithelioid type cells, in the capsule. Thin areas of the capsule may be broken by expansion of the valves and protrusions of the foot. Arey suggested that excystment was accomplished by movements of the juveniles aided by gross sloughing of capsule cells and reverse migration of cells, rather than by sudden terminal thinning or wall weakening. However, in the present study, there was no evidence of gross sloughing or cell migration. Cell junctions were present between cells of the capsule at 14 d, and it is improbable that the fibrous-epithelioid cells composing the capsule could dedifferentiate and migrate back to the filament.

The degree of hyperplasia did not increase throughout the course of infection in walleye; hyperplasia was usually limited to the infected filament and one-two adjacent filaments. By contrast, Karna and Millemann (1978) reported
salmonids infected with the glochidia of *M. margaritifera* develop extensive lamellar fusion to the cyst. The number of fused lamellae increased throughout the course of infection although the capsule underwent thinning. These differences may be attributed to differences between the species of mussel glochidia; *M. margaritifera* increases in size, up to 600% while on the host fish, while *Lampsilis* glochidia do not increase in size.

Several observations indicate that common carp and walleye have very different tissue reactions to *Lampsilis* glochidia. The response of the common carp gill tissue to infestation by glochidia of *L. radiata siliquoidea* indicated it is a non-susceptible host. Capsules were not formed around most glochidia and all were sloughed by 60 hr post-infection. The site of attachment of glochidia varied between the walleye and common carp gill tissue. Glochidia were progressively lost from the filaments of the common carp while those on the gill rakers stayed attached for longer periods. On the walleye gill tissue, glochidia remained attached mainly to the gill filaments, and in samples taken later than 48 hr post-infection, glochidia were not found on the gill rakers. These effects may be attributed to the greater blood supply to the filaments than that to the rakers. To develop to juveniles, glochidia may require nutrients or oxygen in quantities supplied only by the thin
lamellar vessels. In common carp, the larger amount of cellular tissue in the filaments may provide for rapid rejection of glochidia.

The progressive loss of glochidia from the filaments of common carp in the early stages of infection (0-4 hr) and the gaps present between filaments suggest that the glochidia may be actively rejecting the host by releasing. Capsules had not formed around any of the glochidia at this time; thus relaxation of the valves would have accomplished detachment. A chemical factor or factors present in the carp gill tissue may be noxious to the glochidia causing premature release before encapsulation was accomplished.

During later stages of infestation (6-48 hr), the tissue and cellular response of the common carp gill tissue suggested that sloughing may also be due to rejection by the fish. Normal encapsulation did not occur in most cases even by 12 hr post-infection. Capsules started to form around some glochidia but were completed in only a few instances. In most cases, the distal portions of the capsule were composed of necrotic cells and cellular debris, and this probably aided in sloughing of many glochidia. In partially formed capsules, in common carp, the innermost capsule layer was not closely associated with the periostracum and heterophils were more abundant in the immediate vicinity of the shell than in walleye gill tissue. Consistent with these
results, Meyers et al. (1980) found that most glochidia of *M. margaritifera* did not remain attached to the gill of the non-susceptible host, coho salmon, long enough to become completely encapsulated; at 16 hr post-infection, encapsulation of glochidia on the coho salmon either did not occur, or had progressed incompletely, causing a loss of many of the glochidia from the gills. In contrast, Fustish and Millemann (1978) reported that the glochidia of *M. margaritifera* completely encapsulated on coho salmon within 12 hr post-infection. Arey (1932b) also reported complete encapsulation of glochidia on "immune" fishes by 4 hr post-infection. Arey tested largemouth bass which had acquired an immunity to *Lampsilis* glochidia after repeated exposures to the glochidia. In the present study and in the studies of Meyers et al. (1980) and Fustish and Millemann (1978), the non-susceptible fish hosts had not been previously exposed to glochidia and were naturally immune to the glochidia of the tested mussel species. Thus, the previous exposure may alter a fish's immune response to glochidia.

The capsules formed in the common carp gill tissue were similar in size and composition to those in the walleye tissue, except for the extreme degree of thinning which occurred about 24-48 hr post-infection. In contrast, Arey (1932b) reported that capsules of non-susceptible fishes were larger, bulkier, and contained many irregularities including
horizontal spreading "wings." Overgrown encapsulation was attributed to cellular connective tissue in response to the infection. The capsules reached their maximum size at 24 hr and then underwent thinning. The capsules formed by non-susceptible coho salmon infected with glochidia of *M. margaritifera* had a greater diameter than those formed in the susceptible host, chinook salmon (Fustish and Millemann 1978).

Arey (1932b) indicated that *Lampsilis* glochidia may be shed by non-susceptible hosts by several mechanisms. In some cases, glochidia may be destroyed immediately by cytolysis. In others, gradual necrosis of the glochidial cells may cause distension of the valves, and host cells of the stroma may subsequently invade the necrotic cells and destroy the glochidia. Glochidia that are not destroyed internally may be shed intact. In these cases, the capsule may undergo extreme thinning by reverse migration of cells, and the capsule tissue and glochidium are shed together. In the present study, there was no evidence of necrosis of glochidial cells or invasion of glochidia by cells of the stroma. Encapsulated glochidia appeared to be eliminated by premature, extreme thinning of the capsule, and there was no evidence of reverse migration of cells.

Hyperplasia, which is a generalized response to a variety of gill irritants, appeared to play a role in the
elimination of both encapsulated and unencapsulated glochidia in the non-susceptible hosts. Hyperplasia was evident on 2-3 filaments adjacent to infected filaments in common carp; in the walleye tissue, hyperplasia was not evident beyond the filaments immediately adjacent to the glochidium. Fustish and Millemann (1978) also reported a greater degree of hyperplasia in the non-susceptible host, coho salmon, than in the susceptible host, chinook salmon. The greater hyperplastic response and subsequent extreme thinning of the capsule may cause elimination of glochidia that become encapsulated in non-susceptible hosts. Paperna (1964) reported that carp infected with the monogenetic trematode Dactylogyrus vastator experience a similar response. Hyperplasia developed first at the gill filament tips and then progressed to the bases causing elimination of the parasite.

The blood counts showed no significant leucocytosis in the common carp. In contrast, Meyers et al. (1980) found leucocytes increased markedly between days 1 and 2 d post-infection in resistant salmonids when parasite sloughing occurred, and the number remained high throughout most of the infection. Leucocytosis may have occurred in the common carp in this study after glochidia were shed (48-72 hr), however, blood samples were not tested after 48 hr. Isom and Hudson (1984) reported that the plasma from a variety of fish
species, including naturally immune fishes, could be used to successfully transform glochidia of 12 different freshwater mussels. Their results suggest that the plasma component of the blood does not contain factor(s) responsible for host specificity in glochidial infections. In contrast, Meyers et al. (1980) found that the in vitro survival times of glochidia were about 1.5-2x less in plasma of resistant salmon. In addition, antibodies to glochidia were demonstrated in the plasma and mucus of both susceptible and non-susceptible fish species 8 to 12 weeks post-infection.

In light of these two studies, the role of the blood plasma in host specificity of fish to mussel glochidia remains unclear.

Arey (1932b) and Fustish and Millemann (1978) found a greater number of eosinophils in tissues surrounding glochidia in non-susceptible versus susceptible fish. The relative densities of these cells were not reported. Arey also reported eosinophilic plastids and basophilic masses, and he postulated that the latter represented extruded chromatin. Capsular areas in common carp gills, which contained greater numbers of heterophils than seen in controls, indicate a possible mechanism for glochidial rejection, but it is not clear whether heterophils/eosinophils actually play a role in glochidial rejection, tissue repair, or cell clean-up.
The initial response (0-4 hr) of the infected walleye and common carp gill tissue was similar. The general cell changes (elongation of cells and the formation of pseudopodia-like extensions, separation of the epithelia from the basal laminae, compression of the pillar cell, transformation of organelle-rich cells, and the presence of debris cells) may all be induced by various gill irritants and toxicants. Chevalier et al. (1985) and Karlsson-Norrgren et al. (1986) reported that fish from acidified lakes show separation of gill epithelial layers, an increase in the number of chloride cells on the secondary lamellae, and distension of the endoplasmic reticulum and mitochondria. Sticklebacks exposed to zinc in distilled water at doses of 0.5-1.0 mg may exhibit loosening of the epithelium from the basal lamina and pillar cells and vacuolation of the epithelial cells. At 2.0-6.0 mg Zn, the number of chloride cells on the secondary lamellae may increase significantly similar to the early response seen in glochidial infections (Matthiessen and Brafield 1973).

In summary, the present study examined the process of encapsulation and excystment of Lampsilis on a susceptible fish host. Cytological changes leading to an increase in the fibrous content of the capsule were the most pronounced. Several mechanisms for sloughing of Lampsilis glochidia from a non-susceptible host are also demonstrated. The majority
of the glochidia were lost within 6 hr post-infection by simple detachment from the gill tissue. Capsules failed to form or were only partially formed around most remaining glochidia. Complete encapsulation occurred in a few samples by 12-48 hr post-infection, but it was followed by extreme thinning of the capsule and subsequent sloughing of glochidia. A hyperplasia response may have also been responsible for removal of some glochidia. Heterophil type cells were found in greater numbers in the capsule tissue of the common carp, but their role in glochidial rejection has not been determined. The specific cytological/chemical factors responsible for resistance of non-susceptible hosts or for rejection of the host by the glochidium remain unclear. Further examination of individual components of the gill tissue and blood is necessary to determine the role of each in host specificity.
SUMMARY

The early life stages of several *Lampsilis* mussels of the Upper Mississippi River, particularly the endangered species *L. higginisi*, were examined in these studies. The glochidia of *L. higginisi* and the three most similar species were studied using light and scanning electron microscopy in order to find a method for identification of the glochidial stage. The glochidia could only be differentiated using scanning electron microscopy to examine the position of the dorsal hinge ligament and measure the width of the dorsal ridge. The use of host fish by *L. higginisi* in the field could not be feasibly studied using this technique.

Nine species of fishes were found to be suitable hosts for the glochidia of *L. higginisi* in the laboratory. Several of these species including walleye, yellow perch, and smallmouth bass commonly occur in the main channel habitat of *L. higginisi*, suggesting that the availability of a host fish may not be a primary factor in the decline of this mussel species. However, the presence of these fishes over the beds of *L. higginisi* during periods of glochidial release has not been shown.

The "*Lampsilis higginisi* Recovery Plan" (Stern et al. 1982) proposed the introduction of artificially propagated mussels into suitable habitat as one of the steps for
recovery of this species. Host specificity and artificial propagation information will be necessary to implement this project. The host specificity information in this study can be used to determine which species of fish will yield the greatest number of juveniles per infection trial, and it can be used to determine suitable habitat areas for the introduction of individuals. The host fish must be available in the habitat in order for future recruitment to occur.

Propagation studies provided information on the most efficient method for obtaining large numbers of juveniles of the three Lampsilis species. Artificial infection of host fish produced juveniles more consistently than in vitro culture. Juveniles were produced in every infection trial at an average of 3.8-18 juveniles per fish. In vitro culture was much less consistent, producing juveniles in only 13 of 68 replicate plates. However, the average number of juveniles produced per plate (6.0) was similar to that for production on the fish host (3.6-18.2) when mortality of the fish was taken into account. Both methods may be of use depending on the availability of life history information on the mussel species to be cultured, the availability of equipment, and the intended use of the juveniles.

The present study is the first to examine the pathogenesis associated with infection of a susceptible and non-susceptible fish host to glochidia of a Lampsilis mussel
using transmission electron microscopy. Observations of the infected gill tissue of the susceptible host (walleye) using light microscopy were similar to those of earlier investigators: 1) hyperplasia in the interlamellar spaces and proliferation of chloride cells was evident by 2 hr post-infections, 2) the capsule was thinner and more compact by 24-48 hr, 3) the capsule showed little change between 48 hr and 11 d post-infection, and 4) excystment appeared to occur by thinning of the capsule aided by movement of the juvenile. Transmission electron microscopy showed further unreported detail of the capsule: 1) the epithelia of the gills were separated from the basal lamina, increasing the lamellar width and the secondary blood space, 2) transformed cells, containing distended and swollen mitochondria and endoplasmic reticula, became increasingly numerous throughout the first 24 hours of infection, 3) debris-filled cells were found beginning 4 hr post-infection, and 4) fibrous tissue appeared in cells of the capsule at 48 hr and increased in abundance to the end of the infection.

The infected gill tissue of the non-susceptible host (common carp) showed several possible mechanisms for rejection of glochidia: 1) the majority of the glochidia did not fully encapsulate. Partial capsular growth was evident around some glochidia, but the portions of the capsule distal to the bite consisted of necrotic cells and cell debris, 2)
completed capsules, found between 12-48 hr post-infection, underwent extreme thinning and glochidia were subsequently sloughed, 3) hyperplasia was slightly more extensive in common carp than in walleye, and 4) a greater number of heterophil type cells was found in the immediate vicinity of the glochidium than was found in the walleye samples and in control common carp samples.
REFERENCES CITED


Hoggarth, M. A., and K. S. Cummings. 1986. Using light and scanning electron microscopy to identify the host-parasite relationship between Anodonta grandis grandis Say, 1829 (Bivalvia: Unionidae) and Pimephales vigilax (Baird and Girard, 1853) (Pisces: Cyprinidae). Unpublished, Ohio State University, Columbus, OH.


Howard, A. D. 1915. Some exceptional cases of breeding among the Unionidae. Nautilus 29:4-11.


Murphy, G. 1942. Relationship of the fresh-water mussel to trout in the Truckee River. Calif. Fish and Game 28:89-102.


ACKNOWLEDGEMENTS

I wish to acknowledge the assistance of my graduate committee, L. Mitchell, G. Atchison, D. Glenn-Lewin, J. Nickum, and J. Redmond for their suggestions and guidance throughout my graduate studies and for editing this dissertation. The support and ideas of my major advisor, L. Mitchell, are especially appreciated. I would also like to thank the Department of Zoology and the Iowa State Cooperative Education Unit for financial assistance the past four years.

I would like to thank the entire staff at the National Fishery Research Center in La Crosse, Wisconsin, for financial support, use of equipment and personnel, and for the endless assistance in design and implementation of studies provided by numerous people. A special note of appreciation to Dr. Leslie Holland for her assistance in providing a CEA and for her guidance throughout this project.

A special thanks to Donna Spannaus-Martin for use of equipment, materials, her time, knowledge, and moral support. I am grateful to my ISU office mates, Tom Bailey, Glenn Kietzmann, and James Wang for their friendship and support during graduate school. Numerous other Iowa State University staff members and students assisted with statistical analyses, computer programs, specimen preparations, and
scanning electron microscopy. I would like to acknowledge the Iowa State departments of Biochemistry and Biophysics, Botany and Veterinary Pathology for use of their electron microscopes.

Many thanks are extended to family members for their support and encouragement and attempts to understand this study. Finally, but most of all, I am forever indebted to my husband, Bill, and daughter, Haley, for helping me keep my perspective during graduate school. Their patience, sacrifice, and confidence are forever appreciated.