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Simplifying Methods for in Vitro Metamorphosis of Glochidia

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SIMPLIFYING METHODS FOR *IN VITRO* METAMORPHOSIS OF GLOCHIDIA

A Masters Thesis

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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

Biology

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Master of Science

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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% CO_2 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% CO_2. 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

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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 excapsulation 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; Wächtler 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 $\delta^{13}C$ and $\delta^{15}N$ of glochidia, toward the isotope ratios characteristic of the fish host, after metamorphosis. Comparison of the $\delta^{15}N$ 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). 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. Boepple 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_2 (5%) concentrations used for mammalian cell culture (Isom & Hudson, 1982; Keller & Zam, 1990). The high P_{CO_2} and high bicarbonate concentrations used in mammalian cell culture reflect the physiological condition of mammal body fluids. CO_2 and bicarbonate are in equilibrium according to the Henderson-Hasselbach equation:

$$pH = pK' + \log ([HCO_3^-]/ S*P_{CO_2})$$

where pK' is the first dissociation constant of carbonic acid, and S is the solubility of CO_2. The CO_2-bicarbonate system acts to buffer pH because CO_2 is volatile and PCO_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_2. However, the levels of CO_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_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_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, *Lasmigona 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 (L_0) 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 (L_0) 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 CO_2 level on metamorphosis success. In these experiments, the dishes were exposed to filtered air or air+CO_2 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 CO_2 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 CO_2 of the treatment.

In experiment #1 on the Effect of Atmospheric CO_2 Levels, there were 2 treatments, air or 1% CO_2. The CO_2 mixture was prepared using a Matheson DynaBlender® gas mixing system and an infrared CO_2 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, Leibovitz 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 5um 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 5um filtered river water. L_W 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 CO_2 Levels : *Anodonta californiensis* survival *in vitro* (L_{IV}) was significantly higher using air than using 1% CO_2 (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 (L_W), but the difference was insignificant (Figure 3, Table 3). Experiment #2: *Utterbackia imbecillis* L_{IV} was slightly higher in air but not significantly different from 1% CO_2 (Figure 4, Table 4). L_W of *Utterbackia* was not determined. Experiment #3: *Anodonta oregonensis* L_{IV} was similar among different CO_2 levels (Table 5) but juvenile survival in water (L_W) was highest following incubation in air (84%) compared to 1% CO_2 (74%) and 5% CO_2 (68%) (Figure 5, Table 6, P=0.030).

In the experiment Lebovitz versus M199, *Anodonta oregonensis* cultured with an M199 base had a higher L_{IV} than those cultured in Lebovitz's L-15 (P= 0.028, Table 7, Figure 6). However, L_W 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 L_W of *Lampsilis siliquoidea* cultured using rabbit sera from two different suppliers did not differ significantly. This experiment also compared and found no difference in L_W of juveniles "rescued" from contaminated dishes with those from continuously healthy dishes. However, L_W 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

CO_2, pH, and Medium Formulations

For the species tested, it was not beneficial to utilize high levels of atmospheric CO_2 during *in vitro* incubation. Glochidia that were cultured in air (approximately 0.04% CO_2) had equal or higher survival compared to those cultured in 1% or 5% CO_2. Eliminating the need for an expensive CO_2 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% CO_2, or 5% CO_2. The use of lower bicarbonate and CO_2 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% CO2) (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 LC50 (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 I*n 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 (L_{IV}) was measured and again after several days in water (L_W). It is tempting to use the L_{IV} 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. L_W 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_2 and CO_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_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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Table 1. Species and collection localities of mussels used for experiments.

Experiment	Species	Collection site
Effect of CO_2 #1	*Anodonta californiensis*	Pajaro River, CA
Effect of CO_2 #2	*Utterbackia imbecillis*	Swope Park Lagoon, Kansas City, MO
Effect of CO_2 #3	*Anodonta oregonensis*	Hayes Island Upper Columbia River, WA
Lebovitz versus M199	*Anodonta oregonensis*	Hayes Island Upper Columbia River, WA
Serum Source	*Lampsilis siliquoidea*	Silverfork of Perche Creek, Boone Co MO
Duration of Incubation	*Anodonta oregonensis*	Hayes Island Upper Columbia River, WA
Duration in Saline	*Pyganodon grandis*	Tablerock Lake, MO
Long-term Culture	*Pyganodon grandis*	Tablerock Lake, MO
Agitation of Medium	*Leptodea fragilis*	Pomme de Terre River, MO

Table 2 . Effect of CO_2 #1. ANOVA of percent survival of *Anodonta californiensis* in medium (L_{IV}) versus CO_2 concentration.

Source	DF	Adj ss	Adj ms	F-Value	P-Value
CO_2	1	2274	2273.59	58.52	<0.005
Error	32	1243	38.85		
Total	33	3517			

Table 3. Effect of CO2 #1. ANOVA of percent survival of *Anodonta californiensis* in water (L_W) versus CO_2 concentration.

Source	DF	Adj ss	Adj ms	F-Value	P-Value
CO_2 Treatment	1	477.6	477.6	4.39	0.069
Error	8	869.6	108.7		
Total	9	1347.1			

Table 4. Effect of CO_2 #2. ANOVA of percent survival *in vitro* (LIV) of *Utterbackia imbecillis* versus CO_2 concentration.

Source	DF	Adj ss	Adj ms	F-Value	P-Value
CO_2 Treatment	1	1643	1643	0.96	0.335
Error	54888	1715			
Total	56531				

Table 5. Effect of CO_2 #3. ANOVA of percent survival *in vitro* (L_{IV}) of *Anodonta oregonensis* among 3 treatment concentrations of CO_2 and among females within treatments.

Source	DF	Adj ss	Adj ms	F-Value	P-Value
CO_2 Treatment	2	18.16	9.079	1.87	0.173
Female(treatment)	6	44.26	7.377	1.52	0.209
Error	27	130.85	4.846		
Total	35	193.27			

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.

Source	DF	Adj ss	Adj ms	F-Value	P-Value
CO_2 Treatment	2	1447	723.4	3.99	0.030
Female(treatment)	6	1734	289.0	1.59	0.188
Error	27	4900	181.5		
Total	35	8081			

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.

Source	DF	Adj ss	Adj ms	F-Value	P-Value
Treatment	1	79.49	79.49	5.71	0.028
Female(Treatment)	4	125.16	31.29	2.25	0.104
Error	18	250.71	13.93		
Total	23	455.36			

Table 8. Lebovitz versus M199 Medium. ANOVA of percent survival *in vitro* (L_{IV}) of *Anodonta oregonensis* among base media, contaminated versus uncontaminated within medium, and among females.

Source	DF	Adj ss	Adj ms	F-Value	P-Value
Treatment	1	4251	4250.5	6.02	0.025
Female(Treatment)	4	3895	973.8	1.38	0.281
Error	18	12712	706.2		
Total	23	20858			

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

Source	DF	Adj ss	Adj ms	F-Value	P-Value
Treatment	1	1413.0	1412.98	2.95	0.098
Contamination	1	1005.0	1004.98	2.10	0.160
Error	25	11955.8	478.23		
Lack-of-Fit	1	12.0	12.04	0.02	0.878
Pure	Error	24	11943.8	497.66	
Total	27	14127.5			

Table 10. Effect of duration of incubation in medium before transfer to water for *Anodonta oregonensis*. ANOVA of percent survival *in vitro* (L_{IV}) comparing contaminated versus uncontaminated dishes, and duration of incubation in medium.

Source	DF	Adj ss	Adj ms	F-Value	P-Value
Incubation Hours	14	75.16	5.369	0.4	0.96
Error	25	331.89	13.276		
Total	39	407.05			

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

Source	DF	Adj ss	Adj ms	F-Value	P-Value
Incubation Hours	14	22965	1640.3	4.89	<0.005
Error	25	8395	335.8		
Total	39	31359			

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* (L_{IV}) comparing contaminated versus uncontaminated dishes, and duration of incubation in medium before substituting saline.

Source	DF	Adj ss	Adj ms	F-Value	P-Value
Hours in Medium	11	17267	1569.70	4.90	<0.005
Error	36	11537	320.50		
Total	47	28804			

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.

Source	DF	Adj ss	Adj ms	F-Value	P-Value
Hours in Medium	11	17520	1592.70	5.66	<0.005
Error	36	10131	281.40		
Total	47	27651			

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).

Source	DF	Adj ss	Adj ms	F-Value	P-Value
Hours in Medium	6	1.136	0.1893	0.97	0.507
Error	7	1.368	0.1954		
Total	13	2.503			

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).

Source	DF	Adj ss	Adj ms	F-Value	P-Value
Hours in Medium	7	4306	615.2	0.52	0.797
Error	8	9433	1179.2		
Total	15	13740			

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).

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

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.

Source	DF	Adj ss	Adj ms	F-Value	P-Value
Culture Method	1	1092	1092	2.61	0.126
Error	16	6699	418.7		
Total	17	7791			

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.

Source	DF	Adj ss	Adj ms	F-Value	P-Value
Culture Method	1	0.03778	0.03778	0.24	0.628
Error	20	3.12316	0.15616		
Total	21	3.16094			

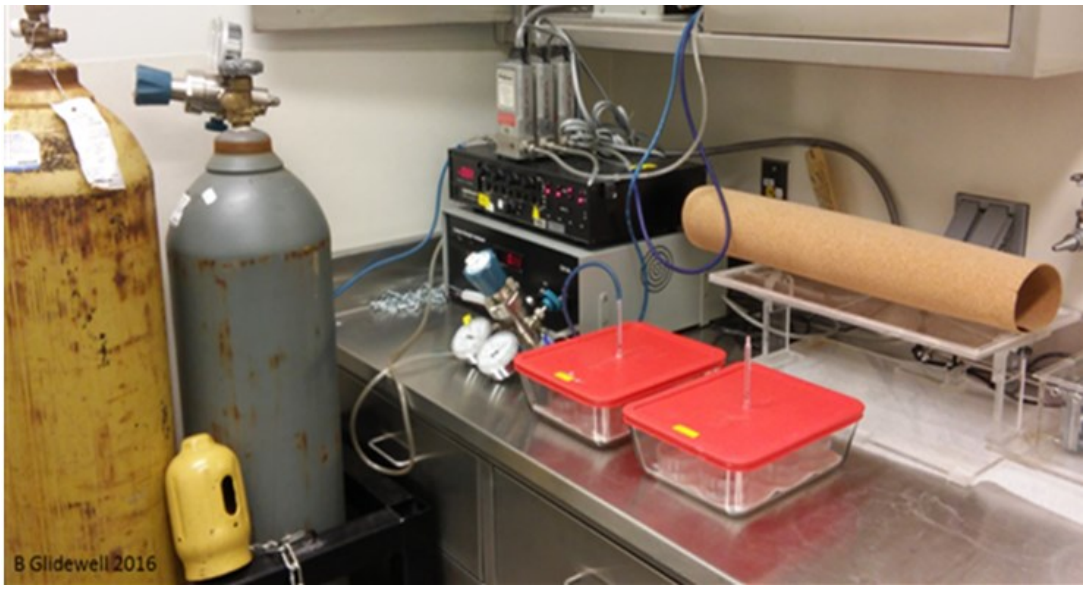

Figure 1. Set-up for incubating dishes in controlled atmosphere. Air-CO_2 mixtures were supplied from mass flow controllers (Matheson) or premixed commercial cylinders. Gas mixtures were passed through ventilated boxes containing the culture dishes. CO_2 fraction was monitored using an Ametek infrared analyzer.

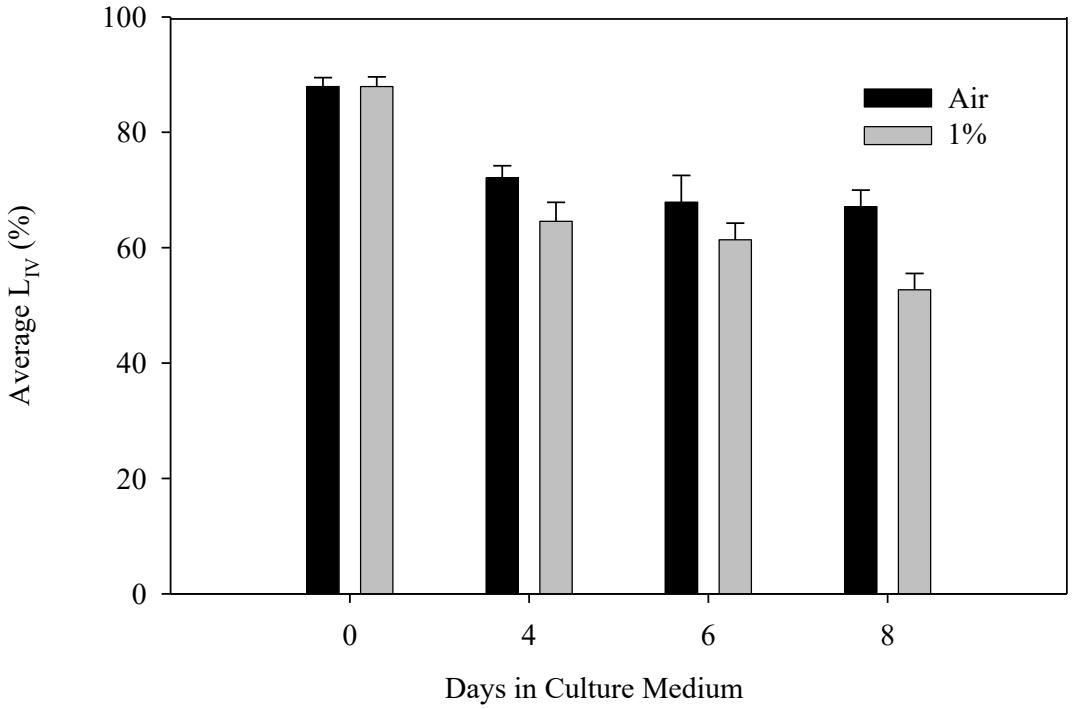

Figure 2. Effect of CO_2 #1. Effect of two levels of atmospheric CO_2 on survival of *Anodonta californiensis in vitro* (L_{IV}) during 8 days of incubation. Bars represent means and 95% confidence intervals, 17 dishes per treatment.

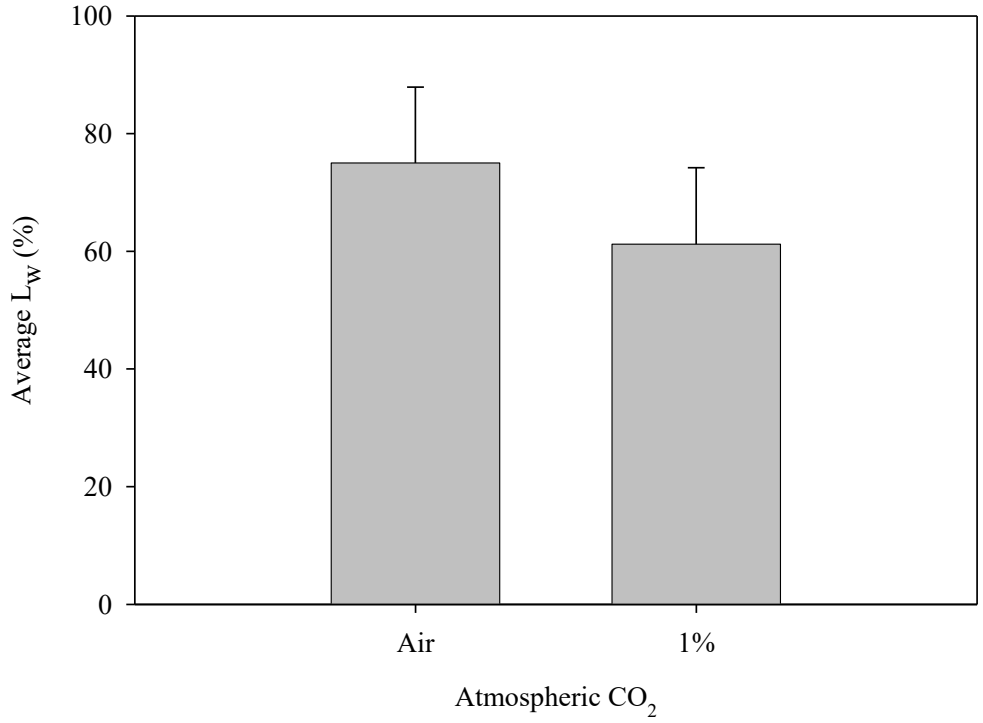

Figure 3. Effect of CO_2 #1. Effect of atmospheric CO_2 used *in vitro* on subsequent 24-h survival of *Anodonta californiensis* in water (L_W). Bars represent 95% confidence intervals (N=5).

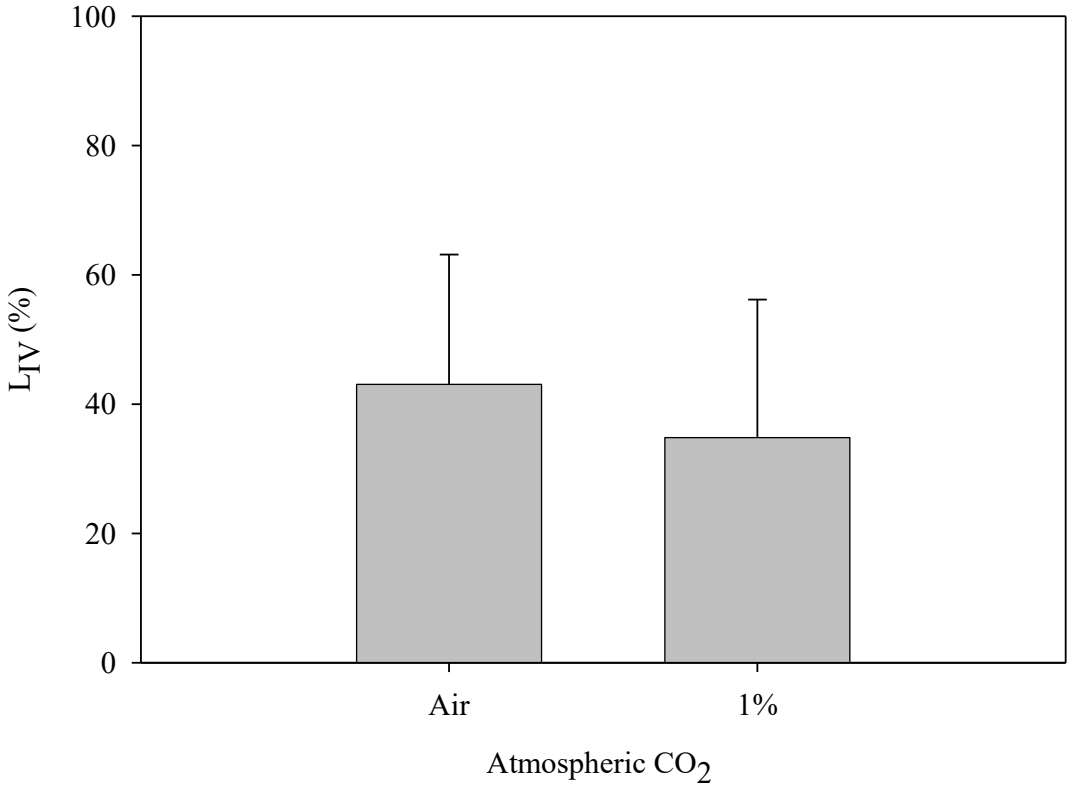

Figure 4. Effect of CO_2 #2. Effect of atmospheric CO_2 on survival of *Utterbackia imbecillis* in culture medium (L_{IV}). Bars represent means and 95% confidence intervals (N=17).

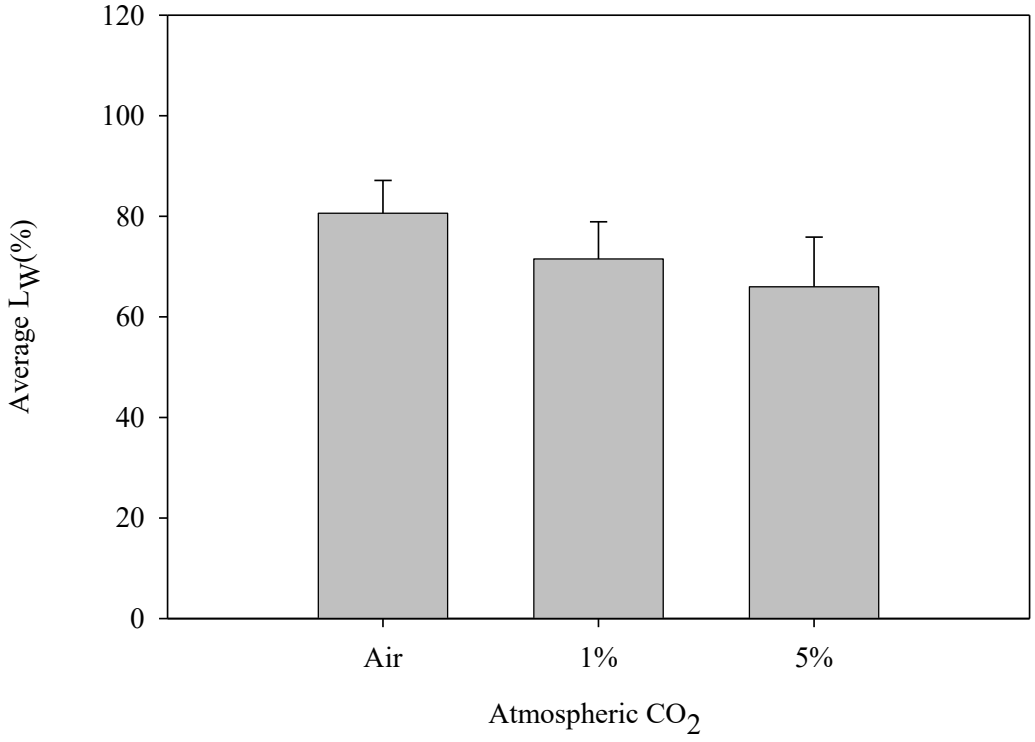

Figure 5. Effect of CO_2 #3. Effect of atmospheric CO_2 used for *in vitro* metamorphosis of *Anodonta oregonensis* on subsequent 24-h survival in river water. Bars represent means and 95% confidence intervals (N=17).

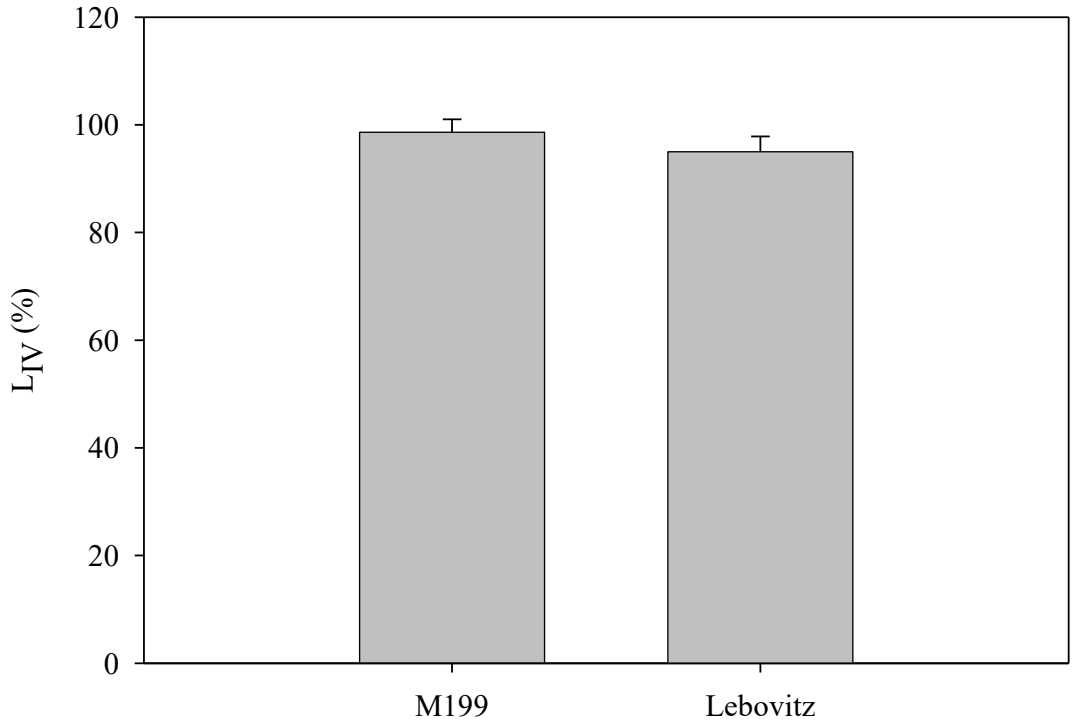

Figure 6. Lebovitz versus M199 Media . Effect of base medium used for *in vitro* metamorphosis of *Anodonta oregonensis* on survival at the end of incubation in culture media (L_{IV}). Bars represent means and 95% confidence intervals (N=13).

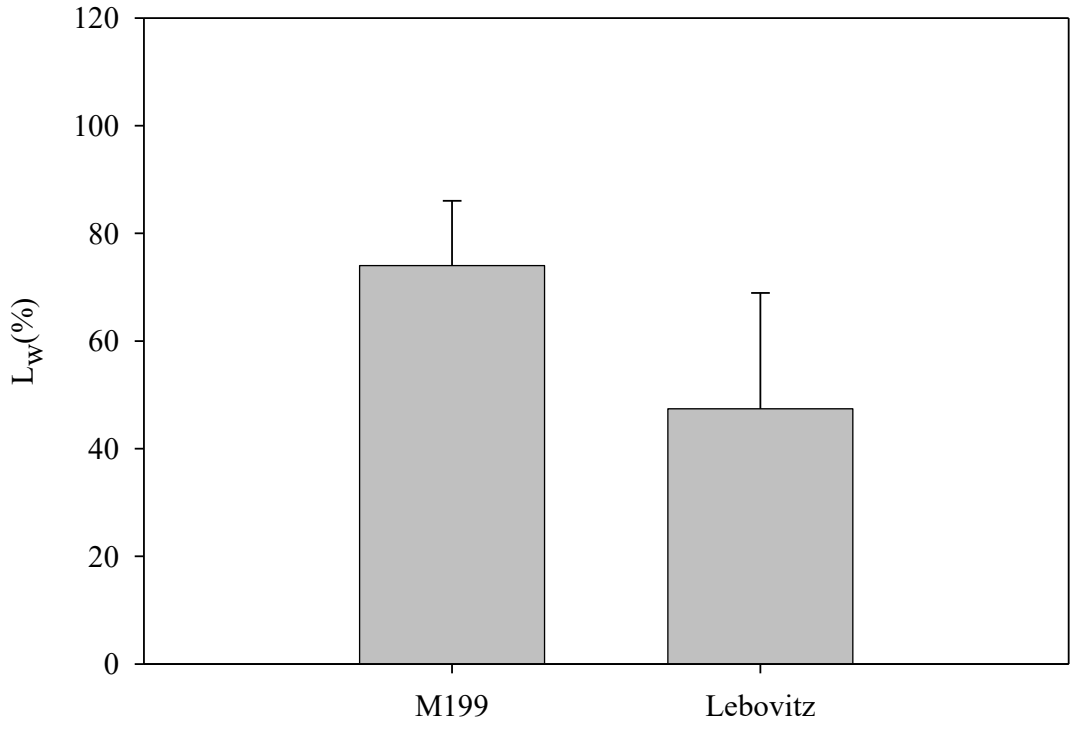

Figure 7. Lebovitz versus M199 Media. Effect of base medium used for *in vitro* metamorphosis of *Anodonta oregonensis* on subsequent 24-h survival in water (L_W). Bars represent means and 95% confidence intervals (N=13).

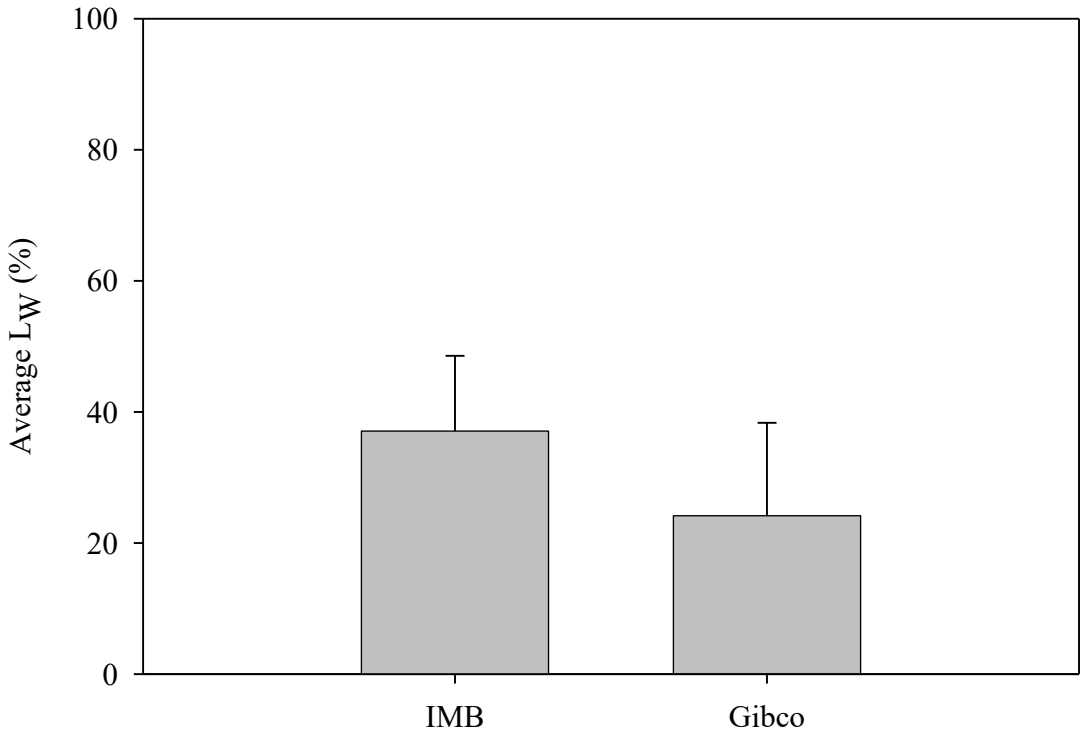

Figure 8. Commercial Sera Source. Effect of sera manufacturer of rabbit sera used for *in vitro* metamorphosis of *Lampsilis siliquoidea* on subsequent 24-h survival in water (L_W). Bars represent means and 95% confidence intervals (N=14).

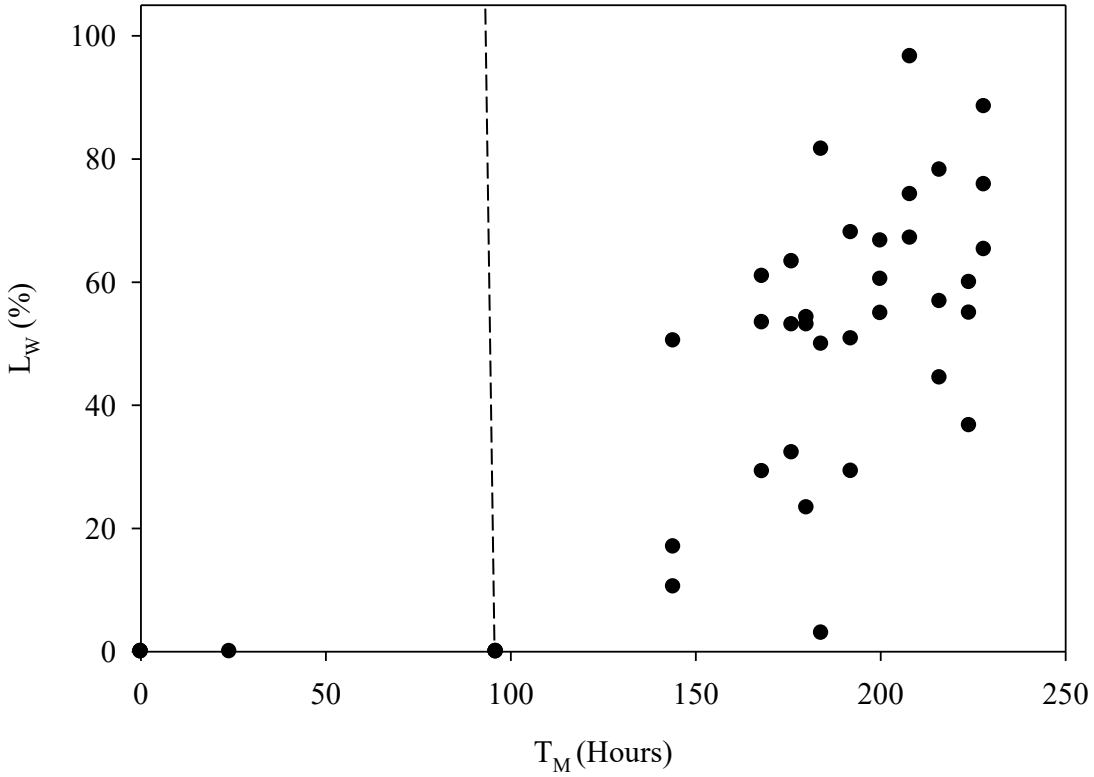

Figure 9. Duration of Incubation. Effect of duration of incubation in culture medium on survival in water (L_W) of *Anodonta oregonensis*. T_M= Time in culture medium. L_W= the percentage surviving after four days in water. Points represents L_W of individual dishes and the dashed line represents the end of stage 1 of development and the beginning of stage 2.

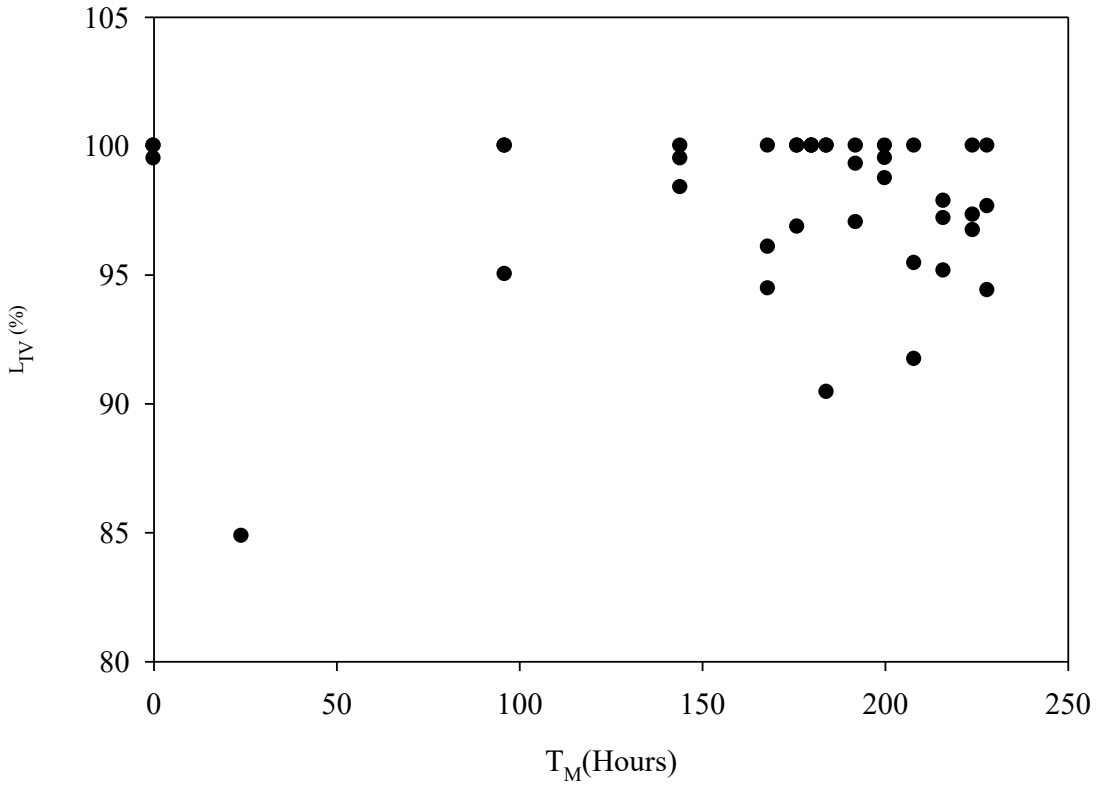

Figure 10. Duration of Incubation. Effect of duration of incubation in culture medium on survival *in vitro* (L_{IV}) of *Anodonta oregonensis* T_M= Time in culture medium. Points represent L_{IV} of individual dishes.

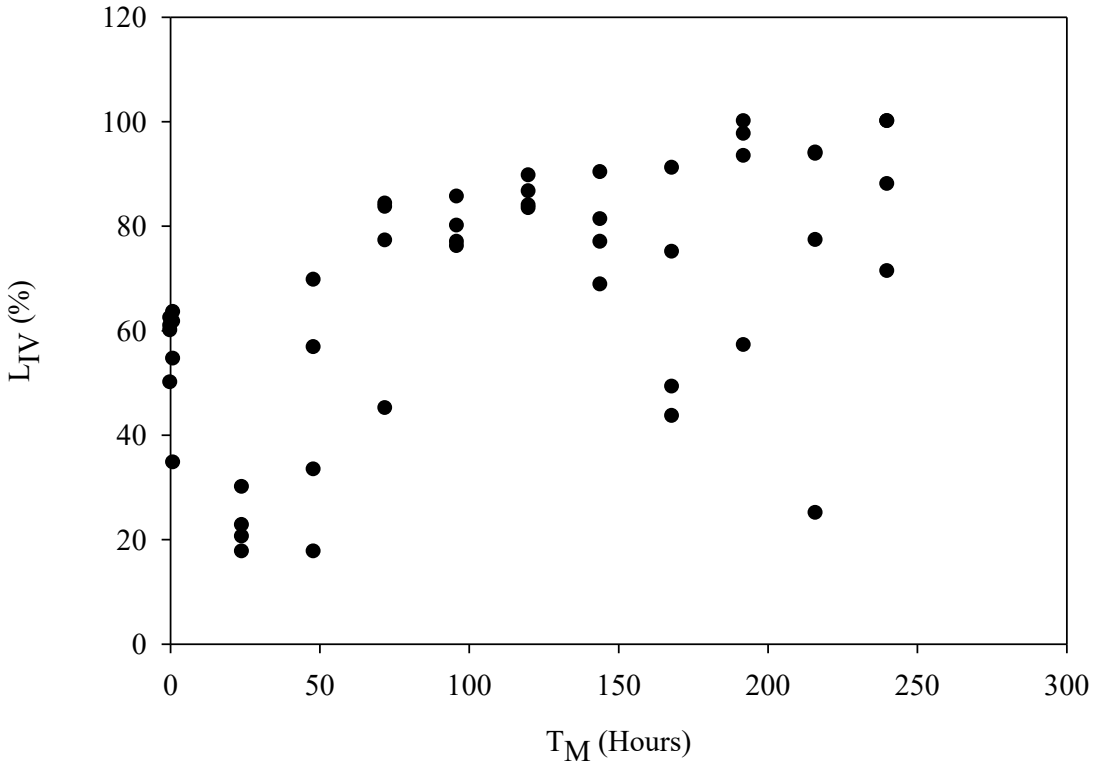

Figure 11. Duration in Saline. Effect of substituting physiological saline for culture medium on survival *in vitro* (L_{IV}) of *Pyganodon grandis.* T_M= Time in culture medium before switching to saline. L_{IV} was measured after 240 hours *in vitro*. Points represent L_{IV} in individual dishes.

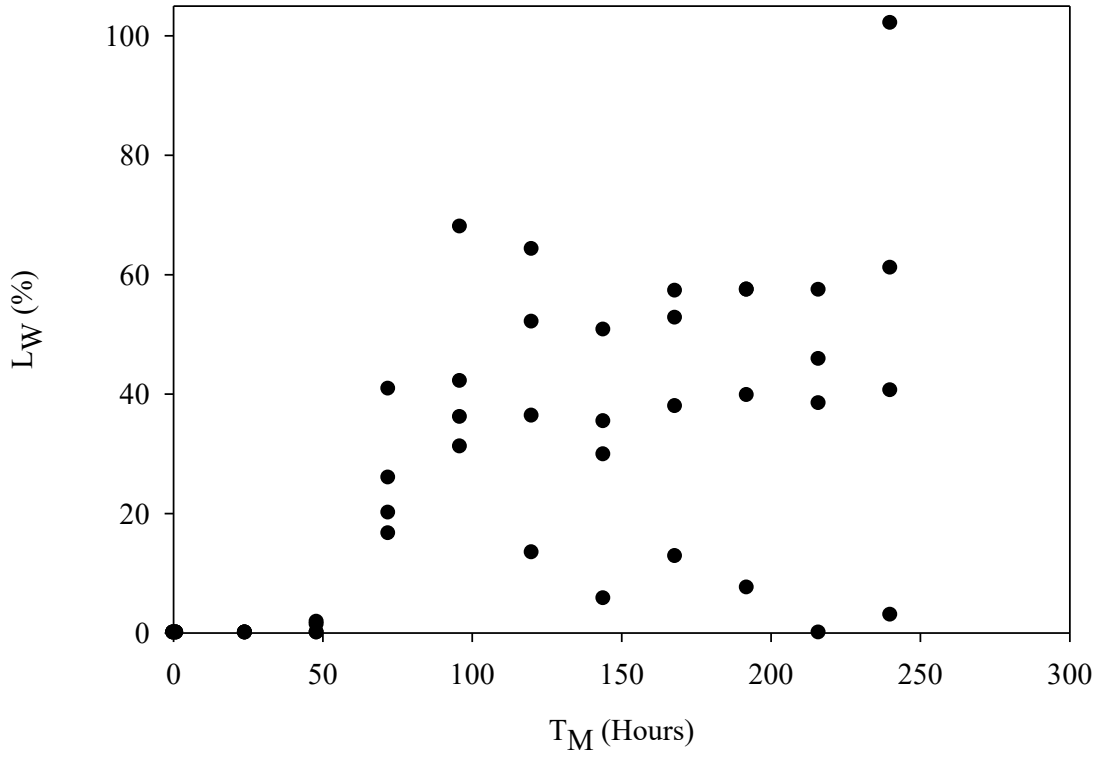

Figure 12. Duration in Saline. Effect of substituting physiological saline for culture medium on post-metamorphic survival in water (L_W). T_M= Time in culture medium before switching to saline. All juveniles were transferred to water after 240 h *in vivo*. L_W= survival of *Pyganodon grandis* after 3 days in water. Points represent L_W in individual dishes.

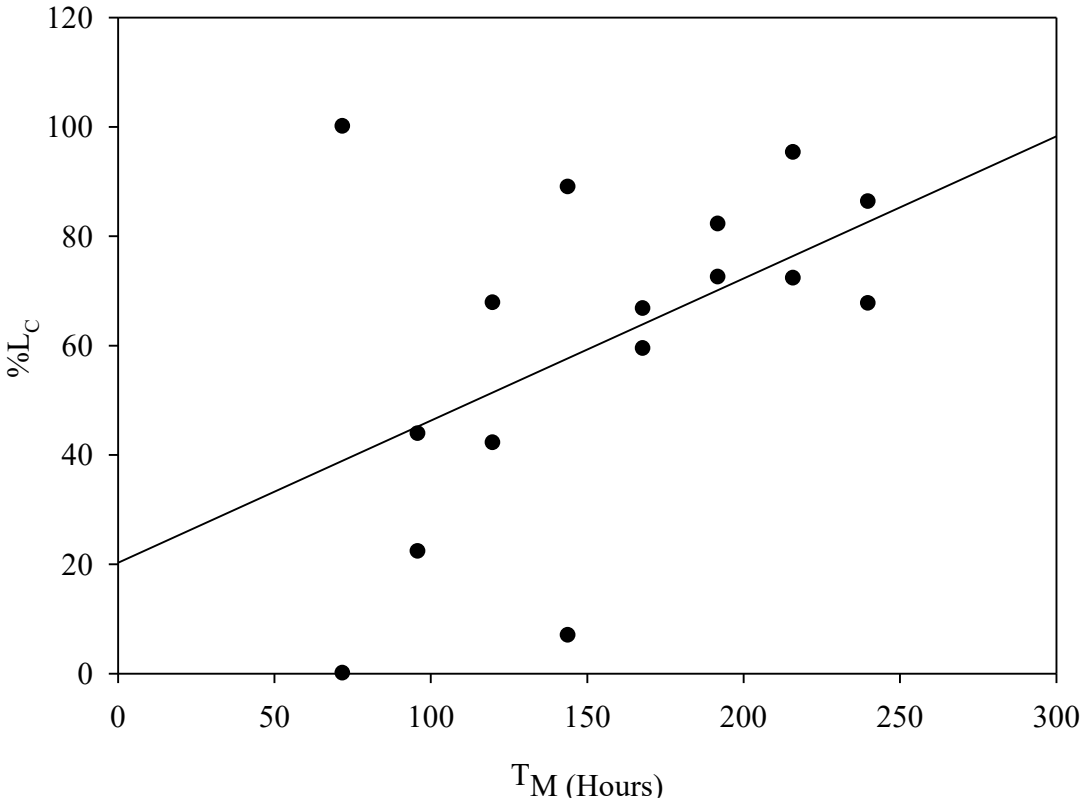

Figure 13. Duration in Saline after Long-term Culture . Survival of saline-incubated *Pyganodon grandis* juveniles after 5 months in laboratory culture (L_C). T_M = time in culture medium before switching to saline. Points represent L_C in replicate groups that were cultured separately.

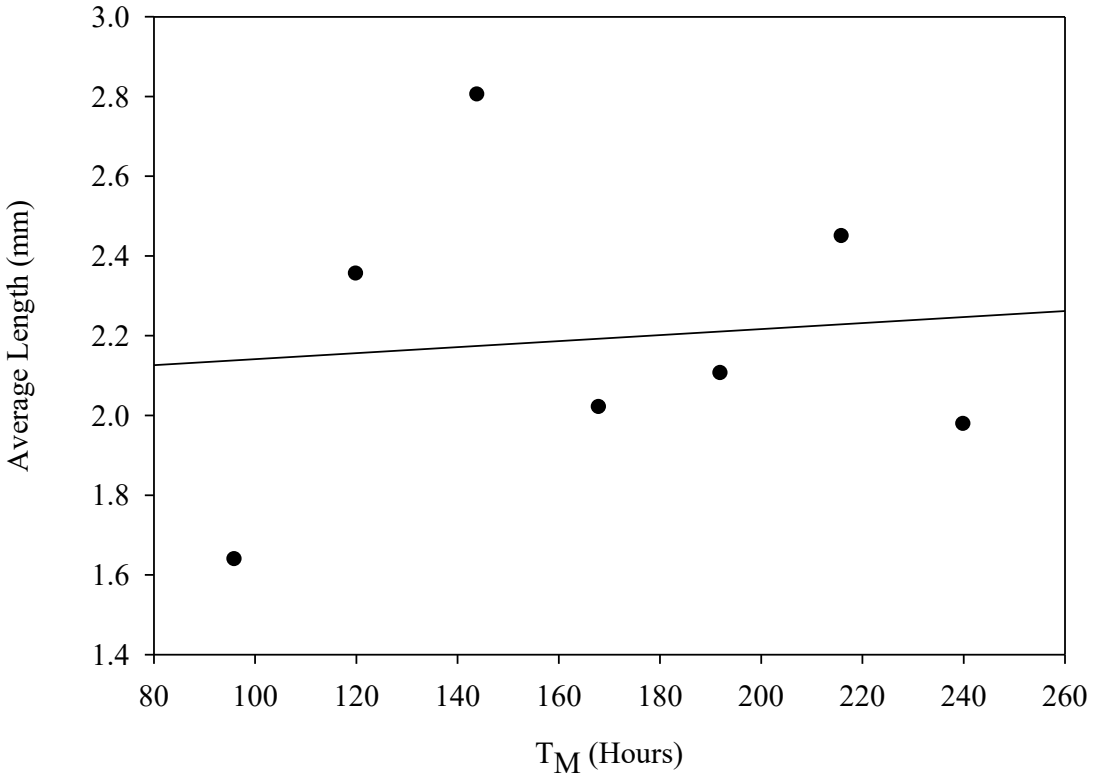

Figure 14. Duration in Saline after Long-term Culture. Growth (average shell length) of saline-incubated *Pyganodon grandis* juveniles after 5 months in laboratory culture. T_M = time in culture medium before switching to saline. Points represent mean shell length in replicate groups that were cultured separately.

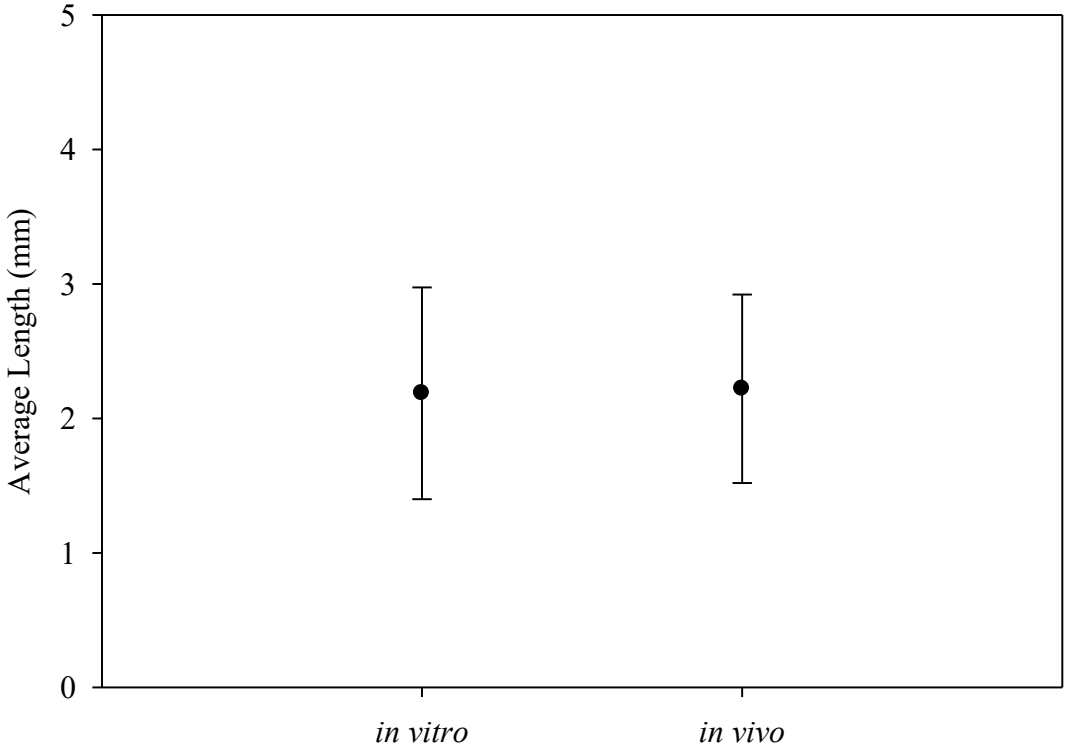

Figure 15. Long-term Culture Between In Vitro & In Vivo (Shell Length). Comparing mean shell length between *P. grandis* juveniles metamorphosed *in vitro* and *in vivo* after 5 months of culture. *In vitro* N=199, *in vivo* N=184 individuals measured.

APPENDICES

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

Salts and nutrients	
M199 Powder	10 g
Sodium Pyruvate	100 mg
Glucose	2 g
D-Galactose	2.5 g
Salmon Oil Extract	1 ml
Menhaden Oil	1 ml
Rabbit serum	500 ml

Amino Acids and Vitamins	
L-Alanine	70 mg
L-Ornithine	20 mg
L-Proline	40 mg
Taurine	80 mg
L-Threonine	20 mg
EAA	1 ml
NEAA	1 ml
MEM Vitamin	1 ml

Anti-microbials	
Rifampicin	150 mg
Gentamicin	150 mg
Carbenicillin	150 mg
Amphotericin B	1.5 ml

Titration solutions
CaOH
NaOH
HCl

Appendix B. Lebovitz's Medium components (ThermoFisher)

Inorganic Salts	**mg/L**
$CaCl_2$ (anhydrous)	140
$MgCl_2$ (anhydrous)	93.7
$MgSO_4$ (anhydrous)	97.7
KCl	400
KH_2PO_4 monobasic	60
NaCl	8000
Na_2HPO_4 dibasic	190

Vitamins	**mg/L**
Choline chloride	1
D-Calcium pantothenate	1
Folic Acid	1
Niacinamide	1
Pyridoxine hydrochloride	1
Riboflavin 5'-phosphate Na	0.1
Thiamine monophosphate	1
i-Inositol	2

Other Components	**mg/L**
D+ Galactose	900
Phenol Red	10
Sodium Pyruvate	550

Amino Acids	**mg/L**
Glycine	200
L-Alanine	225
L-Arginine	500
L-Asparagine	250
L-Cysteine	120
L-Glutamine	300
L-Histidine	250
L-Isoleucine	250
L-Leucine	125
L-Lysine	75
L-Methionine	75
L-Phenylalanine	125
L-Serine	200
L-Threonine	300
L-Tryptophan	20
L-Tyrosine	300
L-Valine	100

Appendix C. Components of M199. (M5017-10X1L - Medium 199 Sigma-Aldrich)

Inorganic Salts	mg/L
$CaCL_2$ (anhydrous)	200
$Fe(NO_3)$-9H2O	0.72
$MgSO_4$ (anhydrous)	97.7
KCl	400
NaCL	6800
NaH_2PO_4-H_2O	140
$NaHCO_3$	2200

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

Vitamins	mg/L
Ascorbic acid	0.05
Biotin	0.01
D-calcium pantothenate	0.01
Choline chloride	0.5
Ergocalciferol	0.1
Folic acid	0.01
i-inositol	0.05
Menadione	0.01
Niacin	0.025
Niacinamide	0.025
PABA	0.05
Pyridoxal HCl	0.025
Pyridoxine HCl	0.025
Riboflavin	0.01
Thiamine HCl	0.01
DL-α-tocopherol phosphate 2Na	0.01
Vitamin A acetate	0.14

Other	
Adenine sulfate dihydrate	10.98
AMP monohydrate	0.2
ATP 2Na trihydrate	1.098
Cholesterol (synthetic)	0.2
2-Deoxy-d-ribose	0.5
Dextrose anhydrous	1000
L-glutathione reduced	0.05
Guanine HCI monohydrate	0.3
Hypoxanthine sodium salt	0.354
Phenol red sodium salt	21.24
D-Ribose	0.5
Thymine	0.3
TweenTM 80	20
Uracil	0.3
Xanthine sodium salt	0.344

Appendix D. Fish saline (Holmes and Stott, 1960)

Components	**(g)**
NaCl	7.41
KCl	0.37
$NaH_2PO_4 \cdot H_2O$	0.40
$NaH_2PO_4 \cdot 2H_2O$	0.20
$NaHCO_3$	0.31
KH_2PO_4	0.17
$MgSO_4 \cdot 7H_2O$	0.31