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Improvement of *in-vitro* culture techniques for unionid bivalves

Diploma thesis

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Declaration

I declare that I have elaborated my diploma thesis “Improvement of in-vitro culture techniques for unionid bivalves" independently under the supervision of the thesis supervisor and using literature and other information sources cited in the thesis and listed in the bibliography at the end of this document. In addition, as the author of the thesis, I declare that I have not infringed the copyrights of third parties.

In Prague, 12/04/2019 _____

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Zlepšení *in vitro* kultivačních technik pro mlže řádu Unionida

Souhrn

Sladkovodní mlži jsou jednou z nejohroženějších skupin živočichů na světě částečně proto, že jejich komplikovaný životní cyklus zahrnuje parazitickou fázi vývoje na hostitelských rybách. Z tohoto důvodu může úbytek hostitelských ryb v mnoha řekách značně ovlivnit populace mlžů. *In vitro* chovy mohou být velmi užitečná metoda pro ochranu druhů, pro které není dostatek znalostí o hostitelských rybách, nebo na lokalitách, kde hostitelské ryby vymizely. In vitro chovy však představují řadu metodických výzev. V současné době je výpočet úspěšnosti metamorfózy do značné míry založen na aktivitě juvenilních mlžů. Faktory, jako je čas a typ ředění média, mohou mít vliv na rychlost pozorované metamorfózy. Kromě toho je potřeba dosáhnout zlepšení složení média u některých druhů, kde nebyla *in vitro* kultura úspěšná. Tato práce prezentuje výsledky našich experimentů provedených s glochidii druhů *Anodonta anatina* a *Margaritifera margaritifera*. V případě druhu *A. anatina* jsme studovali účinky různých časů a ředění na pozorovanou rychlost metamorfózy larev vystavených různým druhům médií a koncentrací antibiotik. V druhém případě, pokus s druhem *M. margaritifera* byl zaměřen na testování účinků na časnou odezvu larev přidáním taurinu, typů séra (kůň nebo novorozené tele) a zdroje lipidů (tresčí játra nebo emulgovaná lipidová směs). Naše závěry poukazují na to, že míra úspěchu metamorfózy v experimentech *in vitro* může být ovlivněna jak ředěním, tak časem vyhodnocení a ukazuje důležitost zvažování dalších metod pro hodnocení úspěšnosti *in vitro* metamorfózy. Alternativně jsme zjistili, že časná reakce larev druhu *M. margaritifera* není zvýšena přidáním taurinu, ale lze pozorovat konzistentní pozitivní vliv emulgované směsi lipidů. Byla zjištěna značná variabilita v kvalitě séra použitá v testu, další studie by měly zahrnovat přidání hostitelské nebo ne-hostitelské plazmy. Žádný z testovaných faktorů neměl významný vliv na konečné rozměry larev od *M. margaritifera*. Výsledky mohou přispět k vývoji nových protokolů a efektivnímu porovnávání *in vitro* technik pro sladkovodní mlže.

Klíčová slova: sladkovodní mlži, ochrana, úspěch metamorfózy, *in vitro, Margaritifera margaritifera, Anodonta anatina.*

Improvement of *in-vitro* culture techniques for unionid bivalves

Summary

The freshwater mussels are one of the most endangered groups of animals in the world in part due to their complicated life cycle which includes a parasitic stage in a host fish. Because of this, the disappearing of host fishes in many rivers can greatly affect mussel populations. The alternative of *in vitro* culture can be extremely helpful for the conservation of species in which there is no sufficient knowledge about the host species or for places where the host fish has disappeared. However, *in vitro* culture poses a series of methodological challenges. Currently, the calculation of metamorphosis rate is largely based on juvenile activity. Factors like time and dilution of medium can affect the observed activity of the juveniles and it is not known if this can have an effect on the observed metamorphosis rate. Additionally the medium composition needs to be enhanced for some species where *in vitro* culture has not been successful. This contribution presents the results from our experiments carried out with the glochidia of *Anodonta anatina* and *Margaritifera margaritifera.* For *A. anatina* we studied the effects of different count times and dilutions on the perceived metamorphosis rate of larvae subjected to different medium types and antibiotic concentrations. While the *M. margaritifera* experiment aimed to test the effects of taurine addition, serum type (horse or newborn calf) and source of lipids (cod liver or emulsified lipid mixture) on the early development of larvae. Our conclusions point out that the measure of metamorphosis success in *in vitro* experiments can be affected by both dilution and time of counting and shows the importance of considering additional methods for assessing *in vitro* metamorphosis success. Alternatively we found that early reaction of larvae from *M. margaritifera* is not enhanced by the addition of taurine, but we observe a consistent positive influence of the emulsified lipid mixture. There was strong variation of the performance of the serums used in the test. Further studies should include the addition of host or non-host fish plasma. Finally, none of the factors tested had any significant effect on the final length of the larvae from *M. margaritifera.* This results can help to develop better protocols for the comparison of *in vitro* culture techniques for unionid bivalves.

Keywords: freshwater mussels, conservation, metamorphosis success, *in vitro, Margaritifera margaritifera, Anodonta anatina.*

Contents

1 Introduction

Biodiversity loss is one of the greatest environmental problems of our time, as many species are declining rapidly in all ecosystems (Butchart et al. 2010). Despite being a generalized problem, some groups seem to be more affected than others (Geist 2010, 2015; Strayer & Dudgeon 2013). This is the case of freshwater mussels (Unionida) considered by some authors to be one of the most threatened groups of animals in the world, with around 40% of all known species listed under some category of threat (Lopes-Lima et al. 2018). The causes for this decline are varied and include habitat loss and degradation, pollution, eutrophication, invasive species and overexploitation (Lopes-Lima et al. 2018). This has led to numerous conservation and restoration plans around the world to preserve these animals (Geist 2010; Patterson et al. 2018; Wen et al. 2018). The plans are focused primarily in restoration of habitats, but for some heavily threatened species that face imminent extinction artificial rearing programs have been developed. The artificial rearing of freshwater mussels is complicated due to its complex life cycle. In the majority of unionid species, larvae (glochidia or lasidia) released from female mussel are obligatory parasites and have to attach to a freshwater fish to complete its development. The encapsulated larvae undergo a process of metamorphosis on the fish and then the individual drop-off from its host to start its life as free living organism (Kat 1984). This makes the artificial rearing programs costly and difficult (Buddensiek 1995), since the host fish need to be kept in a controlled environment for the duration of metamorphosis (up to 90 days in some species). In response to this problems the method of *in vitro* culture of glochidia has been gaining attention recently (Lima et al. 2012; Patterson et al. 2018). In *in vitro* culture the glochidia are placed in artificial medium, typically commercial cell culture mediums, and incubated until metamorphosis is achieved, this precludes the need for host fish and therefore has a great potential for conservation and artificial rearing. However, *in vitro* culture poses a series of methodological challenges. Because of the propagation programs mainly works with rare and endangered species, the number of available glochidia for protocol testing is often limited and it is essential to quickly identify the most effective methods. Currently, the measure used to assess the efficiency of different *in vitro* protocols is the metamorphosis rate (the proportion of individuals that successfully metamorphosed), this measure is largely based on juvenile activity. Factors like time of assessment and dilution of medium can affect the observed activity of the juveniles and is not known if this can have an effect on the observed metamorphosis rate thus obscuring comparison between *in vitro* experiments. Additionally the medium composition needs to be enhanced for some species where *in vitro* culture has not been successful yet. For *M. margaritifera* a successful *in vitro* method has not been developed yet, this is partially due to the great degree of specify for the host that this species show, infecting only certain species of salmonids (Taeubert & Geist 2017) . Previous attempts (Taskinen. et al. 2011) have only managed to have partial success by completing the first part of development on the fish and later finishing the metamorphosis on culture medium. This suggest that some factor is crucial to trigger metamorphosis of this species in *in vitro* conditions and that the current medium composition may lack critical components.

In this work we aimed to study this problematic by first investigating the effects of different approaches to the evaluation of *in vitro* culture success quantification, including the use of post-larval growth as a complementary tool. And, second by testing the early reaction of glochidia from *M. margaritifera* to the addition of new components to the medium that can improve *in vitro* conditions for the species (Wen et al. 2018).

2 Hypothesis and objectives of work

2.1 Objectives of work

1. To perform a complete review of the current knowledge in *in vitro* techniques for unionid mussels culture.
2. Perform pilot experiments with central European species (*Anodonta anatina*, *Margaritifera margaritifera*).
3. Clarify the use of endpoints for the evaluation of *in vitro* technique success.
4. Study the post metamorphosis development of juveniles produced by *in vitro* techniques.

2.2 Hypothesis

1. Dilution has no effect on the metamorphosis rate and growth of *Anodonta anatina*
2. There is a difference between metamorphosis success rates measured at different time points.
3. *Margaritifera margaritifera* reaction to *in vitro* culture can be improved by changes of the medium composition.
4. Taurine have a positive effect on the *in vitro* development of *M. margaritifera.*

3 Literature review

3.1 Freshwater mussels biology and conservation

Biodiversity loss is one of the greatest environmental problems of our time, despite being of concern for scientist and stakeholder alike, current trends reveal that despite the existence of some conservation initiatives, the general trend of the global biodiversity is towards decrease. Buttchart and collaborators (.2010) analyzed the dynamics of several biodiversity indicators worldwide and found that the majority of them report a strong decline in the last decades. In the same study the authors also investigated the dynamics of pressure indices and found steady increments in ecological footprint, alien species and impact of climate change. Despite being a generalized problem, some ecosystems seem to be more affected than others. This is the case of freshwater ecosystems, this ecosystems contain around 5 % of all the species described despite representing just 0.8% of the earth surface area and the vulnerability of freshwater ecosystems is further increase by the high level of endemism, their insular nature and the high dependency of our societies on freshwater resources (Geist 2015; Strayer & Dudgeon 2013).

One example of this are the freshwater mussels (Unionida) considered by some authors to be one of the most threatened group of animals in the world. Freshwater mussels comprehend a diverse groups with around 900 species worldwide, around 40% of all known species are listed under some category of threat, either near threatened, threatened or extinct. The major drivers for this decline include habitat degradation, pollution of rivers and loss of host fish. This problem is further exacerbated by the lack of knowledge on the diversity of some regions like Africa, South America and Asia (Lopes-Lima et al. 2018).

The interest on the conservation of this group has led to numerous conservation and restoration plans around the world (Patterson et al. 2018). This propagation and culture methods of freshwater mussels (order Unionida) are being developed rapidly in rearing facilities worldwide, mainly because of economic (Li, Wu, & Bai 2018) and conservation (Lopes-Lima et al. 2018) concerns.

The conservation efforts have focused mainly on habitat restoration and artificial rearing, however these programs face major challenges due to the complex life cycle of freshwater mussels. In the majority of unionid species, the larvae released from female mussels are

obligatory parasites and must attach to freshwater fish to complete their development. Based on their morphology three major types of larvae exist, glochidia larvae are the most common type of larvae in freshwater mussels, this type of larvae is widespread in all continents. The second type of larvae is the lasidium, the morphology of this larva is very different from the one observed in glochidia and its only found in some species in South America (Wächtler, Dreher-Mansur & Richter 2001). Despite the differences, the general process of the development is similar in both groups. The females keep the eggs in a special cavity inside the gills called the marsupium, the males release sperm into the water and when this sperm reaches the gills of a female fertilizacion occurs. The eggs develop inside the female and when they are fully ripped, the females release the larvae into the water using contraction movements. Once free, the larvae need to find a suitable host fish and attach to it by the use of special structures (teeth in the larval shell for glochidia and elongations of the mantle in lasidia). Once attached the larvae is encapsulated in host tissue and undergoes a process of metamorphosis, when metamorphosis is finished the individual drops off its host to start its life as a free-living organism (Kat, 1984).

3.2 Study species

3.2.1 Anodonta anatina

Anodonta anatina Clessin 1876... is a species that was until recently widely distributed in Europe, with populations being present from Spain to Russia. Despite the decline the species continues to be relatively common and can still be found in most basins in the continent. This species belongs to the tribe Anodontini. Due to high variation in shell morphology many species were described in the past, however current assessments recognize just two species from this tribe in Europe: *A. anatina* and *A. cygnea* (Lopes-Lima et al. 2017).

A. anatina is a great model species for the study of larvae biology, it has long brooding season and its metamorphosis time is very short. Additionally it has been used in the past, in *in vivo* rearing studies as a model for host generalist species (Douda 2015)

3.2.2 Margaritifera margaritifera

In Europe, *Margaritifera margaritifera* (Linnaeus, 1758) has been for many years the main focus of conservation efforts due to its unique position as indicator, flagship, umbrella and key-

stone species (Geist 2010). Thanks to its former importance in the pearl industry, detailed records of the abundance and distribution of *M. margaritifera* throughout the continent are available. Comparison of current populations with these records yield a fall up to 90% on the total abundance of the species by the end of last century (Bauer 1988) with further subsequent declines being more than probable. The species have completely disappeared from many countries and many of the remaining populations show no sign of reproduction and the absence of young individuals (Bauer 1988; Černá et al. 2018; Geist 2010; Gum, Lange & Geist 2011; Österling; Arvidsson & Greenberg 2010). The causes for this decline are not fully understood yet but it seems that habitat change and destruction is the main driving force for many populations (Bauer 1988; Österling et al. 2010) with other aspects like overfishing and disappearance of host fish having only local effects (Hastie 2006). Currently the largest European pearl mussel populations are found in Russia with Scandinavia, United Kingdom and central Europe holding some important populations. Also, relatively large populations have been reported from Northern Spain and Portugal (Geist 2010).

Conservation plans for this species haven taken out in many countries, commonly the main focus has been placed on habitat restoration and rearing under controlled conditions (Gum et al. 2011). The first attempts consisted on the release of hundreds of fish infected with glochidia in different rivers of central Europe this approach had no observable effect on the populations recovery and therefore more sophisticated methods were developed (Buddensiek 1995; Gum et al. 2011). This methods use artificial infection of host fish that then are kept under controlled conditions in specialized facilities until the glochidia are transformed into juveniles. The fish are monitored and the juvenile mussels are collected after they detached from the fish. This juveniles are then kept and fed in a highly controlled environment until they are released into suitable environments (Buddensiek 1995; Preston, Keys & Roberts 2007).

This methods have had limited success. In the Czech Republic, for example, conservation efforts first with the release of fish and then with artificial rearing, have been carried out for over 30 years with only the latter having a slight effect on the increase of juvenile individuals observed (Simon et al. 2015). This approach have the additional withdraw of being expensive and demanding in terms of work and resources. Additionally there is a concern about the genetic viability of the mussels populations recovered though this method. Host fish have shown to have different levels of resistance to pearl mussel glochidia and *M. margaritifera* populations in Europe show a high genetic diversity among populations (Geist & Kuehn 2004; Taeubert et al. 2010).

3.3 *In vitro* culture

Artificial rearing and propagations programs that rely on the use of host fish demand high use of resources, and facilities. Additionally, in many instances the available fish are not suitable for the development of certain species. To circumvent this limitation, an alternative technique of glochidia culture in artificial medium *in vitro* needs to be used, which can successfully metamorphose larvae without the need for host fishes. The *in vitro* culture method is based on works from Isom and Hudson (1982, 1984), the authors used cell culture techniques to successfully transforms several species without the use of a host fish. Initially, culture mediums were very complex (Uthaiwan et al. 2002) and results were difficult to replicate. This changed with the development of simpler and highly efficient methods based on the use of M199 commercial medium mixed with fish plasma (Uthaiwan, Noparatnaraporn, & Machado 2001), which yielded very high transformation rates.

Currently, the medium is typically composed of commercial cell culture medium (e.g., M199 DMEM, L15, etc.) in combination with antibiotics and antifungal mixtures to prevent infections. The medium is normally complemented with different kinds of serums (natural host or non-host fish species, horse, or calf). To date, culture protocols have been developed for more than 50 species (Gąsienica-Staszeczek et al. 2018, Kern 2017; Kovitvadhi & Kovitvadhi 2013, Lima et al. 2012; Lima et al. 2006 , Lima & Avelar 2010; Ma et al. 2018; Wen et al. 2018) with varying success, and the methods for the majority of species are still missing.

The development of standardized, reliable *in vitro* techniques has been crucial for the study of the biology and development of both the glochidia and the juveniles under *in vitro* conditions. Uthaiwan et al. (2002) for example, studied the suitability of different plasma types for the *in vitro* development of *Hyriopsis myersiana* and found great differences up to 61.35% on the survival rate after 2-3 days of incubation for glochidia when exposed to the plasma of four different species, with the common carp plasma having the best performance. Later experiments confirmed this specificity of host in *in vivo* experiments using infected fish and studied the change in amino acids content of fish plasma during glochidial development (Uthaiwan et al. 2003). Additionally temperature during incubation have been found to play a key role in both the length of incubation and the transformation rate (Kovitvadhi & Kovitvadhi 2013; Supannapong et al. 2008), and Uthaiwan et al. (2002) found that survival of glochidia after exposure to the medium is affected by the density of larvae.

Other aspects like medium exchanges, control of contamination and maturity of glochidia have also been suggested as important factors during *in vitro* development (Kovitvadhi et al. 2006; Kovitvadhi & Kovitvadhi 2013). The juveniles obtained through *in vitro* methods have also been used to study aspects of juvenile biology like the development of digestive enzymes (Areekijseree i et al. 2006) or the optimization of feeding protocols (Kovitvadhi et al. 2006).

3.4 *In vitro* culture of study species

A successful *in vitro* method for *M. margaritifera* has not been developed yet, this can be attributed to the complex metamorphosis of this species which grows substantially during its development and to a lesser degree to its high specificity for the host that this species show, infecting only certain species of salmonids (Taeubert & Geist 2017). The experiment of Taskinen et al. (2011) is to our knowledge the only example of an *in vitro* test for this species published. The authors failed to have complete metamorphosis in an *in vitro* setting but obtained partial success when glochidia were first allowed to infect a host fish and were then extracted and transferred to the medium. This suggest that some factor specific to the host is crucial to trigger metamorphosis of *M. margaritifera* in *in vitro* conditions and that the current medium composition may lack critical components.

As an additional contribution, recently Wen and collaborators (2018) achieved *in vitro* metamorphosis of *Potamilus alatus,* an species that also grows during metamorphosis, by the use of a modified *in vitro* protocol and suggested that taurine could be an important amino acid necessary for metamorphosis, this finding may be important in the formulation of protocols for the *in vitro* development of *M. margaritifera.*

In the same study (Taskinen et al. 2011) the authors also achieved the metamorphosis of *A. anatina* under *in vitro* conditions. They used glochidia from different times of the year performing a test for glochidia obtained in spring and another for glochida obtained in autumn. The metamorphosis success rate for glochdia obtained in spring was 100% while for the autumn it was 74.6%. The protocol used by the authors in this study was based in the medium DMEM, alongside newborn calf serum and the addition of antibiotics and antimycotics. They reported that the addition of antimycotics had a significant positive effect on the metamorphosis rate when comparing groups with high and low concentration of antimycotics.

4 Methods

4.1 Experiment 1: Time variation in metamorphosis success

4.1.1 Study species

The Duck Mussel (*Anodonta anatina*) was used as a test organism because it is a widespread European species (Lopes-Lima et al. 2017) with previously published protocol of glochidia *in vitro* culture (Taskinen et al. 2011), long term brooding season and a relatively short metamorphosis period.

4.1.2 Glochidia source and extraction

Gravid females of *A. anatina* were collected in the Vltava River (Czech Republic, 48°56'53" N, 14°27'49" E) and were transferred to an aerated outdoor tank (water volume of 1350 L) at the Czech University of Life Sciences on 7th October, 2017. Female's gravid state were checked using tongs to observe the marsupial color. Before the extraction of the glochidia, debris and algae were removed from the shells. The glochidia were collected by extracting the whole marsupial outer demibranchs, then the gills were cut open using sterilized forceps and scissors, releasing the larva into a beaker of sterilized water. A sub-sample of glochidia was examined under a light microscope (40x) by exposing them to NaCl and counting the proportion that closed to assess glochidia viability, glochidia that periodically open and close their valves were considered suitable for culture (Lima et al. 2006; Roberts & Barnhart 1999). The glochidia from 3 gravid females (shell length 49 – 73 mm) with a glochidia viability exceeding 90% were pooled and used for culture. The larvae were cleaned of any remaining gill tissue by rinsing with sterilized water several times. Within one hour of extraction 100 to 311 cleaned glochidia were placed into each culture dish using a sterile Pasteur pipet. A subsample of glochidia was preserved in ethanol (70%) for initial length measures.

4.1.3 Culture medium composition and incubation phase

Full factorial design (see Table 1) was used to test the effects of three factors (medium type, antibiotic concentration and treatment after metamorphosis), each with two levels resulting in a total of eight treatments The mediums used were DMEM (Sigma Aldrich, product code D5671) and M199 (Sigma Aldrich, product code M4530), each with two concentrations of antibiotics (A = 100 µg/ml penicillin, 100 µg/ml streptomycin and 200 µg/ml neomycin; and B

= 25ug/ml penicillin, 25 µg/ml streptomycin and 50 µg/ml neomycin; both prepared using PSN mixture, Sigma Aldrich, product code P4083). Media were supplemented with horse serum (Sigma Aldrich, product code H1270). The preparation followed a 4:2:1 proportion (Roberts & Barnhart, 1999) for the medium, serum and antibiotics respectively. The mediums were complemented with 5 µg/ml of antimycotics (amphotericin B, Sigma Aldrich, product code A9528) and 50 µL per dish of cod liver oil (Sigma Aldrich, product code 74380). Six petri dishes (15 x 60 mm) were used of each media composition treatment for a total of 24 dishes and each dish was loaded with 5 ml of culture medium. Before the addition of glochidia all dishes were placed in a CO2 incubator (NB-203, N-Biotek, Korea) under UV light for one hour.

Table 1. *Full factorial design of the assessment of metamorphosis experiment, see text for elaboration on concentrations of antibiotics for A and Band dilution treatment.*

Treatment	Medium	Antibiotics concentration	Dilution treatment
1	DMEM	A	Diluted
2	M199	A	Diluted
3	DMEM	B	Diluted
4	M199	B	Diluted
5	DMEM	A	Water
6	M199	A	Water
7	DMEM	B	Water
8	M199	B	Water

In the first phase of the experiment the dishes were kept at an atmosphere of 5% CO2 and 24 °C for ten days. During this phase no fungal or bacterial infection was observed and medium changes were not performed. On the seventh day sterilized (autoclaved at 121 °C) water was added to all dishes in proportion 1:1 (5 ml). On day ten, when the metamorphosis of glochidia was observed, the dishes were divided into two groups for post-metamorphosis treatment. In the first group, sterilized water was added in proportion 1:1 (5 ml of medium removed and 5ml of sterilized water added) and dishes were retained in 5% CO2 atmosphere. In the second group the medium was completely replaced with sterilized water and the dishes were kept in a CO2 non-enriched atmosphere at 24 C°. The incubation was terminated in all treatments on day 13 when the first quantification of metamorphosis success (see below) was performed.

4.1.4 Juvenile culture phase

After the first quantification of metamorphosis success (day 13, see Figure 1) the glochidia and juveniles were removed from the dishes and cleaned by rinsing them with water over a 139 µm mesh. Then the individuals were moved to glass containers filled with 250 ml of water and kept at 20 C° for eight days. In this phase the juveniles were fed daily by adding 150 µl of commercial unicellular algae mixture (Plankto Marine P, Grotech; cell density ~25 x 106/ml) to each glass container. The individuals were cleaned again on the fourth day and new water was added to the containers. At the end of this phase (day 21) a second quantification of metamorphosis success was performed and subsample of each treatment of juveniles from each container was taken and fixed in ethanol (70%) to perform growth measures.

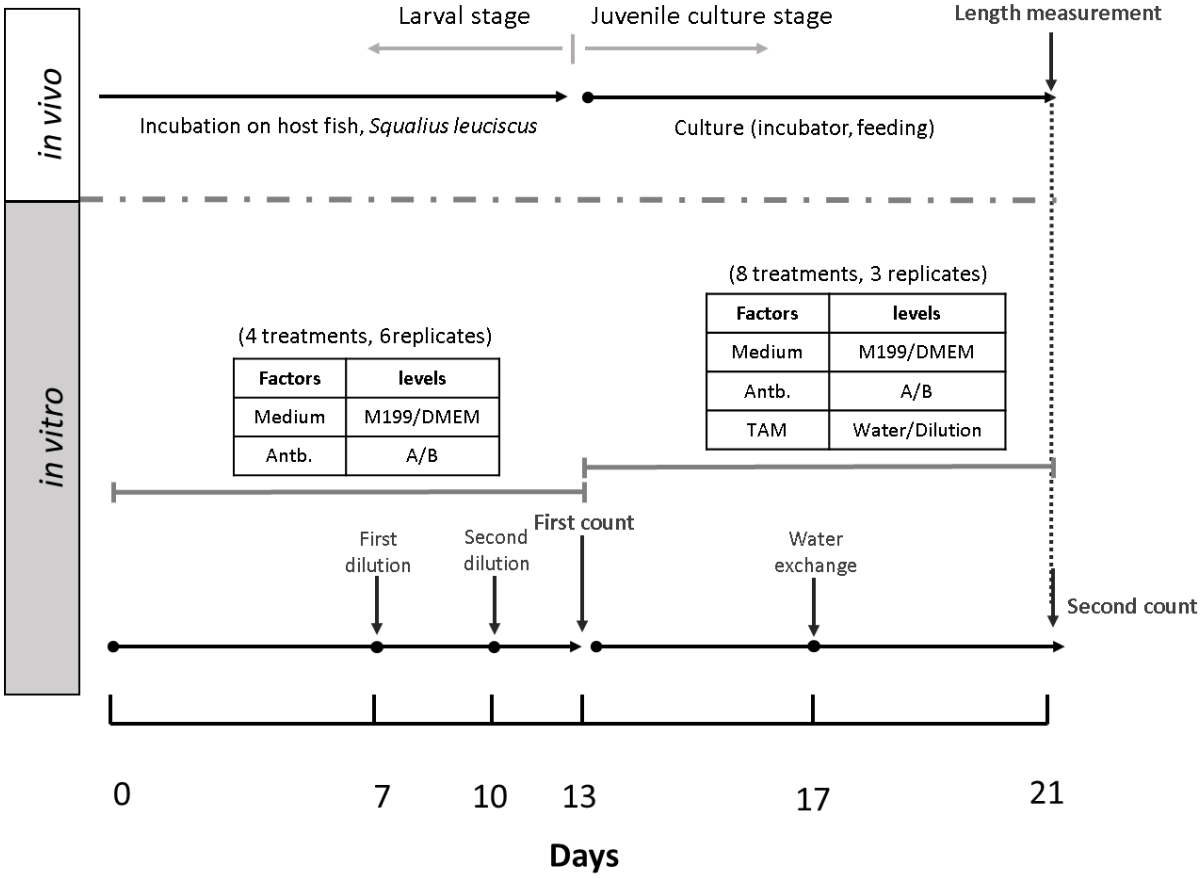

Figure 1. Explanatory diagram of the experiment, the two counts were performed on day 13 and 21. Antb = Antibiotics concentration; A = 100 µg/ml penicillin, 100 µg/ml streptomycin and 200 µg/ml neomycin; and B = 25ug/ml penicillin, 25 µg/ml streptomycin and 50 µg/ml neomycin. TAM = treatment after metamorphosis.

4.1.5 In vivo larval development

Glochidia obtained from *A. anatina* from the same population and collection date as for the *in vitro* experiment were used to infest host fish *Squalius leuciscus* sampled in Vltava River (48°55’18” N, 13°48’25” E) . This fish host species can be considered as a primary host of *A. anatina* with a metamorphosis success rate recorded by a previous study 39.8 % (Douda et al., 2013). Fish were infested for 15 minutes in a bath of 4698 ± 2092 (mean ± SD) glochidia per liter obtained from 10 female mussels. Then the host were kept in a flow through cage in the natural habitat and temperature regime (1-10° C) before movement to individual aquarium system (10° C) to collect juveniles. Juveniles sampled from 9 fish individuals (mean ± SD weight 66.7 ± 7.0 g and body length 164.4 ± 6.7 mm) from three peak drop-off days were used for the evaluation of post larval growth using the same methods as for the *in vitro* produced juveniles.

4.1.6 Metamorphosis success quantification

The individuals observed during each evaluation time point were divided into two groups; viable juveniles displaying movement of the foot outside the shell including juveniles that showed a foot operating but had semi open valves and minimal movement; and non-viable; comprised of closed glochidia with valves tightly closed and no sign of movement or dead individuals with open valves and disintegrating tissues. The metamorphosis success rate was calculated as the sum of viable juveniles over the total count. The same criteria were used for the quantification performed with the juveniles originated from both the *in vitro* and *in vivo* culture method.

4.1.7 Growth increment measures

A subsample of 30 recently extracted glochidia was taken from pooled experimental glochidia before the start of *in vitro* experiment and preserved in ethanol (70%) as a record of initial length. Another subsamples of 30 juveniles were taken at the end of the experiment from each treatment and processed in the same way. A photograph of each subsample was taken using a microscope (magnification 40x) and the measurements were performed using the software ImageJ (Schneider et al. 2012). The length measured corresponded to the longest axis in a direction parallel to the hinge line of the juvenile (see Figure 2). The growth increment was calculated as the length of the individual minus average value of the initial glochidia length.

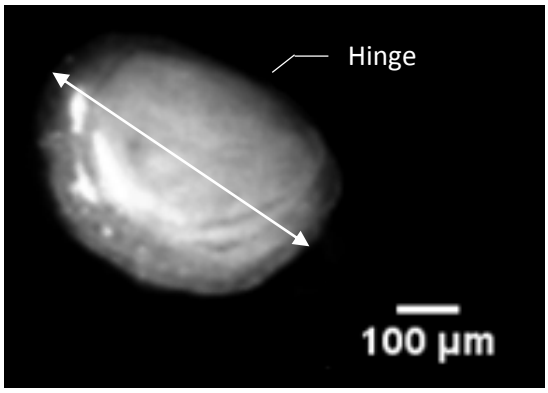

Figure 2. Measured axis in the glochidia and juveniles of *Anodonta anatina*

4.1.8 Statistical analysis

For the effects of the different factors on the metamorphosis rate a generalized linear mixed models (GLMMs) using a binomial distribution and a logit link function was made in R 3.5.0 (R Development Core Team & R Core Team 2017) using the package lme4 (Bates et al. 2015). The response variable was metamorphosis success. Medium type, antibiotic concentration and post-metamorphosis treatment were used as fixed-effect explanatory variables. For the comparison between metamorphosis successes rates in the different time points a GLMM was made with the metamorphosis success as the response variable and the time point of count as the explanatory variable. For the analysis of post larval growth, a linear mixed effects model was used after testing the normality of the data with a Shapiro-Wilk test and quantile-quantile plots. The response variable was the growth increment, and the fixed-effect factors were the medium, the concentration of the antibiotics and the post-metamorphosis treatment – the dish was selected as a random-effect variable for all the analyses.

For the comparison of the potential differences in post larval growth increments of the *in vitro* and *in vivo* produced juveniles, a linear mixed effects model (LME) was used. The response variable used was the growth increment of the juvenile, and the fixed effect used was the method (*in vivo* or *in vitro),* while the random effect was dish (for *in vitro)* or fish (for *in vivo)*; because the juveniles from the fish were obtained on different dates, this was added as a random factor as well.

Homogeneity of variance was tested for all data before the application of statistical analysis. For the metamorphosis rate the assumption was tested on the logit transformation of the data using a Brown–Forsythe test, while for the continuous variable (growth increment) a Levene test was used.

4.2 Experiment 2: Improvement of *in vitro* medium for *M. margaritifera*

4.2.1 Glochidia source and extraction

Two different groups of glochidia were used, the first group was obtained in Blanice River (Czech Republic, 48°55'34"N, 13°58'12"E), and the second group was obtained in the Malše River (Czech Republic, 48°39’01.5” N 14°28’00.3” E), a trial was performed for the glochidia from each location. In the field different individuals were monitored, when glochidia release was observed the individual was extracted and placed into a small container to stimulate further glochidia release, the clogs of glochidia released were extracted with the help of a pipette and observed under a microscope to asses viability, then the glochidia were transferred to 5L containers with river water. After enough glochidia were extracted the females were returned to the same spot on the river where they were initially found, none of the females spent more than 30 minutes outside the river. The bottles with glochidia were then stored in an acclimatized container and transported to the Malacology Laboratory at the Czech University of Life Sciences in Prague. In the laboratory the glochidia were stored in an incubator at 4 C° for one day before being placed on the dishes. Before use the larvae were rinsed with sterilized water to destroy the clogs. The first group of glochidia showed a lower viability than the second one, with a high proportion of underdeveloped glochidia in comparison with the second group (50% in the first group and 5% in the second group). In each dish 1 ml of the dilution of glochidia was placed, previous tests showed that each ml of the dilution would carry 130 to 250 larvae, the cleaned glochidia were placed into each culture dish using a sterile Pasteur pipette. A subsample of glochidia was preserved in ethanol (70%) for initial length measures.

4.2.2 Culture medium composition

For both groups of glochidia a full factorial design was used to test the effects of three factors (taurine addition, serum type and source of lipids), each with two levels resulting in a total of eight treatments. The medium used was M199 (Sigma Aldrich, product code M4530) and was supplemented with two different kind of serums; horse serum (Sigma Aldrich, product code H1270) and newborn calf serum (Sigma Aldrich, product code 12133C). Two different types of lipid sources were used, fish liver oil (Sigma Aldrich, product code F8020) and emulsified lipid mixture (Sigma Aldrich, product code L5146), in each case 50 µl per dish were added. The final factor was the presence or absence of taurine, added in powder form at 42.86 µg/ml (Sigma Aldrich, product code T0625). Additionally, a combination of penicillin (714.2

units/ml), streptomycin (710μg/ml) and neomycin was added to each dish (1430 μg/ml) in each dish (PSN mixture, Sigma Aldrich, product code P4083). The preparation followed a 4:2:1 proportion (Roberts & Barnhart, 1999) for the medium, serum and antibiotics respectively. The mediums were complemented with 5 μg/ml of antimycotics (amphotericin B, Sigma Aldrich, product code A9528). Four petri dishes (15 x 60 mm) were used of each media composition treatment for a total of 32 dishes and each dish was loaded with 5 ml of culture medium. Before the addition of glochidia all dishes were placed in a CO_2 incubator (NB-203, N-Biotek, Korea) under UV light for one hour. The dishes were kept in the CO_2 incubator at 5% CO_2 and 18 C° a change of medium was performed in the fifth day of cultivation, in both trials the experiment was concluded on day 11.

4.2.3 Quantification of reaction to the medium and length measurements

Three counts were performed to assess the amount of closed glochidia after exposure to medium on days two, five and eleven of cultivation. The days of counting were selected to assess the survival of glochidia in the dishes. When the medium conditions are suitable, glochidia will close tightly to start metamorphosis, dead glochidia in the other hand, can be distinguished by their valves completely open. In each count between 50 and 60 random glochidia were counted per dish and the rate of closed individuals was calculated as the amount of closed glochidia over the total number of counted glochidia. In dishes where a large portion of glochidia were lost during cultivation, the counts were performed taking into account all the glochidia observed.

Glochidia of *M. margaritifera* grow substantially during metamorphosis, so we performed a comparison between the initial and final length of the larvae with the aim of trying to detect any differences in the final length of the glochidia in the different treatments. A subsample of 44 recently extracted glochidia was taken from the second group before the start of *in vitro* experiment and preserved in ethanol (70%) as a record of initial length. Another subsamples of between 8 and 25 juveniles were taken at the end of the experiment from each treatment and processed in the same way. A photograph of each subsample was taken using microscope (magnification 40x) and the measurements were performed using the software ImageJ (Schneider et al. 2012). The length measured corresponded to the longest axis perpendicular to the hinge line in the juvenile (Figure ***3***).

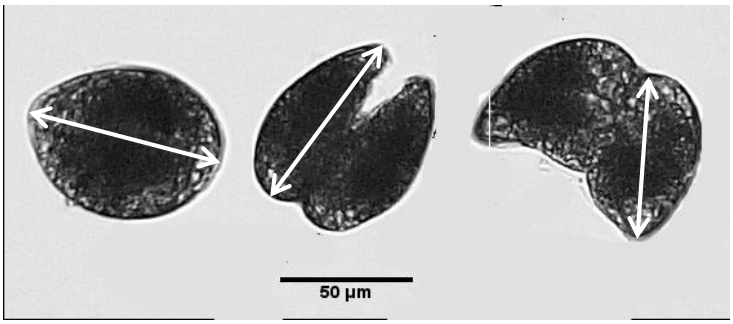

Figure 3. Measured axis in glochidia of *M. margaritifera* in different positions

4.2.4 Statistical analysis

A generalized linear mixed models (GLMM) using a binomial distribution with logit link function was made in R (R Development Core Team & R Core Team, 2017) using the package lme4 (Bates, Maechler, Bolker, & Walker, 2015). The response variable was percentage of closed glochidia. Serum type, lipid source and presence or absence of taurine were used as fixed effects explanatory variables. The dish was selected as a random effect variable. For the analysis of final length a linear mixed effects model was used after testing the normality of the data with a quantile-quantile plot, the response variable was the final length and the fixed effect factors were the serum type, the lipid source and taurine with the dish as random factor. In both models maximum likelihood was used for the estimation of the parameters.

5 Results

5.1 Experiment 1: Time variation in metamorphosis success

5.1.1 Metamorphosis success

The observed metamorphosis success rate was lower in the first (48.54 ± 24.97 %) than in the second (64.49 ± 12.47 %) control (Figure 4), and the difference was statistically significant (GLMM: Z = 18.39, p < 0.001, see Table 2).

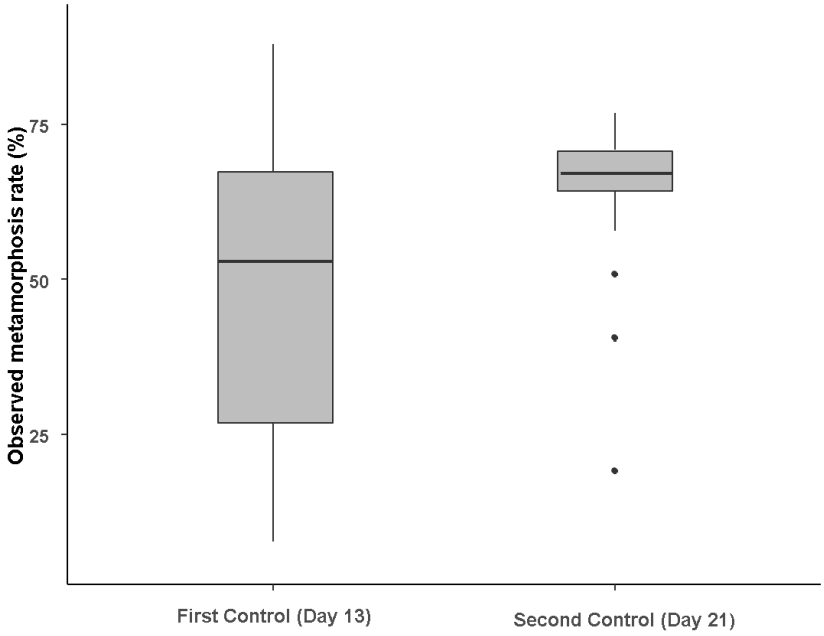

Figure 4. Comparison between observed metamorphosis rates in two different dates of assessment

Table 2 GLMM‘s Coefficient estimate, standard error of the estimate and p-value for the effect of time of counting on the observed metamorphosis rate

Factor	Estimate	Std. Error	z value	p value
Control(Second control, day 21)	0.80781	0.04394	18.385	<0.001

There was a significant effect of the post incubation treatment on the metamorphosis success in the first control (water and medium = 33.97 ± 24.16 %, water only = 63.11 ± 16.03 %, GLMM: Z = 4.85, p = < 0.001), but this effect disappeared in the second control (water and medium = 63.05 ± 15.72 %, water only = 65.92 ± 8.57 %) with no significant effect detected (GLMM: Z = 0.55, p = 0.579) (Figure 5A). A similar result was observed with the effect of medium type. In the first control, the detected metamorphosis success rate was significantly higher for the

M199 medium (60.98 ± 20.20 %) than for the DMEM medium (36.10 ± 23.61 %) (GLMM: Z = 4.09, p < 0.001), but this difference was not significant in the second control (M199 = 63.39 ± 14.67 %, DMEM = 65.59 ± 10.35 %, GLMM: Z = -0.50, p = 0.620) (Figure 5C). The antibiotic concentration showed no effect at any of the two time points (1st control: Z = -1.37, p = 0.171, 2nd control: Z = 0.796, p = 0.452) (Figure 5B). The estimates and p-values for the statistical analysis with the GLMM are presented in

Table 3. GLMM's Coefficient estimate, standard error of the estimate and p-value for the effect on the observed metamorphosis rate of Medium type, antibiotic concentration and treatment after metamorphosis in each count

Factor	First count (day 13)				Second count (day 21)			
	Estimate	**Std. Error**	**z - value**	**p-value**	**Estimate**	**Std. Error**	**z - value**	**p-value**
Medium(M199)	1.25	0.31	4.09	<0.001	-0.11	0.21	-0.50	0.62
Antibiotic concentration (B)	-0.42	0.31	-1.37	0.171	0.17	0.21	0.80	0.43
Treatment after metamorphosis (Only Water)	1.48	0.31	4.84	<0.001	0.12	0.21	0.56	0.58

5.1.2 Growth increment

The mean ± SD initial and final lengths of the glochidia/juveniles in the *in vitro* culture experiment were 358.23 ± 18.88 µm and 417.61 ± 23.01 µm, respectively, resulting in an average growth increment of 14.22 % (59.37 ± 23.01 µm) over the whole experiment. The comparison of the individual factors showed that medium type was the only factor with a significant effect of length increment (M199 = 65.90 ± 24.20 µm, DMEM = 52.90 ± 19.84 µm; LME: t = 3.42, p < 0.001) (Figure 5A and Table 4). The treatment after metamorphosis (LME: t = 1.03 p = 0.3, Figure 5C and Table 4) and the concentration of antibiotics (LME: t = 0.15 p = 0.89, Figure 5B and Table 4) showed no significant differences.

Table 4. LMM's Coefficient estimate, standard error of the estimate and p-value for the effect on the final length increment of Medium type, antibiotic concentration and treatment after metamorphosis

Factor	Estimate	Std. Error	p-value
Medium(M199)	12.90	3.77	<0.001
Antibiotic concentration (B)	0.56	3.77	0.88
Treatment after metamorphosis (Only Water)	3.91	3.77	0.30

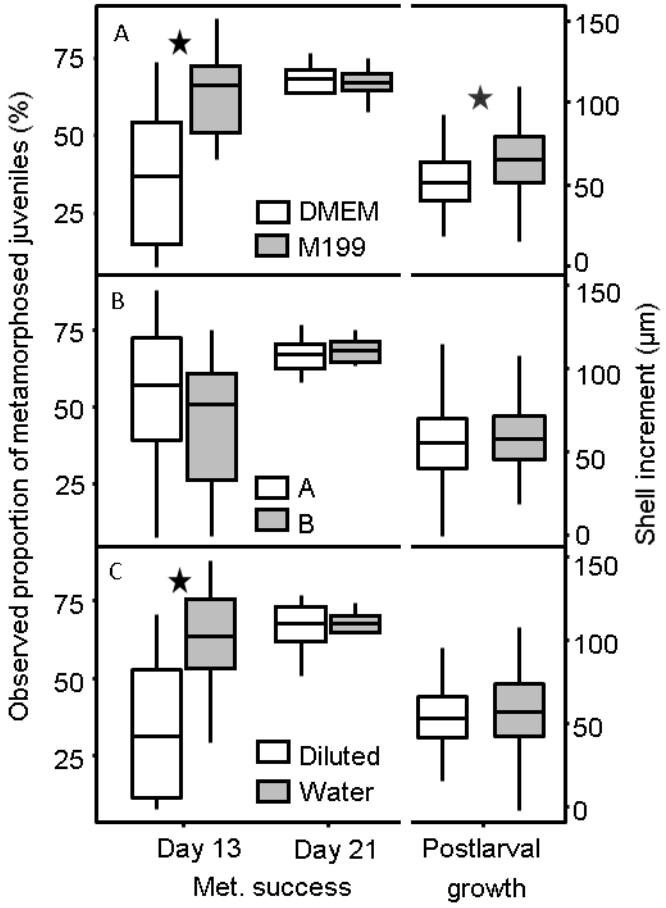

Figure 5 Effects of medium type (top), antibiotics concentration (middle) and dilution (bottom) on the *in vitro* metamorphosis rate assessment of glochidia from *A. anatina* in two different time points. Final growth increment can be found to the right of each plot. The stars denote significant difference between factors ($p<0.001$)

5.1.3 Comparison of *in vitro* and in vivo post larval growth increments

The mean ± SD final length in the juveniles metamorphosed on host fish was 417.03 ± 29.53 µm with an average growth increment of 52.79 ± 29.50, and there was no significant difference in growth increment between in vivo and *in vitro* growth increment. (LME: t = -1.00, p = 0.32).

Table 5. LMM's Coefficient estimate, standard error of the estimate and p-value for the effect on the final length of *in vivo* vs. *in vitro* method.

Factor	Estimate	Std. Error	p-value
Group (in vivo)	-12.24	12.27	0.32

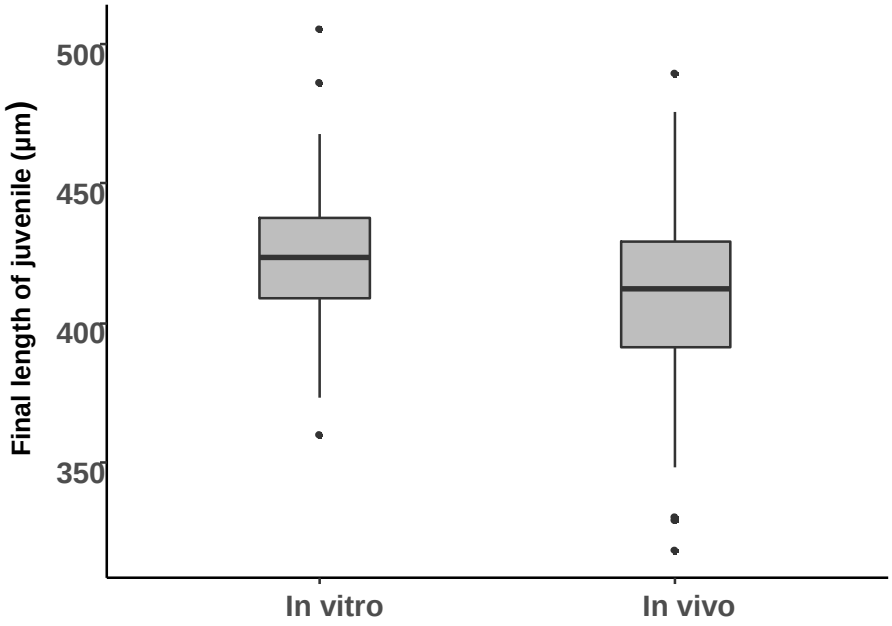

Figure 6. Comparison between the final observed length of juveniles under *in vitro* and *in vivo* settings.

5.2 Experiment 2: Improvement of *in vitro* medium for *M. margaritifera*

5.2.1 The proportion of closed glochidia after exposure

The average proportion of closed glochidia after exposure to the medium during the three counts was similar in both trials (Trial 1 = 32.28 ± 17.39 %, Trial 2 = 33.59 ± 27.67 %). In the first trial there was a significant effect of the serum type in all controls (Table 6) but the effect was not completely coherent (Figure 7B) , in the first control horse serum seemed to perform worse than the newborn calf serum (Newborn-calf = 41.16 ± 16.77 %, Horse = 21.88 ± 13.88 %, GLMM: z = -4.511, p < 0.001), however in the second and third controls this effect was

reversed and the rate of closed glochidia after exposure to newborn calf was significantly lower than the rate for horse serum (Second count, Newborn-calf = 26.46 ± 10.36 %, Horse = 41.38 ± 14.27%, GLMM: z = 3.443, p < 0.001; Third count, Newborn-calf = 21.41 ± 9.42 %, Horse = 42.02 ± 22.12%, GLMM: z = 4.064, p < 0.001).

The ELM had a statistically significant positive effect (Table 6) in the first (ELM = 40.53 ± 18.70 %, Fish Oil = 22.50 ± 12.20 %, GLMM: z = -4.158, p<0.001) and third control (ELM = 39.49± 24.12 %, Fish Oil = 23.94 ± 9.69 %, GLMM: z = -3.235, p = 0.0012), in the second control the average rate of closed glochidia for ELM was slightly higher compared with the fish oil but this difference was not significant (ELM = 39.38 ± 12.03 %, Fish Oil = 33.46 ± 16.87 %, GLMM: z = -0.321, p = 0.749) (Figure 7C). The addition of taurine did not show any significant effect in any of the tree counts of the first trial (Figure 7A and Table 6).

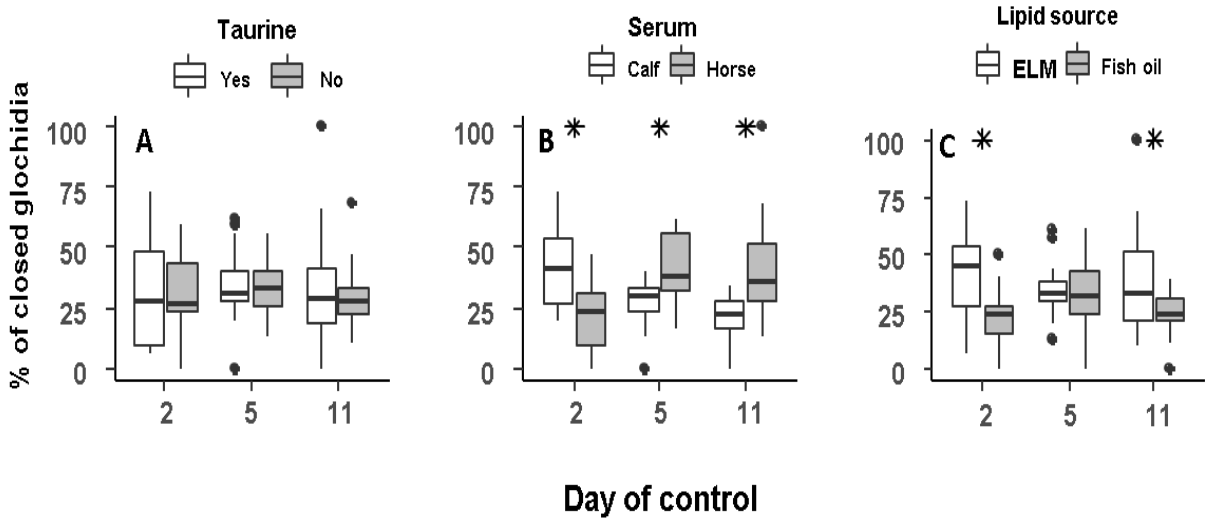

Figure 7. First trial, effects of taurine addition, serum type, and lipid source in the rate of closed glochidia from *M. margaritifera* after exposure to the medium during three different controls. The stars denote statistically significant difference (p<0.01)

Table 6. Summary of the statistical analysis for the first trial; GLMM‘s Coefficient estimate, standard error of the estimate and p-value for the effect on glochidia‘s survival rate of taurine addition, serum type and lipid source in each of the three counts.

Count	Factor	Estimate	Std. Error	z value	p-value
First count (Day 2)	Taurine(Yes)	0.04	0.22	0.19	0.85
	Serum(horse serum)	-0.99	0.22	-4.51	<0.001
	Lipid source (Fish oil)	-0.91	0.22	-4.16	<0.001
Second count (Day 5)	Taurine(Yes)	-0.09	0.20	-0.42	0.67
	Serum(horse serum)	0.70	0.20	3.44	<0.001
	Lipid source (Fish oil)	-0.07	0.20	-0.32	0.75
Third count (Day 11)	Taurine(Yes)	-0.07	0.19	-0.35	0.73
	Serum(horse serum)	0.81	0.20	4.06	<0.001
	Lipid source (Fish oil)	-0.63	0.20	-3.24	<0.01

For the second trial there was also a significant effect of serum type in all three controls (Table 7), however contrary to the first trial, rates of closed glochidia were consistently higher for the newborn calf serum (Figure 8B) with the biggest differences observed in the first (Newborn calf = 75.86 ± 9.49 %, horse = 12.37 ± 14.16, GLMM: z = -808.5, p<0.001) and second control (Newborn calf = 45.31 ± 14.29 %, horse = 21.13 ±22.92, GLMM: z = -5.689, p<0.001). In the third control the difference was less pronounced but still significant (Newborn calf = 23.92 ± 14.94, horse = 16.51 ± 22.16, z = -2.644, p = 0.008).

Regarding the lipid source (Figure 8C), ELM showed significantly higher rate of closed glochidia in the first (ELM = 49.94 ± 33.09, Fish oil = 38.29 ± 35.67, GLMM: z = -228, p<0.001) and second (ELM = 46.27 ± 18.10, Fish oil = 25.20 ± 20.64, GLMM: z = -5.057, p<0.001) controls. In the third control the fish oil had a slightly higher rate of closed glochidia but this difference was not statistically significant (ELM = 18.68 ± 17.26, fish oil = 21.21 ±

22.33, GLMM: z = 0.292, p = 0.77) (Table 7).Addition of taurine did not have any significant effect on the proportion of closed glochidia in any of the tree controls (Figure 8A and Table 7)

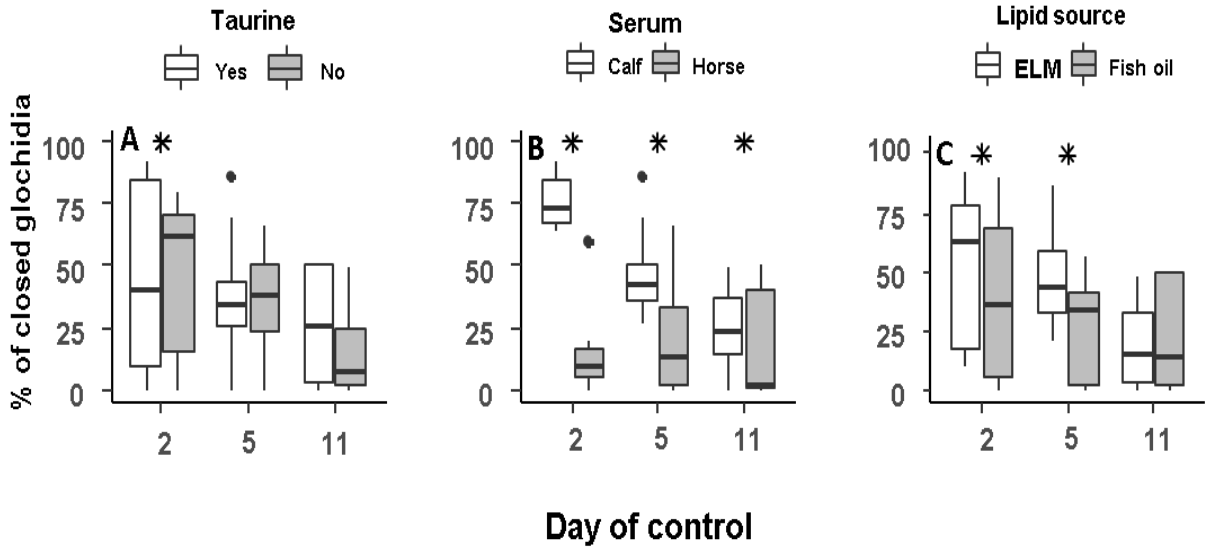

Figure 8. Trial 2, effects of taurine addition, serum type, and lipid source in the rate of closed glochidia from *M. margaritifera* after exposure to the medium during three different controls. The stars denote statistically significant difference (p<0.01)

Table 7. Summary of the statistical analysis for the second trial; GLMM‘s coefficient estimate, standard error of the estimate and p-value for the effect on the glochidia‘s survival rate of taurine addition, serum type and lipid source in each of the three counts.

Count	Factor	Estimate	Std. Error	z value	p-value
First count (Day 2)	Taurine (Yes)	-0.13	0.004	-29.50	<0.001
	Serum (horse serum)	-3.49	0.004	-808.50	<0.001
	Lipid source (Fish oil)	-0.98	0.004	-228	<0.001
Second count (Day 5)	Taurine (Yes)	-0.007574	0.325833	-0.023	0.9815

	Serum (horse serum)	-2.00	0.35	-5.69	<0.001
	Lipid source (Fish oil)	-1.72	0.34	-5.06	<0.001
Third count (Day 11)	Taurine (Yes)	-0.82	0.63	-1.32	0.19
	Serum (horse serum)	-1.682	0.636	-2.644	0.008
	Lipid source (Fish oil)	0.19	0.65	0.29	0.77

5.2.2 Final length

The analysis showed that none of the factors had a significant effect on the final length of the glochidia. The final average length of glochidia exposed to taurine was 72.88 ± 6.10, while for glochidia not exposed to taurine it was 71.70 ± 3.95 um (LMM: χ^2 = 0.1.51, p = 0.22). Regarding the serum type, the final average length was 72.08 ± 4.07 um for the newborn calf serum and 72.24 ± 5.89 for the horse serum (LMM: χ^2 = 0.05, p = 0.91). Lastly, the final average length for the ELM group was 71.72 ± 4.13 while the fish oil group showed an average length of 72.65 ± 5.63 (LMM: χ^2 = 1.01, p = 0.58) (Figure 9 and Table 8).

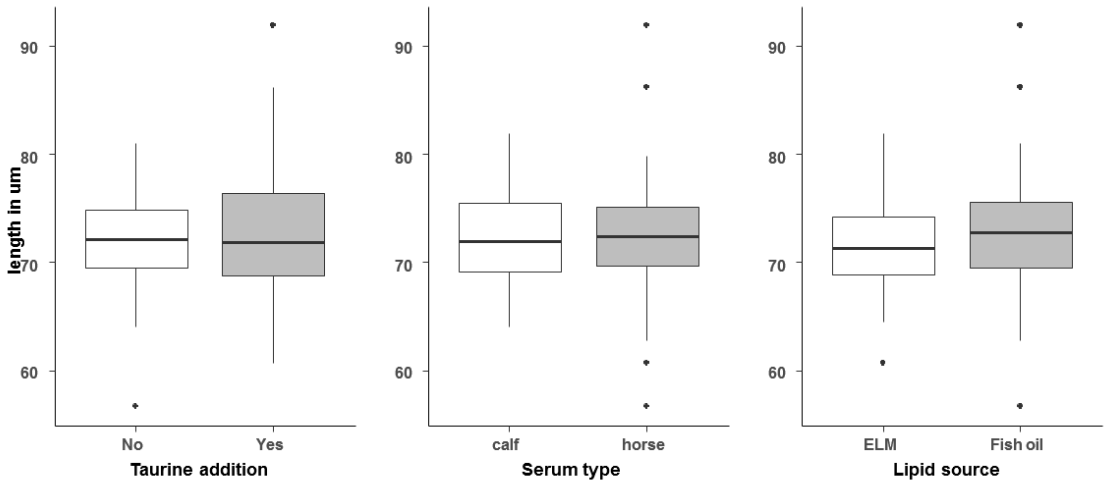

Figure 9. Effects of taurine addition, serum type, and lipid source in the average final length in um of glochidia from *M. margaritifera* after 11 days of exposure to the medium.

Table 8. LMM's coefficient estimate, standard error of the estimate and p-value for the effect on the final length in µm of glochidia from *M. margaritifera* of taurine addition, serum type, and lipid source in the average final length in after 11 days of exposure to the medium.

Factor	Estimate	Std. Error	χ^2	Df	p-value
Serum (horse serum)	0.10	0.92	0.01	1	0.91
Taurine (Yes)	1.14	0.93	1.50	1	0.22
Lipid source (Fish oil)	0.90	0.91	0.99	1	0.32

6 Discussion

6.1 Experiment 1: Time variation in metamorphosis success

This study documents that the metamorphosis success of *in vitro* cultured glochidia assessed by different indicators at different time points can vary substantially in relation to medium composition and other factors. Pedal feeding can have different start times for different treatments, obscuring early comparisons of metamorphosis success. These findings highlight the importance of metamorphosis success indicator selection and timing and are in line with observations of variable developmental times for glochidia on fish hosts (Douda 2015, Khym & Layzer 2000)

Despite the influence that the counting time can have on the observed metamorphosis rate, previous studies vary greatly in the timing of *in vitro* culture success assessment. In some cases, the quantifications were performed just after the incubation period (Roberts & Barnhart 1999; Lima et al. 2006; Taskinen et al. 2011), while in others, a postponed quantification taken at 24 to 48 hours in water was recommended to allow juveniles to become active (Kern 2017). Our study corroborates that postponed counting can be advantageous because of the elimination of false negative viability assessment (and even longer periods than 48 hours can be needed), but on the other hand, the early quantification of juvenile metamorphosis can reveal variable development in different culture media.

The timing and methods of media dilution at the end of incubation are of great importance as well. Various approaches can be found in previous studies. In some cases, there is no report of dilution at the end of the incubation phase before counting (Lima et al. 2006; Uthaiwan, Noparatnaraporn & Machado 2001), while in others, the juveniles were immediately transferred to water after metamorphosis was completed and then counted (Roberts & Barnhart 1999; Fox 2014; Kern 2017). Some studies use gradual dilution (Lima & Avelar 2010; Taskinen et al. 2011) in which a partial exchange or dilution of the medium with distilled or sterilized water is performed before the end of the experiment, and then the medium is replaced completely with water on the final day. Gąsienica-Staszeczek et al. (2018) studied the effects of finishing time and dilution on the *in vitro* development of *Unio crassus* and found that finishing time is critical for successful metamorphosis *in vitro*. Early finishing of the experiment can cause high mortality of glochidia due to the lack of proper development, and extending the *in vitro* phase too much can facilitate the infection of the dishes. They also suggested performing the dilution gradually after the *in vitro* phase culminated, arguing that a sudden change will have negative

effects on glochidia. As observed here, the variety of approaches can greatly affect the calculated metamorphosis rate, especially for counts performed immediately after the end of the incubation period. In our case, the metamorphosis rate was higher in the first count for the dishes that had complete replacement of the medium and addition of water compared to the dishes that just had the medium diluted. This also caused variation in the performance of the methods tested, as was the case with the M199 medium, which showed a significant difference over the DMEM medium in the dishes where the medium was completely replaced, which is in contrast to the dishes where the medium was diluted.

The discrepancies between metamorphosis rates at different time points can be explained by the postponed transition of some individuals to typical juvenile behavior. Healthy larvae close tightly when exposed to the medium and remain so during metamorphosis. After metamorphosis, the valves will be slightly open, and the foot will start operating outside the shell. However, the start of pedal feeding can vary greatly between individuals, even within the same group. This can significantly affect the proportion of metamorphosed juveniles observed, since the criteria for recognizing them are based on the observation of pedal or valve activity. Allowing the juveniles to further develop for a few days after the incubation period can help overcome this limitation.

This study suggests that the risk of biased metamorphosis success assessment can be mitigated by three main approaches. First, the dilution method at the end of the experiment should be standardized or taken into account in the analysis as a source of variation. As shown, studies differ greatly in their approaches for this final step, which can have a considerable effect on the observed metamorphosis rate. Second, multiple time points for the assessment of metamorphosis success should be considered instead of a single early time point, which requires extending the experiment after metamorphosis is first observed. The juveniles should be kept in a suitable environment and counted at least one additional time. This avoids the bias caused by variations in both the start of pedal feeding and juvenile activity. Finally, additional methods should complement traditional counts; in our case, length measures appeared to be a reliable way to measure differences between treatments, but other methods based on physiological indicators and energy reserves, such as lipid content, carbon content or glycogen, can be used, although these methods are methodologically challenging (Fisher & Dimock 2006, Tankersley 2000, Wächtler, Dreher-Mansur & Richter 2001).

In summary, these results demonstrate the importance of further research into glochidia *in vitro* techniques. The optimization of metamorphosis success and viability indicators can be an

important step towards the increased efficacy of juvenile production. Indeed, an increased survival rate during *in vitro* culture can help overcome some of the problems faced currently in the artificial rearing of unionid bivalves, such as the risk of inadvertent selection of genotypes compatible with the host strains used in artificial rearing. The development of efficient methods of *in vitro* larval culture are critical for the conservation of freshwater mussel species, producing juveniles for research, population reinforcements or commercial purposes.

6.2 Experiment 2: Improvement of *in vitro* medium for *M. margaritifera*

This study shows the importance of medium composition for the early survival of glochidia form *M. margaritifera* in *in vitro* conditions. Improving the survival of larvae from this species under *in vitro* settings is critical for future conservation plans. Additionally, due to its long metamorphosis period, the assessment of early survival rates of glochidia of *M. Margaritifera* is of great importance, studying the most suitable conditions that enhance survival of larvae to the medium can be achieved by this short term experiments focusing on the reaction to the medium during the first days of incubation.

Contrary to our hypothesis the addition of taurine did not have a significant positive effect on the survival of glochidia of *M. margaritifera* after exposure to the medium. The effect of taurine was just significant in the first count of the first trial. Serum type showed a significant but inconsistent influence among and within trials. In two of the tree counts performed on the first trial the horse serum showed a statistically significant increase in the amount of closed glochidia after exposure. In contrast, in the second trial the newborn calf serum gave consistently higher rates of closed glochidia in all three counts. One interesting finding of this experiment is the positive effect of the emulsified lipid mixture, in comparison with the fish oil; in four of the six counts performed during both trials, the proportion of glochidia closed was higher when using ELM.

In vitro culture for *M. margaritifera* is especially challenging. Owing to its very long time of development compared to other species of mussels and to the particularity that glochidia of this species grow substantially during metamorphosis. In the past, only partial success has been obtained using and *in vitro* setting. Taskinen et al. (2011) achieved metamorphosis of *M. margaritifera* by using a combination of *in vivo* and *in vitro* techniques. The authors achieved metamorphosis only when the larvae were allowed to develop in the host fish for a long period of time (133 days), after this period the host-fish were killed and the gill arches extracted and placed into culture medium with metamorphosis occurring just 14 days after the start of *in vitro*

phase. Additionally, Taskinen et al. (2011) tried alternative approaches but with no successful metamorphosis observed; for example when the glochidia were placed directly in *in vitro* conditions, without any time spent on the host fishes, they died after 55 days of incubation. Furthermore, glochidia extracted from the host at an earlier stage (56 days) manage to survive in *in vitro* incubation for up to 174 days (total age of glochidia 230 days) but did not manage to metamorphose.

Previous works have also dealt with the survival of the glochidia after exposure to the medium, however most of the works performing survival counts use species with short metamorphosis times. For example Uthaiwan, Noparatnaraporn and Machado (2001) achieved the metamorphosis of *Hyriopsis myersiana* under *in vitro* conditions, the total metamorphosis period was 11 days and the percentage of survival and metamorphosis was studied, the authors found that survival of glochidia from *H. myersiana* is higher when using fish plasma instead of horse serum and this results were consistent with the metamorphosis success rate. Despite this no detailed description of the protocol followed for assessing survival rate was provided in this study. A protocol more similar to the one presented here was used by Uthaiwan et al. (2002) using also *H. myersiana*, the authors tested different modifications of *in vitro* culture conditions to test both the effect of different fish plasma and the density of glochidia during incubation. In this study a survival count was performed after 2-3 days of exposure to the medium finding significant better survival rates for glochidia exposed to common carp plasma and a decrease in survival when the density of larvae per dish was superior to 450 individuals. An interesting finding of these study is that the metamorphosis success rate, obtained as the proportion of surviving glochidia transformed, was 100% in all groups, this suggests that early survival of glochidia after exposure to the medium might be an accurate indicator of the suitability of medium for *in vitro* metamorphosis of larvae from freshwater mussels. However, in this study the total length of the *in vitro* incubation was just 10 days.

This studies used survival rate of glochidia after exposure to the medium mainly as a way to complement the metamorphosis success rate calculation. I argue that this approach might lead to problems in the assessment of *in vitro* techniques and protocols. The first problem arise due to the variable reaction of glochidia to the medium after exposure, in our experiment for example 16 of the 32 dishes in the trial 1 and 14 of 36 dishes in the trial 2 showed higher percentage of closed glochidia in the second count performed in day 5 than in the first count performed in day 2, this might be the effect of delayed closing in some individuals after exposure to the medium. This variability of reaction makes assessments based on a single

survival count more prompt to errors. Additionally, special care needs to be taken when comparing metamorphosis rates obtained after an initial survival count, since this metamorphosis rates can be based just on the amount of surviving individuals and not in the total amount of individuals observed. For example in the study of Uthaiwan et al. (2002) the reported percentage of transformation, this is the metamorphosis rate, was 100% in all groups, in this case the metamorphosis rate was calculated as the proportion of surviving individuals that transformed. In contrast other studies used different approaches, for example Ma et al. (2018) calculated the metamorphosis rate of *Cristaria plicata* under *in vitro* conditions but in this case the rate was based on the total amount of glochidia at the end of the incubation period. Regarding the addition of taurine, our observations reported no significant effect of the addition of taurine in contrast with recent observations of Wen and collaborators (2018), who achieved the metamorphosis of *Potamilus alatus* a highly host-specialist species that also grows substantially during metamorphosis. The authors used a modified *in vitro* protocol and suggested that taurine could be an important amino acid necessary for metamorphosis. In this study a comparison between the effects of plasma from different fish species was performed, this information was complemented with the anlaysis of free aminoacids content in each plasma type. The results showed that common carp plasma produced higher growth rates under *in vitro* conditions (despite having lower metamorphosis rate compared to other kinds of plasma), the authors attributed this behavior to the high content of taurine in the common carp's blood, having 3.5 to 9.1 times the concentration of other fish species. In our case neither the percentage of closed glochidia nor the total length of the glochidia at the end of the experiment was affected by taurine. It is likely that even if taurine is an important amino acid for the development of glochidia that grow during metamorphosis, the effect will be more important at more advanced stages of the process, additionally the observations of Wen and collaborators suggests that it is most likely that the influence, if any, of this amino acid in the development of *M. margaritifera* it's going to be related mainly with growth and not with survival or metamorphosis rate.

In respect to the effect of the lipid source, our experiment found a significant difference between the performance of the ELM and the fish oil regarding the proportion of closed glochidia, with the glochidia exposed to ELM showing higher closing rates. The addition of a lipid source is thought to increase juvenile quality so and addition of 50 µl of fish oil per dish is now common in most studies (Lima et al. 2012). The problem, however, is the insolubility of the traditional fish oil in the medium, this can led to problems especially for longer culture periods. In our first experiment with *A. anatina* we used cod liver oil as a lipid source, during the incubation we

observed that the oil tended to congregate in droplets, this can cause uneven access to the lipid source across the dish affecting the development of the larvae. Additionally, due to the different densities of the oil and the medium, the former tends to form a layer covering the latter, this can affect the gas exchange between the CO_2 enriched atmosphere and the medium and in turn can have an effect in both the available oxygen and the pH of the medium. The addition of an emulsified lipid source can help to overcome this limitations while still providing a good source of lipids to the development of glochidia. Nonetheless, our data only provides information about the early reaction of glochidia of *M. margaritifera* to ELM, more research, probably using species with shorter period of metamorphosis and established *in vitro* protocols, is needed to address the effects of this additive in the final metamorphosis rate.

Finally, we found some inconsistent behavior between the two trial regarding the effects of the two serums tested. Newborn calf serum was used by Taskinen et al. (2011) in their *M. margaritifera* experiment. The authors suggest that other serums should be used, and recommend the use of fish plasma. The addition of fish plasma has proven to be beneficial with many mussel species even when using plasma from a non-host (Lima et al. 2012; Uthaiwan et al. 2002), however some host-specific species may require specific plasma from its host to develop. It is likely that the addition of fish plasma, for example from brown trout, can be beneficial for the *in vitro* development of *M. margaritifera* however this poses a major methodological challenge, because the metamorphosis rate of this species is very long, many changes of medium are required to avoid infection, the amount of serum needed in such cases can be high and obtaining the amounts needed could be very difficult. A possible solution could be the use of a combined mechanism, by using fish serum during the first stages of the development and the changing to another cheapest or more available alternative like rabbit, horse or newborn calf serum.

7 Conclusions

- The method of dilution used at the end of *in vitro* incubation can have a great influence on the observed metamorphosis rate, especially when single earlier counts are performed. In our experiment the observed metamorphosis rate of glochidia from *A. anatina* in the first count was 63.11 ± 16.03 %, when using the just water dilution approach and 33.97 ± 24.16 % when using a partial dilution, with the difference being statistically significant (GLMM: Z = 4.85, p =<0.001). This behavior alongside the lack of standardization on the protocols for dilution after incubation can led to biased conclusions about the performance of *in vitro* protocols.
- Most studies used a single counting point to assess metamorphosis success under *in vitro* conditions, however due to variation in juvenile activity (the main criteria for judging metamorphosis) this counts are subject to high variation and hence can lead to wrong conclusion about the effects of different factors evaluated. In our experiment, for example, we found that the metamorphosis success of on single early count was significantly lower (48.54 ± 24.97 %) than in the metamorphosis observed in the second count (64.49 ± 12.47 %). This can also affect the observed effects of the factors tested, for example when using just a single earlier count the medium M199 showed significantly better percentages of metamorphosis of *A. anatina* than the DMEM medium. This effect disappeared when a second count was performed revealing no significant difference between the two treatments. We suggest that at least two counts should be performed in every *in vitro* culture experiment and this counts should be complemented with additional measures (for example post metamorphosis length measurements).
- The addition of taurine did not influence the proportion of closed glochidia or the final length in any of the two trials performed with *M. margaritifera*. Despite this, the possible effect of this amino acid cannot be discarded completely, since its influence is mainly related with glochidia growth, the effects might be observed in subsequent stages of development.
- The emulsified lipid mixture (ELM) showed a significantly higher proportion of closed juveniles in four of the six counts performed in the two trials of the *M. margaritifera* experiment. ELM can increase lipid availability on the culture dish and avoid possible problems of oxygen depletion and change on pH. However further experiments are required to assess its effects on the metamorphosis process as a whole.
- Serum type can greatly influence the response of glochidia from *M. margaritifera* to the culture medium, in our test the behavior was not consistent between trials suggesting high variability when using horse and newborn calf serum. It is likely that the use of fish serum, especially salmonid fish, will improve the *in vitro* medium conditions for this species, although its long metamorphosis stage makes the use of serum difficult.

8 References

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