Simplifying Methods for in Vitro Metamorphosis of Glochidia

Morgan A. Kern
Kern003@live.missouristate.edu

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SIMPLIFYING METHODS FOR IN VITRO METAMORPHOSIS

OF GLOCHIDIA

A Masters Thesis

Presented to

The Graduate College of

Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Biology

By

Morgan A. Kern

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SIMPLIFYING METHODS FOR IN VITRO METAMORPHOSIS OF GLOCHIDIA

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ABSTRACT

Captive culture of Unionoid mussels is complicated by the parasitic larval stage, which normally requires a host fish for metamorphosis. Alternatively, some mussel species can metamorphose in vitro, i.e. in an artificial medium in Petri dishes. Most workers have used 5% CO₂ atmosphere and bicarbonate to stabilize pH, requiring a specialized incubator. In the present study, in vitro metamorphosis success of Anodonta oregonensis and other species were higher or similar in air than in 1%, or 5% CO₂. The nutritional role of the medium was tested by substituting physiological saline without nutrients at varying intervals before metamorphosis was complete. Pyganodon grandis metamorphosed without external nutrition during more than half of the incubation period, suggesting that development, once triggered, can continue largely on internal reserves. Post-metamorphic growth rates of P. grandis from medium, from saline, and from host fish were similar. Previous studies indicate that species which grow substantially during metamorphosis are unsuccessful in vitro. It was hypothesized that higher nutrient use by these species might result in local diffusion-limited depletion of the growth medium, which might be alleviated by circulation. However, initial attempts to metamorphose Leptodea fragilis glochidia in media circulated by a slow rocker system were unsuccessful.

KEYWORDS: Freshwater mussels, in vitro, development, metamorphosis, Unionoid

This abstract is approved as to form and content

M. Christopher Barnhart, PhD
Chairperson, Advisory Committee
Missouri State University
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Approved:

___________________________
M. Christopher Barnhart, PhD

___________________________
Ryan S. Udan, PhD

___________________________
Dan Beckman, PhD

___________________________
Julie Masterson, PhD: Dean, Graduate College

In the interest of academic freedom and the principle of free speech, approval of this thesis indicates the format is acceptable and meets the academic criteria for the discipline as determined by the faculty that constitute the thesis committee. The content and views expressed in this thesis are those of the student-scholar and are not endorsed by Missouri State University, its Graduate College, or its employees.
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# TABLE OF CONTENTS

**Introduction** ................................................................................................................................. 1
  Life History of Freshwater Mussels ................................................................................................. 1
  Glochidia Development ..................................................................................................................... 3
  Propagation ...................................................................................................................................... 5
    Metamorphosis *In Vivo* on Fish Hosts ......................................................................................... 6
    *In Vitro* Metamorphosis ............................................................................................................ 8
  Goal and Objectives .......................................................................................................................... 11

**Methods** ........................................................................................................................................ 13
  Mussel Collection and Care .............................................................................................................. 13
  Media Preparation .......................................................................................................................... 14
  Loading Culture Dishes .................................................................................................................. 15
  Judging Survival, Metamorphosis and Growth Experiments .................................................................. 16

**Results** ........................................................................................................................................... 22

**Discussion** ..................................................................................................................................... 24
  CO2, pH, and Medium Formulations ............................................................................................... 24
  Optimizing *In Vitro* Incubation ..................................................................................................... 26
  Developmental Triggers and Nutrition during Development .......................................................... 27
  Quality of *In Vitro* vs *In Vivo* Juveniles .................................................................................... 28
  Effect of Agitation of Culture Medium on *L. fragilis* .................................................................... 28
  Measuring Success of *In Vitro* Culture ........................................................................................ 29

**References** ..................................................................................................................................... 31

**Appendices** .................................................................................................................................. 60
  Appendix A. *In Vitro* Culture Medium Composition ...................................................................... 60
  Appendix B. Lebovitz’s Medium Components ................................................................................. 61
  Appendix C. Components of M199 .................................................................................................... 62
  Appendix D. Fish Saline Composition .............................................................................................. 63
LIST OF TABLES

Table 1. Species and collection localities of mussels used for experiments..........................37
Table 2. Effect of CO₂ #1 (L IV)..........................................................................................37
Table 3. Effect of CO₂ #1 (L W)..........................................................................................38
Table 4. Effect of CO₂ #2 (L IV)..........................................................................................38
Table 5. Effect of CO₂ #3 (L IV)..........................................................................................38
Table 6. Effect of CO₂ #3 (L W)..........................................................................................39
Table 7. Lebovitz versus M199 (L IV)....................................................................................39
Table 8. Lebovitz versus M199 (L W)....................................................................................40
Table 9. Effect of Serum Source (L W)....................................................................................40
Table 10. Duration of incubation (L IV)..................................................................................41
Table 11. Duration of incubation (L W)..................................................................................41
Table 12. Duration in Saline (L IV).......................................................................................41
Table 13. Duration in Saline (L W).......................................................................................42
Table 14. Duration in Saline after Long-term Culture (Shell Length).......................................42
Table 15. Duration in Saline after Long-term Culture (L C)....................................................42
Table 16. Average Length of P. grandis After 5 Months in Lab Culture.................................43
Table 17. Long-term Culture Between In Vitro & In Vivo (L C).............................................43
Table 18. Long-term Culture Between In Vitro & In Vivo (Shell Length)...............................44
LIST OF FIGURES

Figure 1. Set-up for incubating dishes in controlled atmosphere ........................................45
Figure 2. Effect of CO₂ #1 (L_{IV}) ...............................................................................46
Figure 3. Effect of CO₂ #1 (L_{W}) ................................................................................47
Figure 4. Effect of CO₂ #2 (L_{IV}) ................................................................................48
Figure 5. Effect of CO₂ #3 (L_{W}) ................................................................................49
Figure 6. Lebovitz versus M199 Media (L_{IV}) .................................................................50
Figure 7. Lebovitz versus M199 Media (L_{W}) .................................................................51
Figure 8. Effect of Commercial Sera Source (L_{W}) ........................................................52
Figure 9. Effect of Duration of Incubation (L_{W}) ............................................................53
Figure 10. Effect of Duration of Incubation (L_{IV}) ..........................................................54
Figure 11. Effect of Duration in Saline (L_{IV}) .................................................................55
Figure 12. Effect of Duration in Saline (L_{W}) ................................................................56
Figure 13. Duration in Saline after Long-term Culture (L_{C}) .........................................57
Figure 14. Duration in Saline after Long-term Culture (Shell Length) .............................58
Figure 15. Long-term Culture Between In Vitro & In Vivo (Shell Length) ......................59
INTRODUCTION

Freshwater mussels (Unionoida) are unique organisms that live unobtrusively in the substrate of rivers and lakes. To the casual observer these living rocks appear to be nothing more than a dirty shell, but these inconspicuous invertebrates contain much more than meets the eye. Mussels hold both historical and ecological significance for the freshwater ecosystems of the world, serving as a crucial trophic link between the water column and the benthos (Vaughn & Hakenkamp, 2001). The North American mussel fauna is of specific concern due to conservation status (Lydeard et. al., 2004). More than 2/3 of the 297 described species of North American freshwater mussels are classified as endangered, threatened, or of special concern (Williams et. al., 1993). Ancient, formerly stable mussel populations throughout North America have been plummeting in the last century. The causes for this decline are complex, but all are anthropogenic at their source. Pollution, damming, and nonindigenous mussel introductions are a few of the factors responsible for the deterioration of mussel populations (Williams et. al., 1993).

Life History of Freshwater Mussels

The life cycle of freshwater mussels makes them sensitive to many changes people have made to fluvial systems. The Order Unionoida is unique among bivalves in having parasitic larvae. Males release sperm into the water column that are then filtered out by females and used to fertilize thousands of eggs. The zygotes are then stored in marsupial portions of the hollow ctenidia (mussel gills) as they develop into the glochidia larvae (Wächtler et. al., 2000). Glochidia are obligate parasites on fish or amphibians.
They are released from the female and attach, by closing their valves, on host gills or skin where they become encapsulated.

The symbiotic relationship between fish and mussels has evolved over the course of 200 million years, resulting in complex mussel-host relationships (Watters, 2000). The impressive diversification of the North American taxa has been driven by adaptations that attract host fish, including many forms of mimicry of host prey by mantle lures and conglutinates. These adaptations act to limit the taxonomic range to that of the fish that glochidia encounter (Haag & Warren, 2003) and probably thereby facilitate the evolution of host specificity (Dodd et al., 2005; Barnhart et al., 2008). Host specificity varies, but most mussels can use only one or a few closely related species of host to complete metamorphosis (Isom & Hudson, 1984; Eckert, 2003).

Glochidia are morphologically simple bivalves with a single adductor muscle. Their function is to close when they contact host tissue, gripping gill or fin tissue between the shell valves (Hoggarth, 1999). Glochidia can be triggered to close by both mechanical and chemical cues. The glochidia that are successful in attachment will become encapsulated by a migration of host epithelial cells, called keratocytes (Rogers-Lowery & Dimock, 2006). Encapsulation can occur within as little as 4 hours of glochidium attachment (Arey 1932a). The ensuing parasitic period varies among mussel taxa from days to months (Arey, 1932b; Kat, 1984; Barnhart et al., 2008). During the parasitic period, glochidia undergo metamorphosis into juvenile mussels, capable of independent existence. Following encapsulation from their hosts, juvenile mussels settle in the substrate and thereafter live independently feeding on bacteria, algae, and fine particulate organic material (FPOM) (Neves & Widlak, 1987).
Glochidium morphology and size vary tremendously among species and reflect the dispersal and attachment strategies of the different species (Hoggarth, 1999; Barnhart et. al., 2008). North American glochidia of the family Unionidae can be placed into three morphological groups: Hooked subtriangular, unhooked semi-elliptical, and axe-head (Lefevre, 1910; Wächter et. al., 2000). These three types are associated with the subfamily Unioninae, most of the subfamily Ambleminae, and the genus Potamilus, respectively (Hoggarth 1999). Hooked glochidia are generally large (300-380µm) and typically attach to fins or other exposed epithelium on the host fish. Semi-elliptical unhooked glochidia are smaller in size (60-280µm) and typically attach to gills. Axe-headed glochidia have an elongated axe or adze-shape and are usually large (Graf, 2006; Lefevre, 1910; Barnhart et. al., 2008).

**Glochidia Development**

Most mussel larvae do not grow during encapsulation. However, taxa with unusually small larvae, including Margaritifera, Leptodea, Truncilla and some Quadrula species, grow 25-100 times in mass while encapsulated (Barnhart et. al., 2008). It is therefore clear that these larvae must derive nutrition from their hosts (Denic et. al., 2015). However, there is evidence that, even in the absence of growth in linear dimensions, glochidia derive nutrition from the host (Fritts et. al., 2013; Denic et. al., 2015). Glochidia of Lampsilis cardium do not grow, but showed significant shifts in δ^{13}C and δ^{15}N of glochidia, toward the isotope ratios characteristic of the fish host, after metamorphosis. Comparison of the δ^{15}N of juveniles, glochidia and fish host suggested that 57.4% of juvenile nitrogen content could be sourced to the host fish (Fritts et. al., 2013; Denic et. al., 2015).
The route of nutrient uptake may be via pores that can be observed in the glochidium shell (Fisher & Dimock 2002a). Secretory granules observed in several studies during the early stages of metamorphosis suggest that extracellular digestion of host tissue may occur (Fisher & Dimock, 2002a; Scharsack, 1994; Mathers, 1972, 1973).

Chester Blystad (1923) was among the first to study the development of Anodonta glochidia. He proposed three stages of development: encapsulation (or encystment), mushroom body formation, and juvenile structure formation. Juvenile structures include the ctenidia, paired adductor muscles, and foot. More recently, Fisher and Dimock (2002a) described two stages of glochidia development in Utterbackia imbecillis. Stage 1 includes breakdown of the larval adductor muscle along with the formation of the mushroom body from the mantle cells during days 3-4 of metamorphosis. Stage 2 involves the generation of juvenile mussel structures including the two separate adductor mussels, the digestive glands, gills, nerves and foot. The mushroom bodies described by these workers are formed as outgrowths of the larval mantle on both sides. They remain throughout the parasitic period, only disappearing one day after the complete metamorphosis. The cells that make up the mushroom body show structural similarities to the digestive gland cells of adult mussels, and it has been suggested that the mushroom body may be instrumental in energy storage and the digestion of the larval adductor muscle and fish host tissues trapped between the shell of the glochidium (Fisher and Dimock 2002b).
Propagation

Work to propagate and culture freshwater mussels has been ongoing for over 100 years. Around the year 1886 an experienced German button cutter named J. F. Boeppele came to the Mississippi River basin in search of North American mussels, a resource that he believed would support the growth of an industry. Mussel harvest on an industrial scale began as early as 1889 resulting in millions of mussels extracted from the rivers of the Mississippi basin (Pritchard 2001). The uncontrolled exploitation of freshwater mussels for shell was exacerbated when competition made it profitable to produce only the highest quality buttons. This meant that fewer buttons could be punched out of one shell and lead to an even faster pace of harvest.

By the year 1899, declines in mussel populations were widely recognized. However, work to conserve the resource did not begin until 1914 with the opening of the U.S. Bureau of Fisheries’ Biological Station at Fairport, Iowa (Coker, 1916; Pritchard, 2001). Mussel propagation and culture was attempted in order to sustain the button industry. As a result, many advances in the knowledge of the basic biology of Unionida were made. Unfortunately, financial support for scientific interest in mussels dried up with the decline of the mussel industry. After lying fallow for decades, interest and research on mussels underwent resurgence with the enactment of the U.S. Endangered Species Act of 1973 (Pritchard, 2001). The conservation of endangered species provided a new source of motivation and funding for research into the biology of Unionida and into captive culture for research and population restoration.

Mussel propagation has been a particularly arduous task due to the parasitic life cycle of Unionida. However, great progress has been made over the last four decades
with advances in knowledge of fish hosts and technical advances in handling and grow-out of juveniles (reviewed by Haag, 2012). Today, propagation has become an important part of conservation efforts aimed at preventing further declines and extinctions of freshwater mussel species. Propagation is used both for population restoration and to provide subjects for toxicology and life history investigations. Nonetheless, glochidia metamorphosis and early juvenile culture remain bottlenecks of most propagation efforts.

**Metamorphosis In Vivo on Fish Hosts.** The earliest efforts at artificially propagating mussels on fish hosts (*in vivo* metamorphosis) began around 1899 at the University of Missouri when Winterton C. Curtis attempted artificially infest carp with *Anodonta* and *Lasmigona*. The biggest advances in mussel culture came when the charismatic George Lefevre began working with Curtis at the University of Missouri and later the Fairport Biological station from 1908 to 1914 (Pritchard, 2001). Further progress was made by Robert E. Coker as the director of the Fairport Biological Station in the late 1920s (Pritchard 2001). These early efforts were focused on producing mussels on an industrial scale by artificially infesting fish with glochidia. Large groups of fish captured by seining from flood backwaters were placed in tanks with glochidia extracted from gravid females. After the glochidia attached, the fish were released in the main body of a river with the expectation that the glochidia could complete their life cycle naturally with this simple assistance (Coker et al., 1921).

*In vivo* metamorphosis on fish hosts is still the predominant propagation method today. Modern techniques focus on efficient use of hosts and glochidia and on accurate measures of metamorphosis success. The number of glochidia is quantified and the intensity of the infection adjusted to an efficient level (Barnhart, 2002). The infested fish
are then housed in specialized recirculating aquarium systems to collect juveniles (Barnhart, 2003; Dodd et al., 2005). These systems allow efficient use of host fish and glochidia, which is important when working with rare mussel species.

*In vivo* metamorphosis works best for mussel species that utilize a robust and easily accessible fish host such as largemouth bass, catfish, or trout. However, in cases where the host fish are small, rare, or hard to house this method is particularly challenging. For example, the California floater (*Anodonta californiensis*) uses Three-spined Stickleback (*Gasterosteus aculeatus*) as their host. These fish are only 3-4 cm in length and are highly aggressive so that they must be isolated from one another. An over-infestation of glochidia can cause the fish host to die before the transformation is complete. One Three-spined Stickleback produces only around 50 juveniles (E. Glidewell and M. C. Barnhart personal communication). Minnows and darters have also been a challenge to use in this method as they are similarly small, sensitive to stress and must be collected in the wild.

A second problem with *in vivo* metamorphosis is that fish that are effective physiological hosts for juvenile production may not be the ecological hosts that the mussel would normally use in nature (Levine, 2009). Hypothetically, mussels that use multiple host species might be genetically polymorphic, with some individuals suited for one host over another. If so, artificial selection might occur during captive propagation, producing individuals that are suited for the production host rather than the ecological host. This is particularly concerning with rare mussel species, as any selected types introduced into a natural population would likely outnumber the native genotypes. Other
challenges that surround *in vivo* methods include; the space required to adequately house the fish host, ability to treat various fish diseases, and acquiring hosts.

**In Vitro Metamorphosis.** Max Mapes Ellis, a physiologist at the University of Missouri, School of Medicine began to work at the Fairport Biological Station in 1925. In 1926, Ellis published a note in Science magazine where he claimed to have developed an artificial method of propagating freshwater mussels without a host. Ellis was attempting to create a controlled environment where glochidia development could be better studied (Ellis, 1926). This early work involved removing encapsulated *Lampsilis. fallaciosa* glochidia from its host fish *Lepisosteus platostomus* after encystment periods of 18 and 96 hours, and placing them in an undescribed nutrient solution. The mussels reared in the best solution had survival rates similar to controls that were left on the fish gills (Ellis, 1926). Shortly after this publication, Ellis claimed that he could transform glochidia taken directly from a brooding female mussel rather than a host fish. He began developing a device that housed the nutrient solution he had developed and would be able to yield, in his estimation, millions of juvenile mussels. He also claimed to have devised a method of sterilizing glochidia to reduce the loss due to microorganism infection.

Archival evidence suggests that Ellis did produce juveniles; however, no pictures, diagrams, or recipes for the artificial culture medium survived (Pritchard, 2001). Mussels at that time were big business, and Ellis might have feared that his work would be stolen by commercial parties. In 1942, the US Bureau of Fisheries stopped funding Ellis’ mussel propagation work possibly due in part to Ellis’ reluctance to document his methods. All detailed knowledge of the artificial propagation technique was lost when Ellis died in 1953.
In the 1980s, Billy G. Isom and Robert G. Hudson began to reexamine Ellis’s work and developed their own culture medium allowing for the metamorphosis of glochidia without the fish host. This culture medium included physiological salts; amino acids, dextrose, antibiotics (carbenicillin, gentamicin sulfate, and rifampin), antimycotic (amphotericin B), and fish blood plasma (Isom & Hudson, 1982). Fish blood plasma was identified as a critical medium component without which metamorphosis would not begin. Isom and Hudson (1984) later reported that the species of fish plasma used was not important, but that use of bovine and fetal bovine serum was unsuccessful. Keller and Zam (1990) tested several commercially available sera and other protein sources, including neonatal calf serum, horse serum, salmon liver, fish plasma, rabbit pancreas, and casein. They found that both neonatal calf serum and horse serum provided higher mean percentage transformation than the fish plasma. Successful use of mammalian sera was also confirmed by Hudson and Shelbourne (1990).

The number one problem plaguing *in vitro* culture is contamination of culture dishes by bacteria or fungus. It is very difficult to isolate glochidia free from microorganisms. Therefore, antibiotic and antimycotic components are needed to minimize growth of contaminant organisms. The original mix of carbenicillin, gentamicin sulfate, rifampin, and amphotericin B, as well as a mix of penicillin, streptomycin and amphotericin B have been effective (Loveless et. al., 1999). However, both penicillin and amphotocerin B are potentially toxic to glochidia (Owen, 2009). Comparisons of juveniles metamorphosed *in vitro* and *in vivo* found that *in vitro* juveniles had lower levels of triglycerides and cholesterol that might be an effect of amphotericin B (Fisher & Dimock, 2006).
Useful improvements were developed by Owen (2009, 2010). These include frequent (2-day) medium changes, lowered concentration of amphotericin B, and added a lipid supplement—menhaden oil following the suggestions of Tankersley (2000). Owen found that mixing serum, menhaden oil, and rifampicin improved incorporation of these components into the mixture.

Early versions of the in vitro method adopted the high bicarbonate and high CO\textsubscript{2} (5\%) concentrations used for mammalian cell culture (Isom & Hudson, 1982; Keller & Zam, 1990). The high PCO\textsubscript{2} and high bicarbonate concentrations used in mammalian cell culture reflect the physiological condition of mammal body fluids. CO\textsubscript{2} and bicarbonate are in equilibrium according to the Henderson-Hasselbach equation:

$$\text{pH} = \text{pK}' + \log \left( \frac{[\text{HCO}_3^-]}{S \times \text{PCO}_2} \right)$$

where pK’ is the first dissociation constant of carbonic acid, and S is the solubility of CO\textsubscript{2}. The CO\textsubscript{2}-bicarbonate system acts to buffer pH because CO\textsubscript{2} is volatile and PCO\textsubscript{2} is held constant by equilibrium with the atmosphere to which the culture medium is exposed. As acid or base equivalents are added by metabolism, the change in pH is less because of conversion of bicarbonate to or from CO\textsubscript{2}. However, the levels of CO\textsubscript{2} and bicarbonate used in cell culture are much higher than would normally occur in fish or mussels, because aquatic organisms operate at much lower PCO\textsubscript{2} and bicarbonate levels than mammals. Keller and Zam (1990) recognized the potential convenience of culturing glochidia in medium equilibrated with air. They compared transformation success of Utterbackia imbecillis in 5\% CO\textsubscript{2} and in air, and with the use of organic buffer systems (HEPES and MOPS). The results were equivocal, but in some tests, equivalent success was obtained with air, and they recommended further testing (Keller & Zam 1990). There
has been a recent change in common culture practices that results in reduced NaHCO$_3$ and CO$_2$ (to 1%) despite the establishment of 5% CO$_2$ as the optimal value by Isom and Hudson (1982) and Hudson and Shelborne (1990).

**Goal and Objectives**

The overall goal for this study is to simplify and improve the efficacy of *in vitro* propagation methods, and to extend the method to a wider variety of species than previously tested. The specific objectives are as follows:

The pH of the culture medium is an important variable. The typical approach to *in vitro* metamorphosis requires a CO$_2$ incubator and presents non-physiological conditions because fish regulate much lower levels of CO$_2$ and bicarbonate than mammals (Dejours, 1981). Therefore, tests were conducted using 5%, 1%, and 0.04% CO$_2$ (ambient air) to develop a low CO$_2$ approach.

Metamorphosis is a developmental process and the time to completion depends on species, temperature, and probably other variables. Glochidia that attached to the host at the same time may exit the host fish over a period of several days or even weeks. In the *in vitro* method, a decision must be made when to transfer all of larvae from the medium to water. Experiments to test effects of duration of incubation on success of metamorphosis were completed. Additionally, morphological and behavioral signs that indicated completion of metamorphosis were identified.

Lebovitz’s L-15 medium is an alternative to M199 that utilizes phosphates and free base amino acids instead of sodium bicarbonate to balance the pH of the solution and
is commonly used in the culture of fish cells. This alternative medium was tested to
determine if it would increase metamorphosis success.

Blood serum is consistently cited as one of the most essential components of the
culture medium. It is a source of protein and perhaps other factors affecting growth.
Several studies compared results using sera from different vertebrates and rabbit serum
generally gives good results (Keller & Zam, 1990; Hudson & Shelborne, 1990; Isom &
Hudson, 1984; Owen, 2009; Owen et al., 2010) Rabbit serum from two different
manufacturers was tested to determine if both were equally suitable, because supplies are
sometimes inconsistent.

The importance of nutrition from the fish host or culture medium during
metamorphosis is unclear and may vary among species. At least one species, *Lasigmona
subviridis*, is capable of direct development within the egg (Barfield & Watters, 1998;
Lellis & King, 1998). A test was performed with *Pyganodon grandis*, in which the
nutrient medium was replaced with physiological saline during development to determine
if development, once initiated, can continue without continued nutrition.

The glochidia of most mussel species do not grow during encapsulation on their
fish hosts. However, several genera with unusually small glochidia (*Truncilla, Leptodea,
Margaritifera, Cumberlandia*) and one with oddly shaped glochidia (*Potamilus*) grow
substantially during encapsulation (Barnhart et. al., 2008). Glochidia resting in a dish, in
static, liquid medium, might experience local nutrient depletion. Therefore, the effect of
agitating the medium during incubation was tested, using *Leptodea leptodon*, a species
that grows during metamorphosis.
METHODS

Mussel Collection and Care

Brooding females were obtained from wild populations. The species tested included *Anodonta oregonensis*, *Anodonta californiensis*, *Pyganodon grandis*, *Utterbackia imbecillis*, and *Lampsilis siliquoidea* (Table 1). The species tested were all long-term brooders that normally carry mature glochidia during the winter months for release in the spring. Mussels were collected by Missouri State University staff or by collaborators and shipped to Missouri State University, where they were housed unfed in a temperature-controlled recirculating aquarium system at 10°C until use. Water used for housing females was filtered (5 μm) James River water collected from Creighton Access. Water chemistry (ammonia, pH, hardness) was monitored and maintained with regular water changes.

Glochidia were extracted from females just before placing the larvae into culture dishes. Several approaches were used during extraction to minimize contamination of the glochidia with bacteria and fungi. Female shells were washed with dilute bleach, taking care to avoid contact of bleach with the mantle margin. Washed females were then placed in 1-2 changes of sterile-filtered (0.45μm) water for up to an hour, to allow the marsupia to perfuse with clean water. Glochidia in most cases were extracted from the female gill by injecting the marsupia with sterile-filtered water from a syringe to flush out the larvae. In some tests, the female was sacrificed and entire marsupial gills were dissected out into sterile-filtered culture medium. The isolated gills continued to perfuse, so that glochidia closed while still within the gill. This procedure seemed to reduce the
proportion of larvae that closed on other larvae. For a few species, glochidia were collected after natural release from the female. Isolated glochidia were rinsed 1-3 times in sterile filtered water after extraction and before placing into culture dishes.

**Media Preparation**

Medium was mixed according to recommendations of Christopher Owen (2009, 2010, and personal communication) (Appendix A). Ingredients were stored frozen (-80°C) or refrigerated (3-5°C) according to product recommendations. Ingredients were kept on ice while preparing solutions. Glassware and other items used were sterile-packaged or were rinsed with 95% ethanol and sterile-filtered water before use. Mixing procedure was as follows:

1) **M199 medium.** Add 100 grams of the dry powder to 900ml of sterile DI water. Mix and then fill to final volume of 1000ml.

2) **Titrate using CaOH to pH 7.6.**

3) **Add the following ingredients (no specific order):** 70mg L-alanine, 20mg L-ornithine, 40mg L-proline, 80mg taurine, 20mg L-threonine, 1ml EAA mixture, 1ml NEAA mixture, 1ml MEM vitamin mixture, 2.25g D-galactose, 2g glucose, 1ml lipid mixture 1, 100mg Sodium pyruvate, 150mg Gentamicin, 150mg Carbenicillin, and 1.5ml Amphotericin B solution.

4) **Stir until all components are in solution, and NaOH is used to titrate the mixture to achieve a pH of 7.65 at 22°C.**

5) **Serum solution.** In a separate 1000 ml flask, mix 500ml of thawed rabbit serum, 1ml menhaden oil and 150mg Rifampicin.

6) **Add the serum solution to the M199 solution while stirring.**

7) **Store the complete medium in 50ml aliquots at -80 °C to be thawed as needed.**
Loading Culture Dishes

Culture was carried out in 3-inch (6 x 1.5 cm) polystyrene Petri dishes (Fisher AS402). Transfers of medium and larvae into dishes were carried out under a class II, type A2 biological safety cabinet (Model no. NU-425-300). Each dish was loaded with 5 ml of medium. Medium was added to each dish from a 60 ml syringe through a sterile 0.45µm syringe filter (Fisher: SCHA033SS). Each dish was loaded with approximately 100-300 glochidia. Larvae were transferred using sterile transfer pipettes (Fisher 13-711-20, 1 ml). It was important to minimize the volume of water transferred into the medium when adding glochidia to the culture dish. This was accomplished by drawing approximately 100-300 larvae into the transfer pipette, then holding the pipette vertically to allow the glochidia to drift to the tip before dispensing. This procedure minimized the volume of suspension added to the medium.

The medium in each dish was changed every other day. Approximately 3ml of medium was removed and 3ml were added to each dish during each change. Records were kept of dishes that became infected at any point during incubation. If a dish became infected the following actions were taken depending on the severity of the contamination. For a light infection, the foreign growth and all medium was removed from the dish and replaced with 5ml of fresh sterile medium. If the foreign growth could not be removed, or if the dish had been previously treated, the healthy glochidia were removed, rinsed 3 times with sterile medium, and placed into a new sterile dish.

Culture dishes were stored in glass boxes with tight fitting lids. The boxes were either unventilated or ventilated with sterile-filtered air or gas mixtures. Incubation temperature was 21-22°C.
Judging Survival, Metamorphosis and Growth

Photographs were taken of each dish of glochidia after loading the culture dishes and before each medium change. The photos were taken through the transparent lid (without opening the dish) on top of a light box using a Canon Eos Rebel T3i camera with a Canon Macro lens EF-S 60mm mounted to a copy stand. Photos were taken using the Eos Utility and were examined to determine the number of open and closed individuals and anatomical features. In some cases, maximum length was measured from photos using Image J software.

The survivorship of glochidia and larvae was determined at 3 times during experiments. First, initial viability of the glochidia (L₀) was determined as the percent of glochidia that closed when initially exposed to the culture medium. Second, the percentage of glochidia that survived during in vitro culture (L_IV) was measured as the percent of individuals that remained closed at the end. Dead larvae usually open because the elastic ligament of the shell opens the valves if not opposed by the adductor muscle. Lastly, the percent survival of juveniles in water (L_W) was measured 1-4 days after transfer to river water. Survival in water was judged by foot movement. Each of these survival counts was calculated as a percent of the previous count. That is, L_IV was calculated based on viable glochidia (L₀) and L_W was calculated based on those that had survived in vitro (L_IV)

During each medium change, reasonable attempts were made to insure that no glochidia live or dead were removed with the extracted media; however, some may have been lost. Some transfer loss of individuals was also possible during the transition of
glochidia to filtered river water from culture media. If a dish was infected, the likelihood of transfer loss was higher as it was deemed more beneficial to remove live or dead glochidia that were in direct contact with the contaminating growth. Any time glochidia were transferred to a new dish there was risk of transfer loss, glochidia can end up stuck to the side of a transfer pipette if it is not carefully inspected. By comparing numbers between counts, these losses were estimated to be less than 3%.

**Experiments**

Three experiments were conducted to test effects of CO₂ level on metamorphosis success. In these experiments, the dishes were exposed to filtered air or air+CO₂ mixtures. The treatment groups of dishes were placed in glass boxes with fitted plastic lids (Figure 1). They were ventilated with treatment gas at about 100 ml/minute via a connection attached to the lid. The lids were tight fitting but vented the gases under slight positive pressure. Gas samples drawn from the boxes by syringe were analyzed with an infrared CO₂ analyzer to confirm the test gas concentrations. Before dispensing to dishes, the medium was equilibrated with the gas mixture for 1-4 hours by vigorous stirring in a ventilated Erlenmeyer flask. The medium was then titrated using NaOH to a pH of 7.65 while continuing stirring with the gas. Medium was considered to be equilibrated if the pH held steady for 30 min at the atmospheric CO₂ of the treatment.

In experiment #1 on the Effect of Atmospheric CO₂ Levels, there were 2 treatments, air or 1% CO₂. The CO₂ mixture was prepared using a Matheson DynaBlender® gas mixing system and an infrared CO₂ analyzer. In each treatment group, there were 17 dishes of glochidia, randomly chosen, from a single *Anodonta*
*californiensis* female. $L_0$ was calculated within 4 hours of introduction to medium. $L_{IV}$ was calculated on the 8th day in media. $L_W$ was calculated after 24hrs in river water for a random selection of five dishes in each treatment. ANOVA was used to compare $L_{IV}$ and $L_W$ of air (0.04% CO$_2$) and 1% CO$_2$.

In experiment #2 on the Effect of Atmospheric CO$_2$ Levels, there were two treatments, air or 1% CO$_2$, similar to experiment #1. In each treatment group, there were 17 dishes of glochidia loaded with glochidia from a single *Utterbackia imbecillis* female. $L_0$ was calculated within 4 hours of introduction to medium. $L_{IV}$ was calculated on the 8th day in media. ANOVA was used to determine differences between the $L_{IV}$ in air and 1% CO$_2$.

In experiment #3 on the Effect of Atmospheric CO$_2$ Levels, there were three treatments, air, 1% CO$_2$, and 5% CO$_2$. The CO$_2$ mixtures were delivered from commercial mixed gas cylinders. In each treatment group, there were 12 dishes of glochidia, with four dishes randomly chosen from each of three *Anodonta oregonensis* females. One day after the appearance of the adductor muscles, $L_{IV}$ was calculated and juveniles were moved into 5um filtered river water after being rinsed. $L_W$ counts were taken after 24hrs unfed in river water. ANOVA was used to compare $L_W$ among the treatments and among females.

In the experiment Lebovitz versus M199, there were two treatments, Lebovitz L-15 and M199 medium, incubated in air (no added CO$_2$). In each treatment group, there was a total of 12 dishes of glochidia, with 4 dishes, randomly chosen, from each of three *Anodonta oregonensis* females. Medium was mixed for this experiment using the same recipe; the only substitution was the Lebovitz (Appendix B) for M199 (Appendix C).
according to the recommendations on the label for mixing 1L of medium. One day after the appearance of the adductor muscles, $L_{IV}$ was calculated and juveniles were moved into 5µm filtered river water after being rinsed. $L_w$ counts were taken after 24-48hrs unfed in river water. An ANOVA was used to determine if there was a difference in $L_w$ between the treatments.

In the experiment Commercial Sera Source, the treatments were media made with rabbit serum from two different commercial sources, Gibco and IMB. In each treatment group, there were a total of 12 dishes of glochidia, with 4 dishes, randomly chosen from each of three *Lampsilis siliquoidea* females. A single batch of medium was mixed through Step 4, and then divided in half before adding serum. One day after the appearance of the adductor muscles, $L_{IV}$ was calculated and juveniles were moved into 5 µm filtered river water after being rinsed. $L_w$ counts were taken after 24-48 hours, unfed, in river water. ANOVA was used to compare $L_w$ between a) treatments and b) infection during incubation on $L_w$.

In the experiment Duration of Incubation, the treatments were duration of incubation in medium before transfer to water. 39 dishes were loaded with glochidia from a single *Anodonta oregonensis* female and incubated in filtered air. Three dishes were randomly chosen for transfer to water at selected time intervals. $L_0$ was calculated within 4 hours of introducing the glochidia to medium. Dishes were removed at intervals that became more frequent after 6 days in media corresponding with an observation of the complete absorption of the larval adductor (stage 2 of development). Records of dishes that became infected at any point during incubation were kept. $L_{IV}$ of each group was
calculated on the last day in media. \( L_w \) was calculated after 4 days in filtered river water. ANOVA was used to test effects of the duration of incubation in medium.

In the experiment Duration in Saline, culture medium was replaced by saline (Appendix D) during metamorphosis of *Pyganodon grandis* glochidia. 48 dishes were loaded with glochidia from a single *Pyganodon grandis* female and incubated in filtered air. Treatments consisted of substituting saline for medium in groups of four dishes, randomly chosen, at time intervals of 0, 1, 24, 48 72, 96, 120, 144, 168, 192, 216, and 240 hours. \( L_{IV} \) was calculated on the 8\(^{th} \) day, before transfer to 5\( \mu \)m filtered river water. \( L \) was calculated after 3 days in water. ANOVA was used to compare among treatment groups.

For Long-term Culture Between *In Vitro & In Vivo*, *Pyganodon grandis* juveniles from the saline experiment above were cultured to determine long-term survival and growth. The four replicates were pooled within each saline treatment and then divided into 2 replicates per treatment for grow out. The replicate groups were kept in separate containers in the culture system. Additionally, a cohort of *in vivo* metamorphosed juveniles was cultured for comparison. These *in vivo* juveniles were derived from the same pool of glochidia and were metamorphosed on host fish (*Betta splendens*). Eight replicate *in vivo* groups (~34 individuals per group) were cultured. After 5 months of culture, replicates were pooled by treatment and survival and growth was recorded for each treatment. ANOVA was used to compare among culture methods.

In the experiment Effect of Agitation of Culture Medium on *L. fragilis*, there were two treatment groups: stirred and static. 24 dishes were loaded with glochidia from a single female *Leptodea leptodon*, a species that grows during metamorphosis. Half of the
dishes were kept in a static condition on the bench top. The other dishes were stirred on a rocker table that was designed to produce a 1-inch tilt over 1 minute. To determine growth and survival in the dishes, a gridded track with three lanes of 6.35 by 6.35 mm boxes was created to span the width of the dish. A random number table was used to select which lane would be counted every 6.35 mm across the dish. In total, 16 boxes were counted for every dish to determine $L_0$, and $L_{IV}$. Image J was used to measure a subset of the dishes in each treatment to determine the average growth of the glochidia.
RESULTS

In Experiment #1 on the Effect of Atmospheric CO₂ Levels: *Anodonta californiensis* survival *in vitro* (LIV) was significantly higher using air than using 1% CO₂ (P= <0.005, Table 2, Figure 2.). The difference was evident after 4 days and increased by 8 days incubation (Figure 2). Juveniles from air-incubation also had higher survival during 24 h in water (L₆), but the difference was insignificant (Figure 3, Table 3).

Experiment #2: *Utterbackia imbecillis* LIV was slightly higher in air but not significantly different from 1% CO₂ (Figure 4, Table 4). L₆ of *Utterbackia* was not determined.

Experiment #3: *Anodonta oregonensis* LIV was similar among different CO₂ levels (Table 5) but juvenile survival in water (L₆) was highest following incubation in air (84%) compared to 1% CO₂ (74%) and 5% CO₂ (68%) (Figure 5, Table 6, P=0.030).

In the experiment Lebovitz versus M199, *Anodonta oregonensis* cultured with an M199 base had a higher LIV than those cultured in Lebovitz’s L-15 (P= 0.028, Table 7, Figure 6). However, L₆ was significantly higher among juveniles cultured in M199 based medium (P=0.025, Table 8, Figure 7).

In the experiment Effect of Serum Sources, the L₆ of *Lampsilis siliquoidea* cultured using rabbit sera from two different suppliers did not differ significantly. This experiment also compared and found no difference in L₆ of juveniles “rescued” from contaminated dishes with those from continuously healthy dishes. However, L₆ generally in the experiment was low and varied widely among dishes (p=0.098, Table 9, Figure 8).
In the experiment Duration of Incubation, $L_w$ of *Anodonta oregonensis* was near zero if the period in medium was less than 144 hours (6 days). $L_{IV}$ was variable among dishes but was generally high throughout the 228 h (9.5 day) incubation (Figure 10, Table 10). $L_w$ increased significantly with longer duration of incubation ($P<=0.005$, Table 11, Figure 9).

In the experiment Duration in Saline, Glochidia that were placed into a physiological saline appeared to produce an exudate after three days. This exudate would obscure the margins of the glochidia making it harder to classify them as closed or open during the $L_{IV}$ count. A similar exudate was noted by Kovitvadhi et. al. 2012 in *Hyriopsis myersiana*. $L_{IV}$ and $L_w$ of *Pyganodon grandis* were significantly higher with time spent in culture medium ($P<=0.005$, Table 12, $P=0.005$, Table 13). However, both $L_{IV}$ and $L_w$ were largely independent of time in culture medium after about 50 hours (Figures 11 and 12). After 5 months of juvenile culture, there was no significant difference in survival or growth of juveniles derived from the different treatments (Table 14, Table 15, Figure 13, Figure 14). Average lengths ranged from 1.64-2.80mm (Table 16).

For Long-term Culture Between *In Vitro* & *In Vivo*, juveniles were cultured post metamorphosis for 5 months and there was no significant difference in survival or growth of the *in vivo* *P. grandis* compared to the *in vitro* derived juveniles from the saline experiment (Table 17, Table 18, Figure 15).

In the experiment, Effect of Agitation of Culture Medium on *L. fragilis*, the glochidia failed to grow in culture after 3 weeks in culture medium. No differences were observed between the glochidia housed on the stable benchtop vs. the rocker table. The experiment was terminated.
DISCUSSION

CO₂, pH, and Medium Formulations

For the species tested, it was not beneficial to utilize high levels of atmospheric CO₂ during *in vitro* incubation. Glochidia that were cultured in air (approximately 0.04% CO₂) had equal or higher survival compared to those cultured in 1% or 5% CO₂. Eliminating the need for an expensive CO₂ incubator makes *in vitro* metamorphosis more economical and accessible. However, it should be noted that all but one of the species reported herein are of one taxonomic tribe (Anodontini) and that tribe is characterized by relatively quick metamorphosis and good success rates *in vitro*. Representatives of two other taxonomic tribes (*Lampsilis siliquoidea* (Lampsilini) and *Quadrula cylindrica* (Quadrulini) were also metamorphosed in air, but survival was low and the juveniles of these species did not thrive in subsequent culture. Future studies should include a wider range of taxa.

Bicarbonate was omitted from the medium, which was titrated to pH 7.65 while equilibrated with air, 1% CO₂, or 5% CO₂. The use of lower bicarbonate and CO₂ concentrations without other buffers might be expected to compromise pH control in the culture medium during incubation. Color change of phenol red indicated that the pH in the culture dishes became more basic but rose no higher than 8.0 between medium changes. Evidently, this change did not have substantial effect on survival or metamorphosis. In contrast, contaminated dishes became more acidic (yellow) presumably because of metabolic products of the contaminant bacteria or fungi. A previous study of *Anodonta suborbiculata* found that success *in vitro* was highest at the
lowest pH (7.6) and the highest level of CO$_2$ tested (5% CO$_2$) (Roberts 1997). However, that study also had good metamorphic success without elevated CO$_2$, if pH was kept low. Lebovitz’s L-15 medium was designed for cell culture without elevated CO$_2$ by including phosphate to buffer pH. The pH changes were less in the L-15 dishes. However, L-15 did not increase juvenile survival in this study. HEPES buffered medium also reduced success in a previous study (Roberts, 1997). Based on these results, medium that includes M199 without sodium bicarbonate presently appears to be the best option for in vitro metamorphosis in air. The M199-based medium used in this study (Appendix A) was supplemented with a number of nutritional components, some of which are present at lower concentrations in the stock M199 (Appendix B). The formulation was based on recommendations but more work could be done to ensure that the extra ingredients are necessary. Lipid accumulation has been used as a metric for metamorphic juvenile health (Lima et. al., 2004; Fisher & Dimock, 2006) and dietary lipid is thought to be important for growth and survival of post-metamorphic juveniles (Gatenby et. al., 1996).

It is interesting that both M199 and L-15 media include 400 mg KCl/liter. Metamorphosed juvenile mussels in water are relatively sensitive to potassium. The 96 hour LC$_{50}$ (concentration lethal to half of newly metamorphosed juveniles) is equivalent to 72-116 mg KCl/liter (Ivey et. al., 2013). It is likely that the parasitic stage of mussels is less sensitive to K, but future studies should test media with lower potassium.

Although there was no difference in results using blood serum from two suppliers, the experiment was equivocal and should be repeated. Other labs have reported trouble with Gibco sera (Monte McGregor personal communications). The utility of fish sera
versus mammalian sera is still debated. A recent study comparing transformation of *Hyriopsis myersiana* among plasma from horse and several fish species found that Carp (*Cyprinus carpio*) serum produced the highest transformation rate and suggested that this might be related to high concentrations of citrulline, glutamine, leucine, proline, threonine and alanine (Uthaiwan et. al., 2001). However, the inconvenience of preparing fish sera may outweigh minor effects on metamorphosis success relative to commercially available mammalian sera. Additionally, not all fish sera sources were equally successful when used in culture. *Hyriopsis myersiana* had lower *in vitro* efficiency when fish species that were not good hosts were used as a plasma source (Uthawain et. al., 2002, 2003; Lima et. al., 2012).

**Optimizing *In Vitro* Incubation**

One of the challenges of *in vitro* metamorphosis is determining the optimum duration of incubation before transferring the juveniles from medium to water. *Anodonta oregonensis* that were removed from media early were relatively slow to “wake up” in water and remained closed tightly in water for 24-48 hours before showing foot movement. Early removal was detrimental to juvenile survival and glochidia that were removed from medium before entering stage 2 of metamorphosis did not survive. Based on this work, the best policy for removing glochidia from the dish is to wait one to two days after the appearance of the juvenile adductor muscles. Some researchers have used foot movement in the culture medium as an endpoint for culture (Uthaiwan et. al., 2001; Owen, 2009; Lima et. al., 2006). In the present study, juveniles allowed to “wake up” in
culture medium did not generally thrive in subsequent culture during routine propagation work (personal observations).

**Developmental Triggers and Nutrition during Development**

Mussels that are long-term brooders retain glochidia for months in a state of developmental arrest. At least one mussel species, the Green Floater (*Lasmigona subviridis*) is able to complete development within the female marsupium without a fish host (Barfield and Watters 1998, Lellis and King 1998). Development and metamorphosis of most species, however, will not occur unless the glochidia attach to a host fish or are placed in a suitable culture medium. The factor(s) that trigger metamorphosis have not been identified. In this study, glochidia of *Pyganodon grandis* did not develop when they were placed directly in a physiological saline solution. However, brief exposure to medium triggered development, and some of the glochidia were able to successfully metamorphose in saline after having spent only 48 hours in culture medium (Figure 11). One or more components of the medium, perhaps in the blood serum, must trigger development and metamorphosis.

The results of substituting saline for medium showed that development of *P. grandis* continued without external nutrition during about 2/3 of the normal development period. Substituting saline for medium could be advantageous in some situations. The most promising application is as a potential treatment for contaminated culture dishes. If a dish becomes contaminated, moving the glochidia to nutrient-free saline would interrupt growth of the microorganisms. Shortening the time spent in culture medium would also reduce the cost of *in vitro* culture. This experiment should be repeated with
other species. Future research should include the rate of the nutrient uptake in glochidia as well as isolating the necessary proteins and growth factors for development so that the medium recipe can be further refined.

**Quality of In Vitro vs. In Vivo Juveniles**

It is important to know whether juveniles produced *in vitro* are of similar quality to those that metamorphose normally on fish hosts. In this study, there was no difference after 5 months of lab culture in the survival or growth of *P. grandis* that were metamorphosed *in vivo* vs *in vitro*. This result supports the suitability of juveniles produced *in vitro* in both restoration and toxicology work.

**Effect of Agitation of Culture Medium on *L. fragilis***

Apparently, no mussel species that grows during metamorphosis has yet been successfully metamorphosed *in vitro*. Possibly species that grow during metamorphosis require high rates of nutrient uptake. The hypothesis that high rates of uptake could lead to local nutrient depletion and diffusion-limitation was tested by stirring the culture medium, but stirring had no evident effect on *Leptodea fragilis*. The glochidia never grew or lost the larval adductor muscle in stirred or unstirred culture. Future studies should investigate the potential stimulus that initiates development in these species. Other labs have reported that the larvae of *Cumberlandia monodonta* will grow during *in vitro* incubation, but none have survived metamorphosis (personal communication Monte McGregor, Kentucky Department of Fish and Wildlife and Diane Waller, USFWS).
Measuring Success of In vitro Culture

There is no standard metric from measuring the success of in vitro culture. Juvenile survival recorded as the percentage of juveniles with foot movement after a number of days in water is the most common metric used in the literature. Uthawain et. al. used a somewhat different metric: in vitro efficiency (the percent of live juveniles after 6-12 days out of the total number of larvae used) in their 2002 study that resulted in 32-94% efficiency for Hyriopsis myersiana using various fish plasma sources. Kovitvadhi et. al. (2008) reported 84% success of Hyriopsis myersiana using fish serum. Lima et. al. (2006) reported juveniles of Anodonta cygnea completed metamorphosis with a mean of 61% using fish serum. Owen (2009) reported nearly perfect metamorphosis success for Utterbackia imbecillis and Anodonta suborbiculata using rabbit serum. The juvenile yield efficiencies of the present study fall within these ranges (A. californiensis 61%, A. oregonensis 70%, 74% and 86%; P. grandis 45-65% and L. siliquoidea 37%).

In this study, survival after incubation (LIV) was measured and again after several days in water (LW). It is tempting to use the LIV count as a measure of metamorphosis success, because it is easy to determine if a glochidium is closed with adductor mussels in a clean dish from a picture that can be processed at any time. Survival in water is more problematic. Juveniles in water may be reluctant to move. LW counts can be highly variable based on the timing of the counts. Counts of foot movement taken immediately upon removal of the dish will be much lower than those taken at 24 to 48 hours in water due to the variation in amount of time it takes for juveniles to “wake up” and begin movement. Another measure of survival is growth of the shell margin, which can be
evident after 24 hrs. The presence of marginal growth after 48 hours could be a better metric for metamorphosis success while a culture period of one month with foot movement would provide information on juvenile survival to account for juveniles that cannot survive the initial transition to water.

In summary, in vitro metamorphosis is a viable and useful method for producing metamorphic juveniles of many mussel species. Elevated CO\textsubscript{2} and CO\textsubscript{2} incubators are not required for the in vitro culture of species of the tribe Anodontini. More work should be done with other taxa to confirm efficacy. Lampsilis siliquoidea and Quadrula cylindrica also completed metamorphosis without elevated CO\textsubscript{2} but the juvenile survival was lower in these species. The method requires good sterile technique and a laminar flow hood for success. Limitation of fluid transfer is the most consistent and successful method for reducing loading contamination. The quality of culture medium may vary depending on manufacturer and lot numbers. Waiting 48 hours after the first appearance of the juvenile adductor mussels is a good end point for Anodonta oregonensis, but this should be investigated across a wider range of taxa. Incubation in physiological saline solution may provide a new method of infection control in culture dishes and a way of reducing use of expensive medium.
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Pritchard, J., 2001. An historical analysis of mussel propagation and culture: research performed at the Fairport Biological Station. Clear Creek Historical Research, Ames, IA. 76.


Table 1. Species and collection localities of mussels used for experiments.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Species</th>
<th>Collection site</th>
</tr>
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<tbody>
<tr>
<td>Effect of CO₂ #1</td>
<td><em>Anodonta californiensis</em></td>
<td>Pajaro River, CA</td>
</tr>
<tr>
<td>Effect of CO₂ #2</td>
<td><em>Utterbackia imbecillis</em></td>
<td>Swope Park Lagoon, Kansas City, MO</td>
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<tr>
<td>Effect of CO₂ #3</td>
<td><em>Anodonta oregonensis</em></td>
<td>Hayes Island Upper Columbia River, WA</td>
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<td>Lebovitz versus M199</td>
<td><em>Anodonta oregonensis</em></td>
<td>Hayes Island Upper Columbia River, WA</td>
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<td>Serum Source</td>
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<tr>
<td>Duration of Incubation</td>
<td><em>Anodonta oregonensis</em></td>
<td>Hayes Island Upper Columbia River, WA</td>
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<tr>
<td>Duration in Saline</td>
<td><em>Pyganodon grandis</em></td>
<td>Tablerock Lake, MO</td>
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<tr>
<td>Long-term Culture</td>
<td><em>Pyganodon grandis</em></td>
<td>Tablerock Lake, MO</td>
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<tr>
<td>Agitation of Medium</td>
<td><em>Leptodea fragilis</em></td>
<td>Pomme de Terre River, MO</td>
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Table 2. Effect of CO₂ #1. ANOVA of percent survival of *Anodonta californiensis* in medium (LIV) versus CO₂ concentration.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Adj ss</th>
<th>Adj ms</th>
<th>F-Value</th>
<th>P-Value</th>
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<tr>
<td>CO₂</td>
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<td>2274</td>
<td>2273.59</td>
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<tr>
<td>Error</td>
<td>32</td>
<td>1243</td>
<td>38.85</td>
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<td></td>
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<td>Total</td>
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<td>3517</td>
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### Table 3. Effect of CO2 #1. ANOVA of percent survival of *Anodonta californiensis* in water (Lw) versus CO2 concentration.

<table>
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<tr>
<th>Source</th>
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<th>Adj ms</th>
<th>F-Value</th>
<th>P-Value</th>
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<tr>
<td>CO2 Treatment</td>
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<td>477.6</td>
<td>4.39</td>
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<tr>
<td>Error</td>
<td>8</td>
<td>869.6</td>
<td>108.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>1347.1</td>
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### Table 4. Effect of CO2 #2. ANOVA of percent survival *in vitro* (LIV) of *Utterbackia imbecillis* versus CO2 concentration.

<table>
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<tr>
<th>Source</th>
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<th>Adj ms</th>
<th>F-Value</th>
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<tr>
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<td>1643</td>
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<td>Error</td>
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<td></td>
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<tr>
<td>Total</td>
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### Table 5. Effect of CO2 #3. ANOVA of percent survival *in vitro* (LIV) of *Anodonta oregonensis* among 3 treatment concentrations of CO2 and among females within treatments.

<table>
<thead>
<tr>
<th>Source</th>
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<th>Adj ss</th>
<th>Adj ms</th>
<th>F-Value</th>
<th>P-Value</th>
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<tr>
<td>CO2 Treatment</td>
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<td>18.16</td>
<td>9.079</td>
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<tr>
<td>Female(treatment)</td>
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<td>44.26</td>
<td>7.377</td>
<td>1.52</td>
<td>0.209</td>
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<tr>
<td>Error</td>
<td>27</td>
<td>130.85</td>
<td>4.846</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>35</td>
<td>193.27</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Table 6.** Effect of CO$_2$ #3. ANOVA of percent survival in water (L$_W$) of *Anodonta oregonensis* among 3 treatment concentrations of CO$_2$ and among females within treatments.

<table>
<thead>
<tr>
<th>Source</th>
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<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO$_2$ Treatment</td>
<td>2</td>
<td>1447</td>
<td>723.4</td>
<td>3.99</td>
<td>0.030</td>
</tr>
<tr>
<td>Female(treatment)</td>
<td>6</td>
<td>1734</td>
<td>289.0</td>
<td>1.59</td>
<td>0.188</td>
</tr>
<tr>
<td>Error</td>
<td>27</td>
<td>4900</td>
<td>181.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>35</td>
<td>8081</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 7.** Lebovitz versus M199 Medium. ANOVA of percent survival *in vitro* (L$_{IV}$) of *Anodonta oregonensis* among base medium, within medium, and among females.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Adj ss</th>
<th>Adj ms</th>
<th>F-Value</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>1</td>
<td>79.49</td>
<td>79.49</td>
<td>5.71</td>
<td>0.028</td>
</tr>
<tr>
<td>Female(Treatment)</td>
<td>4</td>
<td>125.16</td>
<td>31.29</td>
<td>2.25</td>
<td>0.104</td>
</tr>
<tr>
<td>Error</td>
<td>18</td>
<td>250.71</td>
<td>13.93</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>455.36</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 8. Lebovitz versus M199 Medium. ANOVA of percent survival *in vitro* (LIV) of *Anodonta oregonensis* among base media, contaminated versus uncontaminated within medium, and among females.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Adj ss</th>
<th>Adj ms</th>
<th>F-Value</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>1</td>
<td>4251</td>
<td>4250.5</td>
<td>6.02</td>
<td>0.025</td>
</tr>
<tr>
<td>Female(Treatment)</td>
<td>4</td>
<td>3895</td>
<td>973.8</td>
<td>1.38</td>
<td>0.281</td>
</tr>
<tr>
<td>Error</td>
<td>18</td>
<td>12712</td>
<td>706.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>20858</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 9. Effect of Commercial Serum Source. ANOVA of percent survival in water (LW) among serum sources, and comparing contaminated versus uncontaminated dishes, for *Lampsilis siliquoidea*.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Adj ss</th>
<th>Adj ms</th>
<th>F-Value</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>1</td>
<td>1413.0</td>
<td>1412.98</td>
<td>2.95</td>
<td>0.098</td>
</tr>
<tr>
<td>Contamination</td>
<td>1</td>
<td>1005.0</td>
<td>1004.98</td>
<td>2.10</td>
<td>0.160</td>
</tr>
<tr>
<td>Error</td>
<td>25</td>
<td>11955.8</td>
<td>478.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lack-of-Fit</td>
<td>1</td>
<td>12.0</td>
<td>12.04</td>
<td>0.02</td>
<td>0.878</td>
</tr>
<tr>
<td>Pure</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pure Error</td>
<td>24</td>
<td>11943.8</td>
<td>497.66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>27</td>
<td>14127.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 10. Effect of duration of incubation in medium before transfer to water for *Anodonta oregonensis*. ANOVA of percent survival *in vitro* (LIV) comparing contaminated versus uncontaminated dishes, and duration of incubation in medium.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Adj ss</th>
<th>Adj ms</th>
<th>F-Value</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation Hours</td>
<td>14</td>
<td>75.16</td>
<td>5.369</td>
<td>0.4</td>
<td>0.96</td>
</tr>
<tr>
<td>Error</td>
<td>25</td>
<td>331.89</td>
<td>13.276</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>39</td>
<td>407.05</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 11. Effect of duration of incubation in medium before transfer to water for *Anodonta oregonensis*. ANOVA of percent survival in water (LW) comparing contaminated versus uncontaminated dishes, and duration of incubation in medium.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Adj ss</th>
<th>Adj ms</th>
<th>F-Value</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation Hours</td>
<td>14</td>
<td>22965</td>
<td>1640.3</td>
<td>4.89</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Error</td>
<td>25</td>
<td>8395</td>
<td>335.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>39</td>
<td>31359</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 12. Effect of Duration in Saline. Substituting fish saline for medium after varying duration of incubation of *Pyganodon grandis*. ANOVA of percent survival *in vitro* (LIV) comparing contaminated versus uncontaminated dishes, and duration of incubation in medium before substituting saline.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Adj ss</th>
<th>Adj ms</th>
<th>F-Value</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hours in Medium</td>
<td>11</td>
<td>17267</td>
<td>1569.70</td>
<td>4.90</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Error</td>
<td>36</td>
<td>11537</td>
<td>320.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>47</td>
<td>28804</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 13. Effect of Duration in Saline. Substituting fish saline for medium after varying duration of incubation of *Pyganodon grandis*. ANOVA of percent survival in water ($L_w$) comparing duration of incubation in medium before substituting saline.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Adj ss</th>
<th>Adj ms</th>
<th>F-Value</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hours in Medium</td>
<td>11</td>
<td>17520</td>
<td>1592.70</td>
<td>5.66</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Error</td>
<td>36</td>
<td>10131</td>
<td>281.40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>47</td>
<td>27651</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 14. Effect of Duration in Saline after Long-term Culture. Substituting fish saline for medium after varying duration of incubation of *Pyganodon grandis*. ANOVA of average shell length after 5 months in culture among treatments (duration in medium before substituting fish saline).

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Adj ss</th>
<th>Adj ms</th>
<th>F-Value</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hours in Medium</td>
<td>6</td>
<td>1.136</td>
<td>0.1893</td>
<td>0.97</td>
<td>0.507</td>
</tr>
<tr>
<td>Error</td>
<td>7</td>
<td>1.368</td>
<td>0.1954</td>
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<td></td>
</tr>
<tr>
<td>Total</td>
<td>13</td>
<td>2.503</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 15. Effect of Duration in Saline after Long-term Culture. Substituting fish saline for medium after varying duration of incubation of *Pyganodon grandis*. ANOVA of survival after 5 months in culture among treatments (duration in medium before substituting fish saline).

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Adj ss</th>
<th>Adj ms</th>
<th>F-Value</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hours in Medium</td>
<td>7</td>
<td>4306</td>
<td>615.2</td>
<td>0.52</td>
<td>0.797</td>
</tr>
<tr>
<td>Error</td>
<td>8</td>
<td>9433</td>
<td>1179.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>13740</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 16. Average length of *P. grandis* after 5 months in lab culture. *In vitro* hours in medium before substituting saline, or *in vivo* juveniles from fish host. Numbers are mean ± SD (number of individuals measured).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average length (mm)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>In vitro</em>, 5 h</td>
<td>1.62</td>
<td>1</td>
</tr>
<tr>
<td><em>In vitro</em>, 6 h</td>
<td>1.64 ± 0.604</td>
<td>8</td>
</tr>
<tr>
<td><em>In vitro</em>, 7 h</td>
<td>2.35 ± 0.765</td>
<td>24</td>
</tr>
<tr>
<td><em>In vitro</em>, 8 h</td>
<td>2.80 ± 0.696</td>
<td>18</td>
</tr>
<tr>
<td><em>In vitro</em>, 9 h</td>
<td>2.02 ± 0.623</td>
<td>18</td>
</tr>
<tr>
<td><em>In vitro</em>, 10 h</td>
<td>2.11 ± 0.744</td>
<td>44</td>
</tr>
<tr>
<td><em>In vitro</em>, 11 h</td>
<td>2.45 ± 0.712</td>
<td>32</td>
</tr>
<tr>
<td><em>In vitro</em>, 12 h</td>
<td>1.98 ± 0.826</td>
<td>55</td>
</tr>
<tr>
<td><em>In vivo</em>, from fish host</td>
<td>2.16 ± 0.728</td>
<td>55</td>
</tr>
</tbody>
</table>

Table 17. *Pyganodon grandis* survival during 5 months in culture. ANOVA of culture survival between juveniles metamorphosed *in vitro* and *in vivo*. All *in vitro* treatment groups were included. Variability among culture beakers (replicates) was also tested.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Adj ss</th>
<th>Adj ms</th>
<th>F-Value</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture Method</td>
<td>1</td>
<td>1092</td>
<td>1092</td>
<td>2.61</td>
<td>0.126</td>
</tr>
<tr>
<td>Error</td>
<td>16</td>
<td>6699</td>
<td>418.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>7791</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 18. *Pyganodon grandis* growth in mm during 5 months in culture. ANOVA of growth in juveniles metamorphosed *in vitro* and *in vivo*. All *in vitro* treatment groups were included. Variability among culture beakers (replicates) was also tested.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Adj ss</th>
<th>Adj ms</th>
<th>F-Value</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture Method</td>
<td>1</td>
<td>0.03778</td>
<td>0.03778</td>
<td>0.24</td>
<td>0.628</td>
</tr>
<tr>
<td>Error</td>
<td>20</td>
<td>3.12316</td>
<td>0.15616</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>21</td>
<td>3.16094</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. Set-up for incubating dishes in controlled atmosphere. Air-CO$_2$ mixtures were supplied from mass flow controllers (Matheson) or premixed commercial cylinders. Gas mixtures were passed through ventilated boxes containing the culture dishes. CO$_2$ fraction was monitored using an Ametek infrared analyzer.
Figure 2. Effect of CO₂ #1. Effect of two levels of atmospheric CO₂ on survival of *Anodonta californiensis in vitro* (L₄V) during 8 days of incubation. Bars represent means and 95% confidence intervals, 17 dishes per treatment.
Figure 3. Effect of CO$_2$ #1. Effect of atmospheric CO$_2$ used in vitro on subsequent 24-h survival of *Anodonta californiensis* in water (L$_W$). Bars represent 95% confidence intervals (N=5).
Figure 4. Effect of CO$_2$ #2. Effect of atmospheric CO$_2$ on survival of *Utterbackia imbecillis* in culture medium (L$_{IV}$). Bars represent means and 95% confidence intervals (N=17).
Figure 5. Effect of CO$_2$ #3. Effect of atmospheric CO$_2$ used for *in vitro* metamorphosis of *Anodonta oregonensis* on subsequent 24-h survival in river water. Bars represent means and 95% confidence intervals (N=17).
Figure 6. Lebovitz versus M199 Media. Effect of base medium used for in vitro metamorphosis of *Anodonta oregonensis* on survival at the end of incubation in culture media (LIV). Bars represent means and 95% confidence intervals (N=13).
Figure 7. Lebovitz versus M199 Media. Effect of base medium used for in vitro metamorphosis of *Anodonta oregonensis* on subsequent 24-h survival in water (Lw). Bars represent means and 95% confidence intervals (N=13).
**Figure 8.** Commercial Sera Source. Effect of sera manufacturer of rabbit sera used for *in vitro* metamorphosis of *Lampsilis siliquoidea* on subsequent 24-h survival in water (LW). Bars represent means and 95% confidence intervals (N=14).
Figure 9. Duration of Incubation. Effect of duration of incubation in culture medium on survival in water ($L_W$) of *Anodonta oregonensis*. $T_M$= Time in culture medium. $L_W$= the percentage surviving after four days in water. Points represent $L_W$ of individual dishes and the dashed line represents the end of stage 1 of development and the beginning of stage 2.
Figure 10. Duration of Incubation. Effect of duration of incubation in culture medium on survival *in vitro* ($L_{IV}$) of *Anodonta oregonensis* $T_M$= Time in culture medium. Points represent $L_{IV}$ of individual dishes.
Figure 11. Duration in Saline. Effect of substituting physiological saline for culture medium on survival in vitro (LIV) of Pyganodon grandis. T_M = Time in culture medium before switching to saline. LIV was measured after 240 hours in vitro. Points represent LIV in individual dishes.
Figure 12. Duration in Saline. Effect of substituting physiological saline for culture medium on post-metamorphic survival in water (LW). TM = Time in culture medium before switching to saline. All juveniles were transferred to water after 240 h in vivo. LW = survival of Pyganodon grandis after 3 days in water. Points represent LW in individual dishes.
Figure 13. Duration in Saline after Long-term Culture. Survival of saline-incubated *Pyganodon grandis* juveniles after 5 months in laboratory culture (Lc). $T_M =$ time in culture medium before switching to saline. Points represent Lc in replicate groups that were cultured separately.
Figure 14. Duration in Saline after Long-term Culture. Growth (average shell length) of saline-incubated *Pyganodon grandis* juveniles after 5 months in laboratory culture. $T_M =$ time in culture medium before switching to saline. Points represent mean shell length in replicate groups that were cultured separately.
Figure 15. Long-term Culture Between In Vitro & In Vivo (Shell Length). Comparing mean shell length between *P. grandis* juveniles metamorphosed *in vitro* and *in vivo* after 5 months of culture. *In vitro* N=199, *in vivo* N=184 individuals measured.
**APPENDICES**

**Appendix A. In Vitro Culture Medium Composition.** Components are per 1.5 liter of medium.

<table>
<thead>
<tr>
<th>Salts and nutrients</th>
<th>Amino Acids and Vitamins</th>
</tr>
</thead>
<tbody>
<tr>
<td>M199 Powder</td>
<td>L-Alanine</td>
</tr>
<tr>
<td>Sodium Pyruvate</td>
<td>70 mg</td>
</tr>
<tr>
<td>Glucose</td>
<td>L-Ornithine</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>20 mg</td>
</tr>
<tr>
<td>Salmon Oil Extract</td>
<td>L-Proline</td>
</tr>
<tr>
<td>Menhaden Oil</td>
<td>40 mg</td>
</tr>
<tr>
<td>Rabbit serum</td>
<td>Taurine</td>
</tr>
<tr>
<td></td>
<td>80 mg</td>
</tr>
<tr>
<td></td>
<td>L-Threonine</td>
</tr>
<tr>
<td></td>
<td>20 mg</td>
</tr>
<tr>
<td></td>
<td>EAA</td>
</tr>
<tr>
<td></td>
<td>1 ml</td>
</tr>
<tr>
<td></td>
<td>NEAA</td>
</tr>
<tr>
<td></td>
<td>1 ml</td>
</tr>
<tr>
<td></td>
<td>MEM Vitamin</td>
</tr>
<tr>
<td></td>
<td>1 ml</td>
</tr>
<tr>
<td>Anti-microbials</td>
<td>Titration solutions</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>CaOH</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>NaOH</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>HCl</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Components are per 1.5 liter of medium.
### Appendix B. Lebovitz’s Medium components (ThermoFisher)

<table>
<thead>
<tr>
<th>Inorganic Salts</th>
<th>mg/L</th>
<th>Amino Acids</th>
<th>mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂ (anhydrous)</td>
<td>140</td>
<td>Glycine</td>
<td>200</td>
</tr>
<tr>
<td>MgCl₂ (anhydrous)</td>
<td>93.7</td>
<td>L-Alanine</td>
<td>225</td>
</tr>
<tr>
<td>MgSO₄ (anhydrous)</td>
<td>97.7</td>
<td>L-Arginine</td>
<td>500</td>
</tr>
<tr>
<td>KCl</td>
<td>400</td>
<td>L-Asparagine</td>
<td>250</td>
</tr>
<tr>
<td>KH₂PO₄ monobasic</td>
<td>60</td>
<td>L-Cysteine</td>
<td>120</td>
</tr>
<tr>
<td>NaCl</td>
<td>8000</td>
<td>L-Glutamine</td>
<td>300</td>
</tr>
<tr>
<td>Na₂HPO₄ dibasic</td>
<td>190</td>
<td>L-Histidine</td>
<td>250</td>
</tr>
<tr>
<td>Vitamins</td>
<td></td>
<td>L-Isoleucine</td>
<td>250</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>1</td>
<td>L-Leucine</td>
<td>125</td>
</tr>
<tr>
<td>D-Calcium pantothenate</td>
<td>1</td>
<td>L-Lysine</td>
<td>75</td>
</tr>
<tr>
<td>Folic Acid</td>
<td>1</td>
<td>L-Methionine</td>
<td>75</td>
</tr>
<tr>
<td>Niacinamide</td>
<td>1</td>
<td>L-Phenylalanine</td>
<td>125</td>
</tr>
<tr>
<td>Pyridoxine hydrochloride</td>
<td>1</td>
<td>L-Serine</td>
<td>200</td>
</tr>
<tr>
<td>Riboflavin 5’-phosphate Na</td>
<td>0.1</td>
<td>L-Threonine</td>
<td>300</td>
</tr>
<tr>
<td>Thiamine monophosphate</td>
<td>1</td>
<td>L-Tryptophan</td>
<td>20</td>
</tr>
<tr>
<td>i-Inositol</td>
<td>2</td>
<td>L-Tyrosine</td>
<td>300</td>
</tr>
<tr>
<td>Other Components</td>
<td></td>
<td>L-Valine</td>
<td>100</td>
</tr>
<tr>
<td>D+ Galactose</td>
<td>900</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenol Red</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium Pyruvate</td>
<td>550</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Appendix C. Components of M199. (M5017-10X1L - Medium 199 Sigma-Aldrich)

<table>
<thead>
<tr>
<th><strong>Inorganic Salts</strong></th>
<th><strong>mg/L</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl(_2) (anhydrous)</td>
<td>200</td>
</tr>
<tr>
<td>Fe(NO(_3))(\cdot)9H(_2)O</td>
<td>0.72</td>
</tr>
<tr>
<td>MgSO(_4) (anhydrous)</td>
<td>97.7</td>
</tr>
<tr>
<td>KCl</td>
<td>400</td>
</tr>
<tr>
<td>NaCl</td>
<td>6800</td>
</tr>
<tr>
<td>NaH(_2)PO(_4)-H(_2)O</td>
<td>140</td>
</tr>
<tr>
<td>NaHCO(_3)</td>
<td>2200</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Vitamins</strong></th>
<th><strong>mg/L</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>0.05</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.01</td>
</tr>
<tr>
<td>D-calcium pantothenate</td>
<td>0.01</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.5</td>
</tr>
<tr>
<td>Ergocalciferol</td>
<td>0.1</td>
</tr>
<tr>
<td>Folic acid</td>
<td>0.01</td>
</tr>
<tr>
<td>i-inositol</td>
<td>0.05</td>
</tr>
<tr>
<td>Menadione</td>
<td>0.01</td>
</tr>
<tr>
<td>Niacin</td>
<td>0.025</td>
</tr>
<tr>
<td>Niacinamide</td>
<td>0.025</td>
</tr>
<tr>
<td>PABA</td>
<td>0.05</td>
</tr>
<tr>
<td>Pyridoxal HCl</td>
<td>0.025</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>0.025</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.01</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>0.01</td>
</tr>
<tr>
<td>DL-(\alpha)-tocopherol phosphate 2Na</td>
<td>0.01</td>
</tr>
<tr>
<td>Vitamin A acetate</td>
<td>0.14</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Amino Acids</strong></th>
<th><strong>mg/L</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>L-alanine</td>
<td>25</td>
</tr>
<tr>
<td>L-arginine HCl</td>
<td>70</td>
</tr>
<tr>
<td>L-aspartic acid</td>
<td>30</td>
</tr>
<tr>
<td>L-cysteine HCl monohydrate</td>
<td>0.1</td>
</tr>
<tr>
<td>L-cystine 2HCl</td>
<td>26</td>
</tr>
<tr>
<td>L-glutamic acid</td>
<td>67</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>100</td>
</tr>
<tr>
<td>Glycine</td>
<td>50</td>
</tr>
<tr>
<td>L-histidine HCl monohydrate</td>
<td>21.8</td>
</tr>
<tr>
<td>Hydroxy L-proline</td>
<td>10</td>
</tr>
<tr>
<td>L-isoleucine</td>
<td>20</td>
</tr>
<tr>
<td>L-leucine</td>
<td>60</td>
</tr>
<tr>
<td>L-lysine HCl</td>
<td>70</td>
</tr>
<tr>
<td>L-methionine</td>
<td>15</td>
</tr>
<tr>
<td>L-phenylalanine</td>
<td>25</td>
</tr>
<tr>
<td>L-proline</td>
<td>40</td>
</tr>
<tr>
<td>L-serine</td>
<td>25</td>
</tr>
<tr>
<td>L-threonine</td>
<td>30</td>
</tr>
<tr>
<td>L-tryptophan</td>
<td>10</td>
</tr>
<tr>
<td>L-tyrosine 2Na dihydrate</td>
<td>57.7</td>
</tr>
<tr>
<td>L-valine</td>
<td>25</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Other</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine sulfate dihydrate</td>
<td>10.98</td>
</tr>
<tr>
<td>AMP monohydrate</td>
<td>0.2</td>
</tr>
<tr>
<td>ATP 2Na trihydrate</td>
<td>1.098</td>
</tr>
<tr>
<td>Cholesterol (synthetic)</td>
<td>0.2</td>
</tr>
<tr>
<td>2-Deoxy-d-ribose</td>
<td>0.5</td>
</tr>
<tr>
<td>Dextrose anhydrous</td>
<td>1000</td>
</tr>
<tr>
<td>L-glutathione reduced</td>
<td>0.05</td>
</tr>
<tr>
<td>Guanine HCl monohydrate</td>
<td>0.3</td>
</tr>
<tr>
<td>Hypoxanthine sodium salt</td>
<td>0.354</td>
</tr>
<tr>
<td>Phenol red sodium salt</td>
<td>21.24</td>
</tr>
<tr>
<td>D-Ribose</td>
<td>0.5</td>
</tr>
<tr>
<td>Thymine</td>
<td>0.3</td>
</tr>
<tr>
<td>TweenTM 80</td>
<td>20</td>
</tr>
<tr>
<td>Uracil</td>
<td>0.3</td>
</tr>
<tr>
<td>Xanthine sodium salt</td>
<td>0.344</td>
</tr>
</tbody>
</table>
**Appendix D.** Fish saline (Holmes and Stott, 1960)

<table>
<thead>
<tr>
<th>Components</th>
<th>(g)</th>
</tr>
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<tbody>
<tr>
<td>NaCl</td>
<td>7.41</td>
</tr>
<tr>
<td>KCl</td>
<td>0.37</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$ • H$_2$O</td>
<td>0.40</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$•2H$_2$O</td>
<td>0.20</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>0.31</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.17</td>
</tr>
<tr>
<td>MgSO$_4$•7H$_2$O</td>
<td>0.31</td>
</tr>
</tbody>
</table>