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**Resilience of interspecific relationships in the freshwater environment: insights using mussel-
fish interactions**

Doctoral dissertation

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I hereby confirm that doctoral dissertation thesis “Resilience of interspecific relationships in the freshwater environment: insights using mussel-fish interactions” was written independently and it is based on my own work or work I have collaborated on with my colleagues and with help of publications that are properly quoted. The thesis was elaborated under supervision of Ing. Karel Douda, PhD. I agree with publishing this PhD. thesis according to Czech law 111/1998 Sb. about the universities in its current valid wording. This agreement is independent from the results of the defense.

11th March 2024, Prague _____



How beautiful was to know that you
were there, like calm water
-Julio Cortazar.

Resilience of interspecific relationships in the freshwater environment: insights using mussel-fish interactions

Summary

This thesis focuses on the conservation and host parasite relationships of freshwater mussels of the order Unionida. A significant part of the thesis was devoted to methodological developments in the field of conservation, specifically to the artificial rearing under *in vitro* conditions. In this regard, we were successful on identifying key factors that affect the performance of *in vitro* culture protocols. Furthermore, we studied the long-term effects of this technique on mussel populations, including the ability of *in vitro* produced mussels to naturally infect hosts in the wild, and we found that at least for generalist species, the *in vitro* produced mussels have no difference in growth or infection rate when compared with mussels reared on fish.

Other methodological developments are presented including a new field-deployable device for studying host –parasite relationships on species that are difficult to keep in laboratory conditions. We studied the capacity of the device to detect glochidia and juveniles. The device was also successfully used in the field to study host affinity of the endangered *M. margaritifera*, something that was only partially successful in the past.

Finally, a new method for studying bivalve behavior using an automated image analysis technique is also presented. The technique allows tracking changes of behavior based on both the movement of the shell and the soft tissue in contrast with traditional methods that rely solely on the movement of the shell. Additionally, this method shows improvement over similar approaches that also use images to study bivalve behavior, because it can process video automatically and at high speed, whereas other approaches required the user to perform the measurements manually.

In general, I believe this thesis is an important contribution to the field of freshwater mussel research; the insight from our research on *in vitro* rearing will hopefully inform future conservation strategies and show the potential of the technique to study basic mussel ecology. Additionally, the use of image analysis techniques applies to freshwater mussel research opens the door to monitoring programs and better understanding of bivalve behavior against stressors.

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Literature overview

Conservation of freshwater mussels

Biodiversity loss is one of the greatest environmental problems of our time, current trends reveal that despite the existence of conservation initiatives, the general pattern of the global biodiversity is towards decrease. Butchart and collaborators (Butchart et al., 2010) analysed 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, effects of invasive species, and impacts related to climate change. Furthermore, ecosystems are not affected homogeneously across the spectra of pressures, and some of them seem to be more affected than others. This is the case of freshwater ecosystems.

Freshwater ecosystems contain around 5 % of all the species described despite representing just 0.8% of the earth surface area. Apart from the limited extension, their vulnerability is further increased by the high level of endemism these ecosystems have, their high connectivity that allows the rapid expansion of threats like invasive species (Revenga et al., 2005) and the high dependency of our societies on freshwater resources (Geist, 2010, 2015; Strayer & Dudgeon, 2013). All these impacts have resulted in consistently higher extinction rates of freshwater species at different taxonomic levels (A. E. Bogan, 1993; Ricciardi & Rasmussen, 1999), even more some analyses have found that the current rate of decline of some poorly studied faunas like freshwater gastropods exceeds the extinction rates observed during previous mass extinctions (Neubauer et al., 2021).

Another stark example of a declining freshwater fauna are the freshwater mussels (Unionida) considered to be one of the most threatened group of animals in the world. Freshwater mussels comprehend a diverse group with around 900 species worldwide, around 40% of all known species are listed under some category of threat, either near threatened, threatened or extinct (Lopes-Lima et al., 2018). Despite some recent advances (Miyahira, 2017; Pereira et al., 2014), this problem is further exacerbated by the lack of knowledge on the diversity of some regions like Africa, South America and Asia (Bogan, 1993; 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).

These propagation and culture methods of freshwater mussels (order Unionida) are being developed rapidly in rearing facilities worldwide, mainly because of economic (Li et al., 2018) and conservation (Lopes-Lima et al., 2018) concerns.

Most of the conservation efforts have focused on habitat restoration and population increase through 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 (Kat, 1984). Based on their morphology two 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 et al., 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 fertilization occurs. The eggs develop inside the female and when they are fully riped, 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 using 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, 1983).

The complexity of the life cycle makes freshwater mussels especially vulnerable to environmental pressures, these pressure however are manifold and are heavily dependent on the region, although common factors have been identified. For example Lopes-Lima et al. (2017) identified several threats for the European mussel fauna including habitat degradation, invasive species and loss of fish host. Furthermore, these factors don't act in isolation and the presence of one of them often is related with the others, for example habitat degradation due to pollution or infrastructure building can also affect the host fish of freshwater mussels reducing their populations or limiting their distribution.

Recent assessments of freshwater mussel's conservation priorities (Aldridge et al., 2023; Ferreira-Rodríguez et al., 2019a; Modesto et al., 2018) agree that one of the main factors that should be included in research and conservation efforts is the host-parasite interaction. For example Aldridge et al. (2023) stressed the need to increase knowledge not only on host-compatibility but also on the effects that fish extinctions, translocations and introductions can have in the coevolutionary relationships of mussels and their hosts. These relationships are known to have high intraspecific variability with mussel populations having different levels of host affinities even inside the same basin (Douda et al., 2014; Taeubert et al., 2010) making mussels extremely vulnerable to changes in their host communities. This means that not only invasion by non-native species can have a detrimental effect on freshwater mussel populations (Donrovich et al., 2017) but also cryptic intraspecific invasions (the introduction and spread of new lineages of host inside the original host range, Morais & Reichard, 2018) can affect them.

Coextinction and Affiliate species

Affiliate species, defined as those intrinsically reliant on another for their survival or reproduction, such as parasites, commensals, or mutualists (Colwell et al., 2012), face a potentially overlooked threat: coextinction. While this phenomenon, whereby an affiliate species disappears upon the decline or extinction of its host or partner, seems intuitively logical, its true magnitude remained underappreciated until Stork and Lyal (Stork & Lyal, 1993) proposed that parasite extinctions might even equal or surpass those of their hosts.

Quantifying coextinction frequency proved challenging. Koh et al. (2004) were the first to tackle this through modelling, revealing that two key factors govern coextinction rates: host specificity (the degree to which the affiliate relies on a single host) and the host's extinction rate. Studies like Dunn et al. (Dunn et al., 2009) further emphasized the potential impact, predicting over 50 parasite extinctions following the loss of just five North American carnivores.

However, a perplexing discrepancy arises between theoretical predictions and empirical observations. While models project coextinction to be the most prevalent form of extinction (Dunn et al., 2009), real-world evidence presents a contrasting picture, with documented coextinction events appearing relatively rarely.

Part of the challenge lies in defining important aspects of the interspecies relationship, like specificity, a notoriously elusive term (Colwell et al., 2012; Dunn et al., 2009). Often inferred from observed infections, it might not fully capture the inherent adaptability of parasites. Part of the problem might be caused by the current focus on finding and average specificity instead of focusing on the variation.

Further complicating matters, existing conservation assessments often overlook smaller species, particularly insects (Dunn, 2005). For instance, when looking at pythophagous insects Thacker et al (2006) identified 24 aphid or scale species solely restricted to threatened tree species, indicating their potential coendangerment but highlighting their absence from the IUCN Red List.

Beyond its direct consequences, coextinction can trigger cascading extinctions within ecological networks. Diamond et al. (1989) documented such a cascade on Barro Colorado Island, where top predator extinction led to increased mesopredator populations and subsequent extinctions of ground-nesting birds. Moreover, the local extinction of army ants on the same island resulted in the coextinction of a dependent bird species and potentially its host-specific lice (Harper, 1989).

The ubiquity of interdependent species complexes and the inherent complexity of ecological networks (May, 2009) creates a unfortunate dichotomy, it is likely that coextinction are much more common than we anticipate however they are inherently difficult to study and therefore coextinction risks have only been studied for a few groups.

For freshwater mussels, studies have focused on the potential host limitations of specialized freshwater mussels following changes in fish host communities (Strayer, 2008), a phenomenon that its thought to have caused the decline of several species of freshwater mussels in North America and Europe (Bauer, 1988; A. E. Bogan, 1993; Lopes-Lima et al., 2017).

Other studies have given insights on how the dynamics of coextinction could extend beyond the interplay between host specificity and host extinction rate. For example, Douda (2013) found that even changes in the local scale, by the mixing of biotas that were not in contact before, can affect the host availability of generalist species of freshwater mussels, a group

that was thought to be less affected by coextinction. The emphasis on the specificity to local populations and strains have also been raised by other researchers studying host specialist freshwater mussels, finding that different genetic strains of the same host species show different suitability depending on the origin population of the freshwater mussels (Taeubert et al., 2010).

Study species

The papers presented here as part of the thesis revolve around three species, two native and one invasive: *Anodonta anatina*, *Margaritifera margaritifera* and *Sinanodonta woodiana*.

A. anatina was chosen as study species for *in vitro*, because is a great model species for the study of larvae biology, it has long brooding season and its metamorphosis time is short. Additionally it has been used in the past in *in vivo* rearing studies as a model for host generalist species (Douda, 2015). On top of that, previous literature presented an *in vitro* protocol for this species or the closely related *A. cygnea* (Lima et al., 2006; Taskinen et al., 2011). On the same note, this species was also used for the development of the image analysis technique for studying bivalve behaviour because of the ease of capture and care as well as the existence of precedents in the biomonitoring literature that used *A. anatina* with the valvometry technique (Hartmann et al., 2016). This species was until recently widely distributed in Europe, with populations being present from Spain to Russia. Despite the decline the species continues to be common and can still be found in most basins in the continent.

M. margaritifera was the focus of the second *in vitro* experiment (centred on enhancing larvae survival in *in vitro* media). Additionally, the species was also used alongside the floating drop-off particle collector (FDPC) to test intrapopulation variation in host affinity. 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 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 has completely disappeared from many

countries and most of the remaining populations show no sign of reproduction with absence of young individuals (Bauer, 1988; Černá et al., 2018; Geist, 2010; Gum et al., 2011; Österling et al., 2010). The causes for this decline are not fully understood yet but it seems that habitat change and destruction are the main driving forces 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 as well. Also, relatively large populations have been reported from Northern Spain and Portugal (Geist, 2010).

Conservation plans for this species haven't 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). These methods use artificial infection of host fish that are then 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 kept and fed in a highly controlled environment until they are released into suitable environments (Buddensiek, 1995; Preston et al., 2007).

The approach described above has had mixed results. 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 has the additional drawback 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 through this method.

S. woodiana was used in the study of the long term effects of *in vitro* culture, the reason behind this choice was the rapid growth that the species can achieve, reaching sexual maturity within two years, (Chen et al., 2015), additionally *S. woodiana* is a generalist and its hosts in Czech Republic are known (Donrovich et al., 2017; Douda et al., 2012). The

species is originally from East-Asia, having a widespread distribution there. In recent years the range of *S. woodiana* has expanded and has become invasive in Europe and Asia with some reports also in United States (Beran, 2008; A. Bogan, 2011; Dobler et al., 2022; Labecka & Czarnoleski, 2021), genetic evidence suggest that the species entered Europe thanks to the Asian carp trade coming to eastern Europe, although subsequent invasions in other places have likely occurred as well (Konečný et al., 2018).

In vitro culture of freshwater mussels

Due to the parasitic nature of the larvae, the culture of freshwater mussels can be problematic (Patterson et al., 2018) and complications are further increased in situations where the fish host species are rare, not suitable for culture in captivity or unknown.

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 (Isom & Hudson, 1982, 1984), and it has expanded to a variety of applications (Kovitvadhi et al., 2006; P. Lima et al., 2012a). Initially, culture media were highly complex (Uthaiwan et al., 2002a) and results were difficult to replicate. This changed with the development of simpler and more efficient methods based on the use of commercial media mixed with fish plasma (Uthaiwan et al., 2001a). Currently, the medium is typically composed of commercial cell culture medium (e.g., M199 and DMEM) in combination with antibiotics and antifungal mixtures to prevent infections. The medium is normally complemented with various 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-Staszczek et al., 2018; Kern, 2017; Kovitvadhi and Kovitvadhi, 2013; Lima et al., 2012; Lima et al., 2006; Lima and 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* protocols is a time and labour-intensive process of testing of a variety of culture technique parameters. The number of factors that affect the success of a culture are manifold and depend on the intrinsic characteristics of the species cultured as well as the formulation of the media. Some factors that have been recognized to affect the success of *in vitro* culture are the source of plasma source and amino acid content (Uthaiwan et al., 2002; Uthaiwan et al., 2003), the temperature during

incubation (Kovitvadhi et al., 2012; Supannapong et al., 2008), the culture atmosphere composition (Roberts & Barnhart, 1999), the density of larvae and medium exchange regime (Kovitvadhi et al., 2006; Owen et al., 2010; Ryan et al., 2022; Uthaiwan et al., 2002a), control of contamination and maturity of glochidia (Kovitvadhi et al., 2006, 2012). Also, because propagation programs work with rare and endangered species, the number of available glochidia for protocol testing is often limited.

The standard in vitro culture

In what follows, to clarify the experiments and results, I will make a brief description of what I call the “standard” *in vitro* culture workflow. Most *in vitro* protocols follow some variation of this protocol with the main changes relating to the composition of the media and the regime of culture care.

As for media composition, the most common approach is to use commercial cell culture media with the most popular choices being the Medium 199 (M199) or Dulbecco’s Modified Eagle’s Medium (DMEM). The use of this media was established by Keller & Zam (1990) as a way to simplify *in vitro* culture that, until that moment, relied on labour intensive, manual preparation of a modified Eagle’s media proposed by Isom & Hudson (1982).

The media is complemented with a source of protein, usually some kind of serum or plasma, some authors insist that the use of fish plasma is beneficial (Kovitvadhi et al., 2012; P. Lima et al., 2006a; Uthaiwan et al., 2002a; Wen et al., 2018a) although this means that the plasma has to be extracted in the lab since commercial fish plasma is hard to acquire. Other authors suggest that commercial protein sources like bovine, rabbit or horse serum have comparable or even better results with lower cost and ease of use (Keller & Zam, 1990). Regardless of the sources of protein is has become a standard to add it in a 1/3 or 33 % proportion to the media, a value that is attributed to Isom & Hudson (1982).

The final two components of the media are a mixture of antibiotics and antimycotics and the addition of a lipid source in a small quantity, normally fish oil (P. Lima et al., 2012a). Bacterial and fungal infections in the culture dishes are one the main problems for this type of culture (Owen et al., 2010; Ryan et al., 2022). Most experiments use one of two antibiotics combinations, either Gentamycin sulphate, Carbenicillin, and Rifampicin or Penicillin,

Neomycin and Streptomycin. The first combination was more common in the first studies while the latter is more recent. Finally, a fungicide is also added to the culture media. The most common choice for fungicide is amphotericin B, although as far as I am aware, no strong justification exists for this preference. Furthermore, Owen et al. (2010) reported that amphotericin B in the concentrations traditionally used was toxic for the glochidia and affected negatively the development. This was also the case with other fungicides evaluated by the author. Because of this, some alternative ways of controlling culture infections have been proposed, these include the constant changing of culture media, the removal of death glochidia and the increase in the volume of the culture in each dish. How well do these measures work is still debated with some authors suggesting that increasing culture manipulation (by constant exchanges and removal of death glochidia) increases rather than decreases the risk of infection (Ryan et al., 2022)

After the media has been prepared, glochidia are added to each dish and placed into a CO₂ incubator chamber. The CO₂ atmosphere is used to maintain a stable pH on the culture media (Keller & Zam, 1990; Roberts & Barnhart, 1999), some protocols have also used media that doesn't require a CO₂ incubator like L15 (Wen et al., 2018a) but this is much less common. I will refer to the period from the addition of glochidia to the media until the observation of the first metamorphosis as "incubation phase". During this phase, the dishes are checked periodically to assess glochidia survival in the media, glochidia that adapt well to the media remain closed, while death glochidia open completely. Following the survival rate in the early incubation phase of the *in vitro* culture of *M. margaritifera* was one of the main goals in one of our experiments.

The time until the first observation of metamorphosis depends on the species, for some species it can be from 6 to 10 days (*A. anatina* and *S. woodiana*) while for others it can take several weeks (e.g. *Unio crassus* see Gąsienica-Staszeczek et al., 2018) or even months (e.g. *M. margaritifera* see Taskinen et al., 2011). It is a common practice to dilute the media with sterilized water at the end of the incubation phase, however several different approaches are used in this "dilution phase" and no single standardized form exists, furthermore the effects of these variations are not well studied (Gąsienica-Staszeczek et al., 2018a). In some cases, there is no report of any kind of dilution at the end of the incubation phase before counting (Lima et al., 2006; Uthaiwan et al., 2001), while in others the juveniles were immediately transferred to water after the metamorphosis was completed and then

counted (Kern, 2017; Roberts & Barnhart, 1999). A final approach consist on some form of stepped dilution, replacing the media for water over the course of several days (Gašienica-Staszeczek et al., 2018a; R. C. Lima & Avelar, 2010; Taskinen et al., 2011).

I want to make a final note on the method for assessing metamorphosis, the main theme of one of our papers. The main way for measuring metamorphosis consists on performing counts of active juveniles, if a juvenile has successfully metamorphosed it will start a stage of pedal feeding in which it uses its foot both to move around and to transport food particles to the mouth using cilia (Wächtler et al., 2001b). However, the start of pedal feeding can vary between individuals even within the same group, this can significantly affect the proportion of metamorphosed juveniles observed depending on the time in which the metamorphosis assessment is performed. Most studies use a single quantification of metamorphosis success immediately after dilution (Kovitvadhi et al., 2012; Roberts & Barnhart, 1999) while other propose an initial assessment soon after the end of the incubation phase and then a posterior assessment a few days later to allow all the juveniles to activate (Kern, 2017). This different approaches can strongly influence the observed metamorphosis specially when other sources of variation are added like when juveniles are exposed to different dilution approaches at the end of the experiment (Gašienica-Staszeczek et al., 2018a).

Effect of in vitro techniques on the long run

The earlier discussion was focused mostly on technical details of the *in vitro* culture of freshwater mussels, now I turn into the application of the method in the conservation of freshwater mussels. *In vitro* culture has been used with great success in conservation programs in North America. However not much was known about these *in vitro* produced juveniles will do in the wild in terms of survival and reproduction, a topic that is related to the broader theme of captive breeding techniques. The next part of the thesis dealt with this.

Captive breeding techniques are an integral part of many conservation programs (Ebenhard, 1995; Seddon et al., 2007), but they may produce individuals poorly adapted to the wild, and releasing those individuals can compromise the fitness of wild populations (Araki et al., 2007; Davis et al., 2020; Snyder et al., 1996). Furthermore, multiple captive breeding methods exist for some organisms, but the fitness of individuals produced by these methods

may differ (Davis et al., 2020). Finally, captive breeding and its effects are well-studied for some groups, including mammals (Pinder & Barkham, 1978), fishes (Attard et al., 2016; Fraser, 2008), and amphibians (Griffiths & Pavajeau, 2008). Consequences of captive breeding are less well-studied for invertebrates (Witzenberger & Hochkirch, 2011), but some evidence suggests that these groups also may experience negative outcomes related to this technique (Davis et al., 2020; Lewis & Thomas, 2001; Pearce-Kelly et al., 1998).

Despite the advantages of in vitro methods, they represent a major modification of the natural process of metamorphosis. Furthermore, in vitro methods require antibiotic treatment to control bacterial growth in the media and a CO₂-enriched atmosphere to regulate pH (Roberts & Barnhart, 1999). Consequently, in vitro methods have the potential to produce mussels that differ substantially from those produced in the wild or in vivo. In 96-hour laboratory toxicity tests, in vitro juvenile mussels were slightly more sensitive to several toxicants than in vivo juveniles, but the magnitude of the differences was within normal toxicity test variation, suggesting that juveniles produced by both methods responded similarly (Popp et al., 2018).

Freshwater mussels' behaviour

An important part of studying the reproductive biology of mussels is also monitoring their behaviour. Mussels, in their interaction with fish, employ a wide range of physiological and behavioural adaptations. These parameters initially began to be studied within ecotoxicology but are also making their way into other areas, including species conservation.

In the case of bivalves, studies have concentrated on the study of valve movement, also known as “valvometry” tests. Some of the first studies extracted the information about valve gaping behaviour by the use of monofilaments attached to the edge of the valve and connected to a displacement transducer (Byrne et al., 1990), muscle transducers (Doherty et al., 1987), strain gauges attached to analogous chart recorders (Djangmah et al., 1979; Higgins, 1980; Shumway & Cucci, 1987), and later, by the use of electromagnetic induction-based systems (Jenner et al., 1989). These methods were complicated to implement and required the bivalve to be fixed by one valve to a stable surface while measuring the free valve's movement, limiting the studies to highly artificial laboratory settings. This changed with the development of light-weight sensors, based on either impedance (Tran et al., 2003) or Hall effect (Nagai et al., 2006) that do not require the animals to be fixed. The small and

less invasive sensors have been used to allow the experimental individual to remain in free, more natural settings in the laboratory or directly by installing them into bivalves in the field. More recently, the coupling of the sensors with electronic units has extended the system's capabilities by permitting remote data acquisition and transfer to the internet (Schmitt et al., 2011; Sow et al., 2011). The valvometry approach has been used successfully to evaluate the response of bivalves to trace elements (Curtis et al., 2000; Doherty et al., 1987; Kádár et al., 2001; Tran et al., 2003), parasites (Chambon et al., 2007), toxic algal blooms (Shumway & Cucci, 1987), dispersed crude oil (Redmond et al., 2017), as well as a water quality biomonitoring system (Borcherding, 2006; W.-Y. Chen et al., 2012; Sluyts et al., 1996).

In toxicological studies based on valvometry, valve behaviour is used as a proxy of the changes in filtering induced by a stressor (Hopkins, 1933; Riisgård, 2001; Robson et al., 2009). The main idea is that pollutants disrupt the bivalve filtering behaviour, causing changes in the observed gaping activity (Riisgård, 2001). However, despite its importance, valvometry-based assessments ignore the mussel soft tissue behaviour involved in the filtration process. In particular, the inhalant and exhalant siphons are highly mobile and sensitive structures, furnished with receptors capable of detecting changes in water quality (Davenport, 1984) and can be protruded or retracted to control filtration rate (Riisgård, 2001). In fact, some authors consider siphon activity the best proxy for the bivalve pumping rate (Foster-Smith, 1976).

There is an increasing interest in the use of image analysis or computer vision in the field of ecology (Weinstein, 2018). The image analysis approach has the advantage of being highly efficient and, low on cost, allowing to gather high volumes of data with relative ease, especially for experimental laboratory systems (Pennekamp & Schtickzelle, 2013). Moreover, the breadth of open-source libraries and software available has made the adoption of image analysis techniques easier for ecologists worldwide. However, despite some previous studies, the image analysis approach in bivalve behaviour has been limited mainly to manual analysis of images taken with low frequency (Carroll & Clements, 2019; Maire et al., 2007; Rodland et al., 2006) or used as an alternative to magnetic sensors to extract valvometry data (Dzierżyńska-Białończyk et al., 2019) with almost no attention paid to the potential of this method to study the movement of soft parts.

Image analysis in bivalve behaviour

The use of images to track bivalve behaviour is not new. Sequences of digital images have been used to measure mussel activity in several studies in the past because it provides a useful understanding of their long-term patterns of undisturbed feeding behaviour in situ, especially in situations when there is no filtering but the valves are open (Newell et al., 2001). For example, Thorin et al. (2001) used video records of the siphon area to test the effect of water-current direction and velocity on the siphon aperture of *Mya arenaria*. A similar approach was used by Newell et al. (2001) and Riisgård et al. (2003) to study the effect of algal concentrations and velocity in both valve opening and siphon aperture. Finally, Sénéchal et al. (2008) used a custom-made program to measure valve aperture and siphon area using high definition pictures to study the effects of mussel density in culture socks of *Mytilus edulis*. In these studies, the measurement of the siphon area was done manually in a set of frames taken at specified intervals that varied from a few seconds to even 1 minute, limiting the amount of data available for analysis. An attempt to the automatic extraction of information was done by Rodland et al. (2006). To achieve this, the authors tracked the changes in brightness (defined as changes in values of grey-scale pixels) of a region of interest to extract points of change of valve and siphon movement on sequences of images obtained every 20 seconds. The authors also report that because the method is based on brightness level it requires either constant lighting or user calibrations specific to each video. Using this approach, the authors found consistent periodical patterns in three different species of marine bivalves living under different environmental settings, suggesting that valve and siphon activity are physiologically rather than environmentally controlled. Other studies (Maire et al., 2007) attempted to adapt software used for behavioural studies in other groups of organisms to bivalve behaviour. In this case, the authors were able to automatically extract the area of the exhalant siphon and the aperture of the valves in *Mytilus galloprovincialis* using image sequences obtained at 15 seconds intervals. More recently, Dzierżyńska-Białończyk et al. (2019) developed a method for automatic processing of videos with a larger number of frames per second (1 fps) applying plasticine colour marks in *Dreissena polymorpha* shells and tracking gaping behaviour using Noldus Ethovision (Noldus et al., 2001). However, the automatic method developed by the authors was aimed primarily to study movements of the valves with siphon movements being studied by visual inspection. Interestingly, this visual assessment of the videos revealed that no siphon extension was observed when valve closure was superior to 80%, furthermore this period of incomplete valve closure and inactive siphons were more common in the individuals

subjected to alarm cues, this suggests that even stress events might not induce a complete closure of the valves and that siphon activity might be a more sensitive way of studying bivalve behavioural responses to environmental stressors. As mentioned previously, most methods discussed previously depended on pictures taken at intervals that vary from 15 seconds to 1 minute, limiting the capacity of detecting complex or rapid responses. Indeed, the selection of the sampling frequency is critical because it can have strong effects on the observed behaviour, moreover, the use of long intervals influence the number and duration of events detected (Robson et al., 2009; Ropert-Coudert & Wilson, 2004).

Objectives and hypotheses

The aim of this dissertation was to provide new insights into freshwater mussel ecology, with special focus on the larval stage development of freshwater mussels of the Unionida order, which could have a clear (and practical) impact on species conservation. During my doctoral studies, I engaged in a broad array of topics within this area. Here, I summarize the most significant objectives, ordered by their expected contribution, and linked with five completed publications included into the thesis.

The main goals of the thesis are related to the *in-vitro* breeding techniques of freshwater bivalve's parasitic larvae (glochidia) and can be also used to study host-parasite relationships and their dynamics in a changing freshwater environment. We produced a series of experiments that relate to each one of these objectives. Because of the complexity of some the experiments planned, some relevant results remain unpublished although all the necessary experiments have already been performed. The itemized list of all the experiments performed, and the goal to which they are related to is presented below.

A total of 5 papers are already published, and one more is in the final stages of preparation, but will not be included to this thesis.

Objective 1:

The primary objective was to contribute to the understanding of the larval requirements of mussels and provide new knowledge on the development of in-vitro techniques for their cultivation. This goal is not intended to move unionid conservation more towards the replacement of natural reproduction with artificial breeding methods. Rather, it is seen as an important last-resort technique for species where no other option exists. At the same time, these techniques can provide us with crucial information about the biological essence of the relationships between mussels and fish.

This was the goal where I had more publications in, it was based on three experiments that are already published (Douda et al., 2021; Escobar-Calderón et al., 2019; F. Escobar-Calderón & Douda, 2019) and described in great detail in the following pages.

Scientific hypotheses tested.

The hypothesis of the studies included in the thesis are as follows:

Study 1: *In vitro* *A. anatina* and the effect of time of counting in observed metamorphosis:

H1: The observed metamorphosis rate in *in vitro* cultures is different when using a single endpoint than when using multiple endpoints.

Study 2: Early survival *In vitro* of *M. margaritifera*

H2: The addition of different lipid sources, serum types and the presence of taurine enhances the early survival of *M. margaritifera* glochidia in *in vitro* media culture.

Study 3: Effects of *in vitro* in the long-term survival and reproduction of *S. woodiana*

H3: Survival, infection capacity of hosts and growth of *S. woodiana* individuals produced under *in vitro* conditions do not significantly differ from the ones of individuals produced using fish hosts.

Objective 2:

Objective 2 is somewhat thematically distinct from the other studies included in this dissertation, but I decided to incorporate it because it deals with the development of a new promising technique that can be utilized in studies of mussel ecology, including research on reproductive behavior. It involves high-frequency monitoring of siphon activity through video recording.

For this goal I was involved (and led data processing and analysis) in an experiment whose results are presented here and is already published (Escobar-Calderón et al., 2022). The experiment is related with the development of an automatic method for extracting bivalve behaviour from video records, something that was done manually until recently.

Scientific hypotheses tested.

Study 4: Image analysis of mussel behaviour and comparison with valvometry

H4: Image analysis will be more sensible and specific than valvometry in detecting instances of mussel behavioural reaction.

H5: Reaction time of mussels to the nitrate-nitrogen pollution will be smaller when using image analysis data that can detect the contraction of the soft tissue in opposition to valvometry that only can detect valve movement.

Objective 3:

The third objective was to further contribute to the development of methods for research focused on the larval stage of mussels. Specifically, this goal addresses the issue of lacking research infrastructure in remote areas and offers a solution through the use of inexpensive floating pontoons. This is a new technique suitable for researching the success of glochidia metamorphosis and other related questions.

For this experiment, I was involved in the implementation of all parts of the experiment, both in the laboratory and in the field. The output (Douda et al. 2020) has already been published and is included here in full text.

Scientific hypothesis tested.

Study 5: Device for in situ and low-cost monitoring of particles falling from freshwater animals: from microplastics to parasites.

H6: Using a simple flow-through device, it's possible to make a breakthrough advance in field-based fish–glochidia interaction studies by addressing questions previously tractable only under laboratory conditions (collection of glochidia and juveniles)

Published works

Objective 1:

Early responses of in vitro cultured fish-growing glochidia: The effects of taurine, lipids and sera on *Margaritifera margaritifera*

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Abstract

In vitro culture has great potential for the propagation of freshwater mussels in both commercial and conservation aquaculture. The use of in vitro techniques precludes the need for host fish, thus decreasing costs and increasing efficiency. However, protocols are still lacking for many species that grow substantially during the parasitic stage. In this study, we tested the effects of taurine addition, serum type and source of lipids on the survival rate and increase in length of *Margaritifera margaritifera* larvae during the initial stage of culture (first 11 days of exposure to media). Our results show that taurine has no significant effect on the early survival rate of glochidia; however, the possible importance of this amino acid in subsequent stages is discussed. The use of an emulsified lipid mixture instead of traditional fish oil showed significantly higher rates of survival. Finally, the addition of serum showed variable effects, with both horse serum and newborn calf serum having higher survival in trials using mussel populations from different sources. These findings can contribute to the invention or improvement of in vitro protocols for species growing during infection and at the same time show the potential that early survival assessments could have for the development of in vitro methods in species with long parasitic stages.

KEYWORDS

freshwater mussels, growth during metamorphosis, in vitro, *Margaritifera margaritifera*, taurine

1 | INTRODUCTION

The farming of freshwater bivalves (order Unionida) is practised worldwide for economic and conservation reasons (Patterson et al., 2018; Sicuro, 2015). Additionally, the culture of freshwater mussels has great potential in the near future, with studies showing the prospective use of freshwater mussels in biomaterial applications (Liao, Mutvei, Hammarström, Wurtz, & Li, 2002) and the long-term monitoring of freshwater pollution (Anderson, Bell, Hodson, Marsalek, & Watt, 2004; Koistinen, Herve, Ruokojärvi, Koponen, & Vartiainen, 2010; Rodrigues et al., 2012). Therefore, culture methods of freshwater mussels are being developed rapidly in rearing facilities worldwide.

However, the culture of freshwater mussels can be problematic during the larval stage (Patterson et al., 2018), as most unionid

species possess parasitic larvae (glochidia or lasidia). Female mussels keep the larvae in modified gills called marsupia, and once the larvae are released from the female, they need to find and attach to a freshwater fish to complete their development. Once encapsulated in the host tissue, larvae undergo a process of metamorphosis that can take several days or even months in some species. After metamorphosis is completed, the individual drops off its host to start its life as a free-living organism (Kat, 1984). This process makes the host fish stage in culture programs particularly problematic, especially in situations where the fish host species are rare, not suitable for culture in captivity or unknown.

An alternative for artificial rearing of larvae is in vitro culture. In the in vitro culture of freshwater mussels, the glochidia are placed in artificial medium, thus precluding the need for a host fish. The medium is typically composed of commercial cell culture media (e.g.

M199 and DMEM) in combination with antibiotics and antifungal mixtures to prevent infections. Additionally, different kinds of serums (natural host or nonhost fish species, horse, and calf) are normally used to complement the media. To date, culture protocols have been developed for more than 50 species (Gąsienica-Staszczek, Zając, Zając, & Olejniczak, 2018; Kern, 2017; Lima et al., 2012; Lima & Avelar, 2010; Ma et al., 2018; Wen et al., 2018) with varying success, and the methods for the majority of species have not yet been established. This is especially true for species that grow substantially during development. In the majority of species, larvae metamorphose without any significant change in their size; however, some species are known to grow substantially during the parasitic stage. Typically, these species have small glochidia and long developmental times. In vitro culture of these fish-growing larvae has proven to be very difficult, and only a few successful protocols have been developed for them.

One of the freshwater mussel species with the most intensive growth during the parasitic stage is *Margaritifera margaritifera* (Barnhart, Haag, & Roston, 2008). Similar to several other freshwater mussel species, *M. margaritifera* has been the main focus of conservation efforts for many years due to its unique position as an indicator, flagship, umbrella and keystone species (Geist, 2010). The species has 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). Conservation programs for this species have commonly focused on habitat restoration and rearing under controlled conditions (Gum et al., 2011) using artificially infected host fish (Buddensiek, 1995; Gum et al., 2011). However, this method has had limited success (Simon et al., 2015) and has the additional disadvantage of being expensive and demanding in terms of work and resources. Additionally, there is a concern about the genetic viability of the mussel populations recovered through this method. Novel culture methods of *M. margaritifera* can represent a significant contribution to conservation programs of this endangered species and the culture methods of unionids in general.

A successful in vitro method for *M. margaritifera* has not yet been developed, partially because this species grows substantially during the parasitic stage, which could be related to high nutritional demands. Additionally, *M. margaritifera* possesses a great degree of specificity, infecting only certain species of salmonids (Taeubert & Geist, 2017). The experiment of Taskinen, Saarinen-Valta, Vällilä, Mänpää, and Valovirta (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; the glochidia were then extracted and transferred to the medium. This suggests that some factor specific to the host is crucial to trigger metamorphosis of *M. margaritifera* under in vitro conditions and that the current medium composition may lack critical components.

Recently, Wen et al. (2018) achieved in vitro metamorphosis of *Potamilus alatus*, a species that also grows during the parasitic stage,

by the use of a modified in vitro protocol and suggested that taurine could be an important amino acid necessary for metamorphosis. Taurine is an organic solute and a nonprotein amino acid (Welborn & Manahan, 1995). Because it can accumulate to high intracellular concentrations without perturbing macromolecules, it serves an important function in osmoregulation (Yancey, Clark, Hand, Bowlus, & Somero, 1982). Taurine functions as an osmolyte used in osmotic regulation in many animal groups (Yancey et al., 1982). In molluscs, taurine is found in high concentrations (Allen, 1961) and seems to play an important role during the development and growth of marine species larvae. For example, Welborn and Manahan (1995) studied the taurine concentrations during metamorphosis from veliger to juvenile in the gastropod *Haliotis rufescens* and the bivalve *Crassostrea gigas*. Larvae from *H. rufescens* do not grow substantially during development, and the authors found that taurine concentrations remained stable during the entire experiment. In contrast, the size of veliger from *C. gigas* increased by 360% during metamorphosis, and the authors observed a large increase in taurine, with the final concentration being 43 times the initial concentration. Rapid growth imposes new osmotic challenges to developing mollusc larvae, and the increase in taurine concentration can be a response to the osmotic stress caused as a consequence of larger body size in a hyperosmotic marine environment. Glochidia of freshwater bivalves, which grow substantially during metamorphosis, are likely to be subjected to the same kind of osmotic pressures and consequent requirements for taurine; this and the fact that many of the most commonly used media (M199, DMEM or L15) do not contain taurine would explain the current lack in success of in vitro protocols for species growing during metamorphosis.

The goal of this study was to test the hypothesis that the addition of taurine will influence the early reaction of glochidia of *M. margaritifera* to the culture medium. Furthermore, the addition of taurine was tested alongside the use of different types of serums (horse and newborn calf), as the kind of serum used has been identified as a crucial aspect of in vitro protocols (Lima et al., 2012; Uthaiwan, Pakkong, Noparatnaraporn, Vilarinho, & Machado, 2002). Finally, we also tested the effects of different sources of lipids, namely fish liver oil and emulsified lipid mixtures, because lipid uptake plays a key role during the development of glochidia, and it has been observed that, for some species, this effect is directly linked to post-larval vitality and growth (Douda, 2015).

2 | METHODS

2.1 | Glochidia source and extraction

Glochidia of *M. margaritifera* were obtained from female mussels sampled in the Vltava River basin (Czech Republic). Two trials were performed using glochidia sampled at two different sites (Trial 1, Blanice River, 48°55'34"N, 13°58'12"E; Trial 2, Malše River, 48°39'01.5" N 14°28'00.3" E). In the field, different individuals were monitored, and when glochidia release was observed, the individual

was extracted and placed into a small container to stimulate further glochidia release. The clumps of glochidia released were extracted with the help of a pipette and observed under a microscope to assess viability. Then, the glochidia were transferred to 5-L containers with river water. A mixture of glochidia obtained from 35 and 3 female mussels was used in Trials 1 and 2 respectively. After glochidia were extracted, the females were returned to the same spot on the river where they were initially found, and none of the females spent more than 30 min outside the river. The containers with glochidia were then transported immediately to the laboratory. In the laboratory, the glochidia were stored in an incubator at 4°C for one day before the start of in vitro culture. Before use, the larvae were rinsed with sterilized water to disintegrate the clumps. Glochidia used in Trial 1 showed lower initial viability estimated by NaCl reaction (Lefevre & Curtis, 1910; Roberts & Barnhart, 1999), with a high proportion of underdeveloped glochidia (50%), in comparison with Trial 2 (5%). A total of 130 to 250 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.

2.2 | Culture medium composition

A full factorial design was used to test the effects of three factors (serum type, source of lipids and taurine addition), each with two levels. Eight different treatments, with four replicas per treatment, were performed for each trial, resulting in a total of 32 dishes per trial. Two millilitres of M199 medium (Sigma-Aldrich, product code M4530) was added to each dish for all treatments. Then, 1 ml of either horse serum (Sigma-Aldrich, product code H1270) or newborn calf serum (Sigma-Aldrich, product code 12133C) was added to each dish, followed by 50 µl of lipid source, which was added either as fish liver oil (Sigma-Aldrich, product code F8020) or emulsified lipid mixture (ELM) (Sigma-Aldrich, product code L5146). The final factor was the presence or absence of taurine, added in powder form at a concentration of 150 µg per dish (final concentration in dish = 42.86 µg/ml, Sigma-Aldrich, product code T0625). To control infections, 0.5 ml of a stock mixture of antibiotics was added to each dish, and the mixture consisted of a combination of penicillin, streptomycin, neomycin (concentrations in stock solution: penicillin = 5,000 units/ml, streptomycin = 5 mg/ml, neomycin = 10 mg/ml) and antimycotics (concentration in stock solution = 7 µg/ml). The stock solution was prepared using PSN mixture (Sigma-Aldrich, product code P4083) and amphotericin B (Sigma-Aldrich, product code A9528). The preparation of the culture media followed a 4:2:1 proportion (Roberts & Barnhart, 1999) for the medium, serum, and antibiotic mixture respectively. Before the addition of glochidia, all dishes were placed in a CO₂ incubator (NB-203, N-Biotek, Korea) under UV light for one hour. After inoculation with glochidia, the dishes were kept in the CO₂ incubator at 5% CO₂ and a temperature of 18°C. The medium was changed on the fifth day of cultivation. Larvae of *M. margaritifera* are known to survive up to 10 days without a host at comparable temperatures (Jansen, Bauer, & Zahner-Meike, 2011). Because

of this and since the goal was to test the survival of the larvae after exposure, the experiment was concluded on day 11 in both trials to ensure that the observed survival of glochidia was due to a positive reaction to the medium composition.

2.3 | Quantification of reaction to the medium and length measurements

The closure of glochidia was used as a proxy for the successful start of the metamorphosis process because when the larvae are exposed to host tissue, they will close tightly and remain closed (Jansen et al., 2011). In contrast, glochidia induced to close on nonsuitable surfaces have been observed to open after a few minutes (Wood, 1974). Additionally, because of the elasticity of the hinge, dead glochidia will tend to open completely after the relaxation of the adductor muscle (Roberts & Barnhart, 1999). Based on this criterion, glochidia were classified as 'developing' if they were closed and 'nondeveloping' if they were open. Three quantifications were performed to assess the amount of developing and nondeveloping glochidia after exposure to medium on days two, five and eleven of cultivation. Survival quantification on day two has been used in previous in vitro experiments (Uthaiwan et al., 2002). The quantifications on days five and eleven were added to track the survival of the glochidia until the maximum known survival of free larvae was reached (Jansen et al., 2011). In each quantification, between 50 and 60 random glochidia were counted per dish, and the rate of developing individuals was calculated as the number of closed glochidia over the total number of counted glochidia. In dishes where a large portion of glochidia was lost during cultivation, the quantifications were performed taking into account all the glochidia observed.

To complement the survival data, we performed length measurements of the glochidia before and after the experiment. A subsample of 44 recently extracted glochidia was taken in Trial 2 before the start of the in vitro experiment as a record of initial length. Subsamples of between 8 and 25 juveniles were taken at the end of the experiment from each treatment and preserved in ethanol (70%). A photograph of each subsample was taken using a microscope (magnification 40x), and the measurements were performed using ImageJ software (Schneider, Rasband, & Eliceiri, 2012). The length measured corresponded to the longest axis perpendicular to the hinge line in the individual.

2.4 | Statistical analysis

Because of the strong difference in the viability of the larvae in both populations, the following analysis was performed independently for each trial. Accordingly, the results presented are also grouped by each trial. Generalized linear mixed models (GLMM) using a binomial distribution with the logit link function were made in R version 3.6.1 (R Core Team, 2019) using the package lme4 (Bates, Maechler, Bolker, & Walker, 2015). The response

variable was the percentage of developing 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 all analyses. 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 a random factor. In both models, the maximum likelihood was used for the estimation of the parameters.

To compare the difference between the initial and final length and to assess whether the average change in the length was different from zero, one-sample *t* tests were performed for each level and each factor. The *p*-values obtained from this multiple testing were corrected using a sequential Bonferroni procedure (Holm, 1979).

3 | RESULTS

3.1 | Proportion of developing glochidia after exposure

The average proportion of developing glochidia after exposure to the medium (mean \pm SD) during the three quantifications was similar in both trials (Trial 1 = 32.38 \pm 17.39%, Trial 2 = 33.59 \pm 27.67%). In Trial 1, there was a significant effect of serum type on all quantifications, but the effect was not completely coherent. In the first quantification, horse serum seemed to perform worse than the newborn calf serum (newborn calf = 41.16 \pm 16.77%, horse = 21.88 \pm 13.88%, GLMM: $z = -4.511, p < .001$); however, in the second and third quantifications, this effect was reversed, and the proportion of developing glochidia after exposure to newborn calf serum was significantly lower than the proportion for horse serum (second quantification, newborn calf = 26.46 \pm 10.36%, horse = 41.38 \pm 14.27%, GLMM: $z = 3.443, p < .001$; third quantification, newborn calf = 21.41 \pm 9.42%, horse = 42.02 \pm 22.12%, GLMM: $z = 4.064, p < .001$). The ELM had a statistically significant positive effect in the first (ELM = 40.53 \pm 18.70%, fish oil = 22.50 \pm 12.20%, GLMM: $z = -4.158, p < .001$) and third quantification (ELM = 39.49 \pm 24.12%, fish oil = 23.94 \pm 9.69%, GLMM: $z = -3.235, p = .0012$). In the second quantification, the average rate of developing glochidia for ELM was slightly higher compared with the fish oil, but this difference was not significant (ELM = 39.38 \pm 12.03%, fish oil = 33.46 \pm 16.87%, GLMM: $z = -0.321, p = .749$). The addition of taurine did not show any significant effect in any of the three quantifications of Trial 1 (Figure 1).

In Trial 2, there was also a significant effect of serum type on all three quantifications (Figure 1); however, contrary to Trial 1, the proportions of developing glochidia were consistently higher for the newborn calf serum, with the largest differences observed in the first (newborn calf = 75.86 \pm 9.49%, horse = 12.37 \pm 14.16%,

GLMM: $z = -808.5, p < .001$) and second quantifications (newborn calf = 45.31 \pm 14.29%, horse = 21.13 \pm 22.92%, GLMM: $z = -5.689, p < .001$). In the third quantification, the difference was less pronounced but still significant (Newborn calf = 23.92 \pm 14.94%, horse = 16.51 \pm 22.16%, $z = -2.644, p = .008$). Regarding the lipid source, ELM showed a significantly higher rate of developing glochidia in the first (ELM = 49.94 \pm 33.09%, fish oil = 38.29 \pm 35.67%, GLMM: $z = -228, p < .001$) and second (ELM = 46.27 \pm 18.10%, fish oil = 25.20 \pm 20.64%, GLMM: $z = -5.057, p < .001$) quantifications. In the third quantification, the fish oil had a slightly higher rate of developing glochidia, but this difference was not statistically significant (ELM = 18.68 \pm 17.26%, fish oil = 21.21 \pm 22.33, GLMM: $z = 0.292, p = .77$).

3.2 | Final length

The analysis showed that none of the factors had a significant effect on the final length of the glochidia. The final length (mean \pm SD) of glochidia exposed to taurine was 72.88 \pm 6.10 μm , while for glochidia not exposed to taurine, it was 71.70 \pm 3.95 μm (LMM: $\chi^2 = 1.50, p = .23$). Regarding the serum type, the final average length was 72.08 \pm 4.07 μm for the newborn calf serum and 72.24 \pm 5.89 μm for the horse serum (LMM: $\chi^2 = 0.01, p = .91$). Last, the final average length for the ELM group was 71.72 \pm 4.13 μm , while the fish oil group showed an average length of 72.65 \pm 5.63 μm (LMM: $\chi^2 = 0.99, p = .32$).

The final length of the glochidia was smaller than the average initial length (average decrease in length = $-1.95 \pm 4.88 \mu\text{m}$), and the decrease was significantly distinct from zero (one-sample *t* test: $t = -4.33, df = 116, p < .001$). For the taurine, the difference in length was $-2.39 \pm 3.95 \mu\text{m}$ for the individuals without taurine addition, with the decrease being significantly different from zero (one-sample *t* test: $t = -5.18, df = 72, p < .001$), and $-1.23 \pm 6.10 \mu\text{m}$ for the individuals exposed to taurine, with the decrease not being significantly different from zero (one-sample *t* test: $t = -1.33, df = 43, p = .19$). The newborn calf serum ($-2.02 \pm 4.07 \mu\text{m}$) and the horse serum ($-1.86 \pm 5.89 \mu\text{m}$) also showed a slight decrease, but this decrease was only significantly different from zero for the former (horse, one-sample *t* test, $t = -2.18, df = 47, p = .10$; newborn calf, one-sample *t* test, $t = -4.13, df = 68, p < .001$). Finally, the ELM ($-2.39 \pm 4.13 \mu\text{m}$) and fish oil ($-1.45 \pm 5.63 \mu\text{m}$) also showed a decrease, but this decrease was only significantly different from zero for the ELM (ELM, one-sample *t* test, $t = -4.59, df = 62, p < .001$; fish oil, $t = -1.90, df = 53, p = .13$).

4 | DISCUSSION

Contrary to our hypothesis, the addition of taurine did not have a significant positive effect on the survival or growth of glochidia of *M. margaritifera* after exposure to culture medium. The effect of taurine

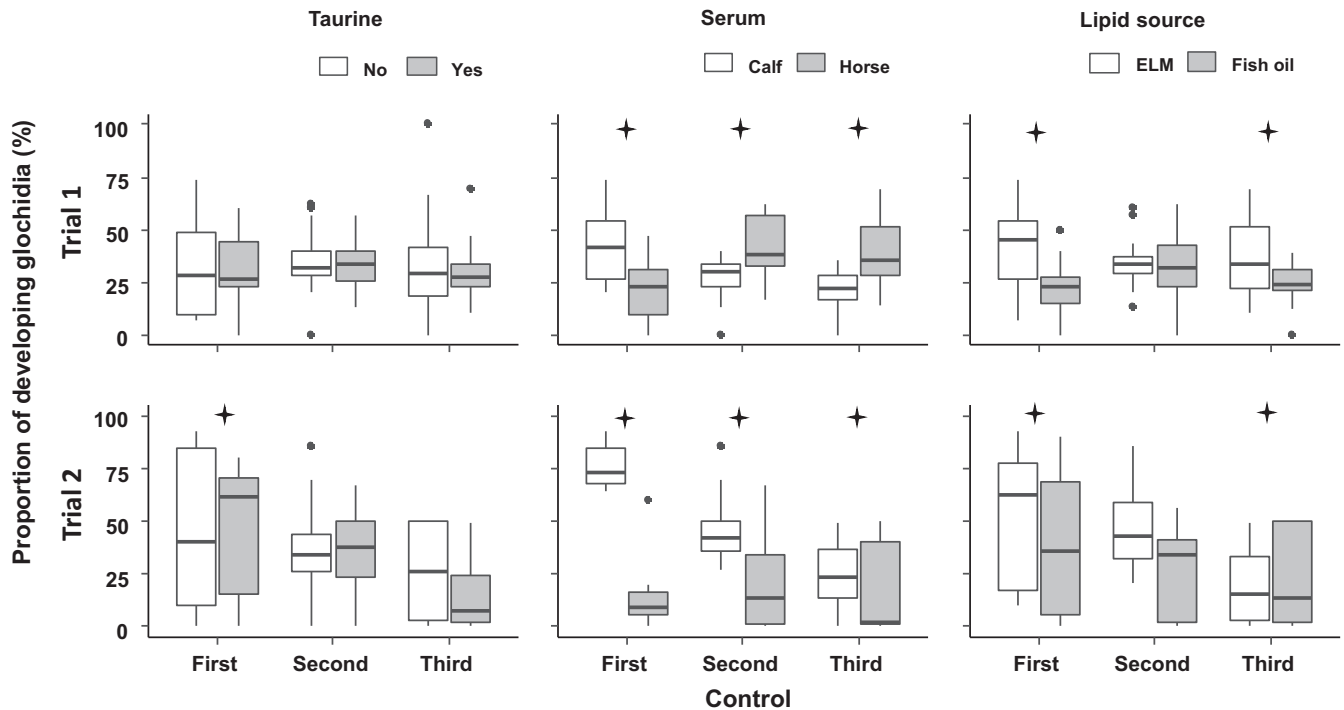


FIGURE 1 Effects of taurine addition, serum type and lipid source on the rate of developing glochidia from *M. margaritifera* after exposure to the medium during three different quantifications. The stars denote a statistically significant difference ($p < .01$)

was only significant in the first quantification of Trial 1. Serum type showed a significant but inconsistent influence among and within trials. An interesting finding of this study is the positive effect of the ELM, in comparison with the fish oil; in four of the six quantifications performed during both trials, the proportion of developing glochidia was higher when using ELM.

In the majority of unionids, glochidia do not grow during metamorphosis; however, for some species, considerable increases up to seven times the initial length have been observed (Barnhart et al., 2008). Glochidia growth during the parasitic stage typically occurs in species with small glochidia sizes and extended periods of metamorphosis (Barnhart et al., 2008; Howard & Barry, 1922), where current in vitro culture methods are not able to achieve metamorphosis of such species. The reasons for this are currently unknown, but growth during metamorphosis has been linked to higher nutritional demands of the glochidia (Kern, 2017; Wen et al., 2018). The use of stable isotopes has added some support to this claim. For example, Denic, Tæubert, and Geist (2015) found that the intake of stable isotopes from the host of glochidia of *M. margaritifera* was higher (change in $\delta^{15}\text{N} = 8\%$) in comparison with species not growing during metamorphosis (change in $\delta^{15}\text{N} = 2\%$). For *M. margaritifera*, as mentioned before, the only partial success under in vitro culture was obtained by Taskinen et al. (2011) by using a combination of in vivo and in vitro techniques. In this case, metamorphosis was observed only when the larvae were first allowed to grow in the host fish for a long period of time (133 days). After this period, the gill arches were extracted and placed in culture medium, and metamorphosis occurred after 14 days. In contrast, glochidia placed directly under

in vitro conditions, without any time spent on the host fishes, died after 55 days of incubation. These findings seem to support the idea of higher nutritional demands for species that grow substantially during metamorphosis.

Regarding the addition of taurine, our observations do not document any significant effect of this factor in the early reaction of larvae from *M. margaritifera*. This is in contrast with recent observations of Wen et al. (2018), who achieved the metamorphosis of *Potamilus alatus*, a highly host-specialist species that also grows substantially during metamorphosis. The authors suggested that taurine could be an important amino acid necessary for metamorphosis. This claim is based on the observation of a higher growth rate when using fish plasma with a higher content of taurine. In our case, however, neither the percentage of developing glochidia nor the total length of the glochidia at the end of the experiment was affected by taurine. Despite this, we believe that the effect of taurine cannot be completely dismissed since it can be more important in later stages and influence mainly the growth of glochidia. The importance of taurine to freshwater mussels can be dependent on the osmotic conditions to which glochidia are exposed during infection. Freshwater environments are predominately hypoosmotic, and adult freshwater molluscs possess very low concentrations of taurine in their tissues when compared with their marine relatives (Huxtable, 1992; Simpson, Allen, & Awapara, 1959). Some experiments have even found that unionid bivalves no longer display the capacity to use changes in taurine concentration as a way to cope with osmotic stress caused by increased salinity, a characteristic still present in other freshwater bivalves such as Corbiculidae (Matsushima, Katayama, & Yamada, 1987). Nonetheless, it is possible that because

of their parasitic nature, glochidia are subjected to hyperosmotic environments during infection in the form of the host plasma and fluids. This point should be the focus of further research to elucidate the osmotic conditions of encysted glochidia and the influence on the glochidia osmotic regulation of both fish blood and exterior freshwater.

With 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 developing glochidia, with the glochidia exposed to ELM showing higher closing rates. Lipids play a key role during the development of larvae from marine bivalves (Bayne, 1976), and in freshwater mussels, it has been found that the growth rate of the juvenile after metamorphosis is congruent with the lipid reserves acquired during the development of glochidia (Douda, 2015), suggesting a similarly important role of lipids in the development of freshwater species. However, some of the first formulations of in vitro culture media for freshwater mussels did not include the addition of a lipid source (Dimock & Wright, 1993; Fisher & Dimock, 2006; Isom & Hudson, 1982), resulting in decreased lipid reserves in the metamorphosed juveniles (Fisher & Dimock, 2006; Tankersley, 2000). Because of this, the addition of 50 µl of fish oil (normally cod liver or menhaden) per dish is now common in most studies (Lima et al., 2012). The problem, however, is the insolubility of traditional fish oil in the medium, which can lead to complications, especially for longer culture periods. Previous experiments using cod liver oil as a lipid source have shown that the oil tends to congregate in droplets (Douda & Escobar-Calderon, unpublished data), which 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 can form a layer covering the latter, which can affect the gas exchange between the CO₂-enriched atmosphere and the medium and in turn affect both the available oxygen and the pH of the medium. Other studies have suggested additional approaches to solve this problem. Owen (2009) used a mixture of serum, menhaden oil and rifampicin to obtain positive results (Kern, 2017; Lima et al., 2012). According to our results, the addition of an emulsified lipid source can also help to increase lipid availability during the in vitro development of glochidia. Nonetheless, our data only provide information about the early reaction of glochidia of *M. margaritifera* to ELM. More research, probably using species with a shorter period of metamorphosis and established in vitro protocols, is needed to address the effects of this additive on the final metamorphosis rate. Additionally, we found an inconsistent behaviour between the two trials regarding the effects of the two serums tested. In two of the three quantifications performed on Trial 1, the horse serum showed a statistically significant increase in the number of developing glochidia after exposure. In contrast, in Trial 2, the newborn calf serum gave consistently higher rates of developing glochidia in all three quantifications. Newborn calf serum was used by Taskinen et al. (2011). The authors suggested that other serums should be

used and recommend the use of fish plasma. The addition of fish plasma has been shown to be beneficial with many mussel species even when using plasma from a nonhost (Lima et al., 2012; Uthaiwan et al., 2002); however, some stenoparasitic species may require specific plasma from their hosts to develop (Lima et al., 2012). 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 of this species is very long and many changes in the medium are required to avoid infection. The amount of serum needed in such cases can be high, and obtaining sufficient amounts of serum can be very difficult. Finally, this work demonstrates that the use of early survival assessment of larvae under in vitro culture conditions can simplify the study of the reaction of glochidia to different medium compositions without having to achieve complete metamorphosis. Previous works have also addressed the survival of glochidia after exposure to the medium; however, most of these works use species with short metamorphosis times. For example, Uthaiwan, Pakkong, Noparatnaraporn, Vilarinho, and Machado (2003) studied the survival and metamorphosis rate of *Hyriopsis myersiana* under in vitro conditions, and the authors found differences in the survival of glochidia related to the kind of serum used during cultivation. Interestingly, these results were consistent with the metamorphosis success rate. A protocol similar to the one presented here was used by Uthaiwan et al. (2002); using *H. myersiana*, the authors tested different modifications of in vitro culture conditions to test both the effect of fish plasma and the density of glochidia during incubation by quantifying the survival after 2–3 days of exposure to the medium. The authors found that the metamorphosis success rate, obtained as the proportion of surviving glochidia transformed, was 100% in all groups, which suggests that early survival of glochidia after exposure to the medium can be an accurate indicator of the final metamorphosis rate. In our experiment, we performed additional assessments of early survival (on days 5 and 11) and found a variable reaction of glochidia to the medium after exposure. Sixteen of the 32 dishes in Trial 1 and 14 of the 36 dishes in Trial 2 showed a higher percentage of developing glochidia in the second quantification performed on day 5 than in the first quantification performed on day 2. Such a result can be the effect of delayed closing in some individuals after exposure to the medium. Because of this result and recent findings demonstrating the variability of in vitro success indicators at different time points (Escobar-Calderón & Douda, 2019), we suggest including more quantifications when assessing the survival of glochidia under in vitro conditions, especially for mussels with long parasitic stages that can take more time to react to the culture media. This study shows the importance of the medium composition of in vitro media for the early survival of glochidia that grow during metamorphosis. Despite the fact that we were unable to find a significant effect of taurine on the glochidia reaction to media in the first 11 days of exposure, we believe that the evidence supporting the importance of taurine

for the development of freshwater larvae is well established. It can be expected that taurine becomes more important in later stages of development, especially as an osmolyte when glochidia begin to grow. However, more research focused on the osmotic conditions of larvae during infection is needed to clarify this. Additionally, we found that the use of ELM can improve glochidia survival in the medium, possibly due to higher lipid availability, but the effect on the long-term development and on juvenile lipid reserves after metamorphosis has yet to be confirmed. Regarding serum, further experiments should focus on identifying the best serums to use under *in vitro* settings, which might be dependent on the species; the use of host fish serum in combination with other alternatives that have yielded consistently good results in other experiments (e.g. rabbit serum) can enhance the survival of glochidia in the medium and keep the cost of culture acceptable. Finally, due to the long metamorphosis period of many species that grow during infection, the assessment of early survival rates can be of great importance. Testing different media compositions to find the most suitable conditions that enhance survival can be supplemented by short-term experiments focusing on the reaction to the medium during the first days of incubation.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest concerning this article.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Short communication

Variable performance of metamorphosis success indicators in an *in vitro* culture of freshwater mussel glochidia

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ABSTRACT

The *in vitro* cultivation of parasitic larvae (glochidia) is becoming an important constituent of culture methods of freshwater mussels (order Unionida) worldwide. Despite rapid methodological progress, published methods for the vast majority of freshwater mussel species do not exist. In the present study, we tested the performance of current approaches to evaluate *in vitro* culture success quantification. A full factorial design was used to culture *Anodonta anatina* glochidia *in vitro* in separate media (M199 and DMEM), antibiotic concentrations and post-incubation treatments (dilution methods). The glochidia metamorphosis success (the proportion of successfully transformed glochidia) in each treatment was quantified at two different time points, and the postlarval growth (8 days) of the juveniles was also estimated. The results showed a significant effect of medium type on metamorphosis success at the first time point (day 13) (M199 = $60.98 \pm 20.20\%$, DMEM = $36.10 \pm 23.61\%$; generalized mixed effect models, GLMMs, $p < .001$) but not at the second (day 21). Similarly, the dilution approach showed a significant variation in metamorphosis success only at the first quantification time point (water and medium = $33.97 \pm 24.16\%$, water only = $63.11 \pm 16.03\%$; GLMM $p < .001$). In contrast, the postlarval growth quantification showed an increased growth increment in the juveniles in the M199 medium (M199: $65.90 \pm 24.20 \mu\text{m}$, DMEM = $52.90 \pm 19.84 \mu\text{m}$; linear mixed effect model $p < .001$). The study shows that with current methods, the metamorphosis success assessment of *in vitro* culture protocols can be influenced by the effects of assessment time and dilution approach, two aspects that are not well standardized in the current methodology. Greater emphasis should be placed on the optimization of indicators with direct links to the final criteria, such as postlarval growth or physiology-based parameters. The optimization of metamorphosis success and viability indicators can be an important step towards the increased efficacy of current juvenile production by *in vitro* techniques and the development of new protocols.

1. Introduction

Propagation and culture methods of freshwater mussels (order Unionida) are being developed rapidly in rearing facilities worldwide (Patterson et al., 2018), mainly because of economic (Li et al., 2018) and conservation (Lopes-Lima et al., 2018) reasons. The larval stage of freshwater mussels is one of the most problematic life-history phases during culture (Patterson et al., 2018). In the majority of unionid species, the larvae (glochidia or lasidia) released from female mussels are obligatory parasites and must attach to freshwater fish to complete their development. The encapsulated larvae undergo a process of metamorphosis on the fish, and then the individual drops off its host to start its life as a free-living organism (Kat, 1984). The host fish stage in a culture program can be particularly problematic in situations where the fish host species are rare, not suitable for culture in captivity or unknown.

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 (1984, 1982) and it has expanded recently to a variety of applications (Kovitvadi et al., 2006; Lima et al., 2012). Initially, culture media were highly complex (Uthaiwan et al., 2002) and results were difficult to replicate. This changed with the development of simpler and more efficient methods based on the use of commercial media mixed with fish plasma (Uthaiwan et al., 2001). Currently, the medium is typically composed of commercial cell culture medium (e.g. M199 and DMEM) 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 > 50 species (Gąsienica-

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Staszeczek et al., 2018; Kern, 2017; Kovitvadhi and Kovitvadhi, 2013; Lima et al., 2012; Lima et al., 2006; Lima and 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* protocols is a time and labor-intensive process of testing of a variety of culture technique parameters. Plasma source and amino acid content (Uthaiwan et al., 2002, 2003), temperature during incubation (Kovitvadhi and Kovitvadhi, 2013; Supannapong et al., 2008), culture atmosphere composition (Roberts and Barnhart, 1999), density of larvae and medium exchange regime (Kovitvadhi et al., 2006; Owen et al., 2010; Uthaiwan et al., 2002), control of contamination and maturity of glochidia (Kovitvadhi et al., 2006; Kovitvadhi and Kovitvadhi, 2013) were identified by the previous studies as the most influential factors. Also, because propagation programs mainly work 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.

A critical issue is the selection of proper indicators to compare the performance of particular protocols. Most studies use a single quantification of the metamorphosis success rate of glochidia immediately after *in vitro* culturing, such as the proportion of living juveniles over the total number of individuals (Kovitvadhi and Kovitvadhi, 2012; Roberts and Barnhart, 1999). The observation of pedal feeding is typically used as the main indicator of metamorphosis success. In contrast, recent studies have indicated that survival measured immediately after incubation can be different from the results measured after several days in water (Kern, 2017). This variation can be further increased when juveniles are exposed to different dilution approaches at the end of the experiment (Gąsienica-Staszeczek et al., 2018). Furthermore, it is unclear whether the simultaneous early quantification across treatments can provide consistent information on metamorphosis success. If not, there is a risk of making biased decisions regarding the selection of the most effective protocols, especially protocols that aim to compare the effects of different factors on medium performance (medium composition, serum type, the role of additions, etc.).

In this report, we investigated the effects of different approaches for the evaluation of *in vitro* culture success quantification. We tested whether quantification at different time points and using different dilution procedures at the end of *in vitro* culture can unequally affect the observed level of activity in the juveniles. Our goal was to determine whether these factors might compromise the reliability of *in vitro* culture protocol success assessment. Postlarval juvenile growth quantification was used as a complementary method for comparing metamorphosis success.

2. Materials & methods

2.1. Study species

Duck mussel (*Anodonta anatina* Linnaeus, 1758) was used as a test organism because it is a widespread European species (Lopes-Lima et al., 2017) with a previously published protocol for glochidia *in vitro* culture (Taskinen et al., 2011), a long-term brooding season and a relatively short metamorphosis period.

2.2. 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 transferred to an aerated outdoor tank (water volume of 1350 L) at the Czech University of Life Sciences in Prague on 7 October 2017. The gravidity state of the females was 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 demibranch, and then the gills were cut open using sterilized

forceps and scissors, releasing the larvae into a beaker of sterilized water. A subsample of glochidia was examined under a light microscope (40×) by exposing them to NaCl and counting the proportion that closed to assess the glochidia viability. The glochidia that periodically opened and closed their valves were considered suitable for culture (Roberts and Barnhart, 1999; Lima et al., 2006). 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 them 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 the initial length measures.

2.3. Culture medium composition and incubation phase

A full factorial design (see Appendix A, Table A1) 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 with three replicates per treatment (i.e. combination of factors). The media used were DMEM (Sigma Aldrich, product code D5671) and M199 (Sigma Aldrich, product code M4530), each supplemented with two different solutions of antibiotics (A = 100 U/mL penicillin, 100 µg/mL streptomycin, 200 µg/mL neomycin and 5 µg/mL amphotericin B; and B = 25 U/mL penicillin, 25 µg/mL streptomycin, 50 µg/mL neomycin and 1.25 µg/mL amphotericin B; both were prepared using PSN mixture and antimycotic amphotericin B, Sigma Aldrich, product codes P4083 and A9528). The media were supplemented with horse serum (Sigma Aldrich, product code H1270). The preparation followed a 4:2:1 proportion (Roberts and Barnhart, 1999) for the medium, serum, and antibiotics solution, respectively. The media were complemented with 50 µL per dish of cod liver oil (Sigma Aldrich, product code 74380). Six Petri dishes (15 × 60 mm) were used for 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 the glochidia, all the dishes were placed in a CO₂ incubator (NB-203, N-Biotek, Korea) under UV light for one hour. The experiment consisted of two phases (see Appendix A, Fig. A1), 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. The two post metamorphosis treatments differed in both the type of dilution used and the use of CO₂ enriched atmosphere. In the first group, sterilized water was added in proportion 1:1 (5 mL of medium removed and 5 mL of sterilized water added), and dishes were retained in a 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. In both cases, the dishes were kept at the same temperature of 24 °C. The incubation was terminated in all the treatments on day 13 when the first quantification of metamorphosis success (see below) was performed (see Appendix A, Fig. A1).

2.4. Juvenile culture phase

After the first quantification of metamorphosis success (day 13), 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 × 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 a subsample of each treatment of juveniles from each container was taken and fixed in ethanol (70%) to perform the growth measures.

2.5. *In vivo* larval development

A. anatina glochidia from the same population and collection date as those in the *in vitro* experiment were used to infest the host fish *Leuciscus leuciscus* (Linnaeus, 1758), sampled in the Vltava River (48°55'18" N, 13°48'25" E). This fish host species can be considered a primary host of *A. anatina* with a metamorphosis success rate of 39.8% recorded in a previous study (Douda et al., 2013). The fish were infested for 15 min in a bath of 4698 ± 2092 (mean \pm SD) glochidia per liter obtained from 10 female mussels. Then, the fish with encapsulated glochidia were kept in a flow-through cage (90 \times 60 \times 45 cm) in a natural habitat and temperature regime (1–10 °C) before movement to the laboratory aquarium system (10 °C) to collect the juveniles. Each fish individual was placed in a separate tank (33 \times 19 \times 25 cm) with continuous aeration (through airstone), daily water exchange (~20%) and feeding (live dipteran larvae). Filtration of tank water was performed daily to collect and count juvenile mussels that drop-off from the fish. Juveniles sampled from 9 fish individuals (mean \pm SD weight 66.7 ± 7.0 g and body length 164.4 ± 6.7 mm) from three peak drop-off days (92–101 days after infection) were used for the evaluation of postlarval growth using the same methods as those for the *in vitro* produced juveniles.

2.6. Metamorphosis success quantification

The individuals observed during each evaluation time point (days 13 and 21) were divided into two groups: 1) 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 2) nonviable juveniles comprised of closed glochidia with tightly closed valves and no sign of movement or dead individuals with open valves and disintegrating tissues. The metamorphosis success rate was calculated as the sum of the viable juveniles over the total count. The same criteria were used for the quantification performed with the juveniles originating from both the *in vitro* and *in vivo* culture methods.

2.7. Growth increment measures

A random subsample of 30 recently extracted glochidia was taken from the pooled experimental glochidia before the start of the *in vitro* experiment and preserved in ethanol (70%) as a record of the initial length. Another randomly selected subsample of 30 juveniles was 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 40 \times), and the measurements were performed using ImageJ software (Schneider et al., 2012). The length measured corresponded to the longest axis in a direction parallel to the hinge line of the juvenile. The growth increment was calculated as the length of the individual minus the average value of the initial glochidia length.

2.8. 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 were made in R 3.5.0 (R Core Team, 2018) 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 success 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 postlarval 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. In all cases, models with and without interactions were calculated.

For the comparison of the potential differences in postlarval 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 a Levene's test was used for the continuous variable (growth increment).

3. Results

3.1. Metamorphosis success

Because interactions were not significant after performing model selection (See Appendix B) the results presented here correspond to the models without interactions. The observed metamorphosis success rate was lower in the first ($48.54 \pm 24.97\%$) than in the second ($64.49 \pm 12.47\%$) control, and the difference was statistically significant (GLMM: $Z = 18.39, p < .001$). There was a significant effect of the postincubation treatment on the metamorphosis success in the first control (water and medium = $33.97 \pm 24.16\%$, water only = $63.11 \pm 16.03\%$, GLMM: $Z = 4.85, p < .001$), but this effect disappeared in the second control (water and medium = $63.05 \pm 15.72\%$, water only = $65.92 \pm 8.57\%$) with no significant effect detected (GLMM: $Z = 0.55, p = .579$) (Fig. 1C). 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 ($60.98 \pm 20.20\%$) than for the DMEM medium ($36.10 \pm 23.61\%$) (GLMM: $Z = 4.09, p < .001$), but this difference was not significant in the second control (M199 = $63.39 \pm 14.67\%$, DMEM = $65.59 \pm 10.35\%$, GLMM: $Z = -0.50, p = .620$) (Fig. 1A). The antibiotic concentration showed no effect at any of the two time points (1st control: $Z = -1.37, p = .171$, 2nd control: $Z = 0.796, p = .426$) (Fig. 1B).

3.2. Growth increment

The mean \pm SD initial and final lengths of the glochidia/juveniles in the *in vitro* culture experiment were $358.23 \pm 18.88 \mu\text{m}$ and $417.61 \pm 23.01 \mu\text{m}$, respectively, resulting in an average growth increment of 14.22% ($59.37 \pm 23.01 \mu\text{m}$) over the whole experiment. The comparison of the individual factors showed that medium type was the only factor with a significant effect on length increment (M199 = $65.90 \pm 24.20 \mu\text{m}$, DMEM = $52.90 \pm 19.84 \mu\text{m}$; LME: $t = 3.42, p < .001$) (Fig. 1A). The treatment after metamorphosis (LME: $t = 1.03, p = .3$) and the concentration of antibiotics (LME: $t = 0.15, p = .89$) showed no significant differences. The mortality of juveniles during juvenile culture phase for all treatments varied between 0.0% and 8.1%.

3.3. Comparison of *in vitro* and *in vivo* postlarval growth increments

The mean \pm SD final length in the juveniles metamorphosed on host fish was $417.03 \pm 29.53 \mu\text{m}$ with an average growth increment of

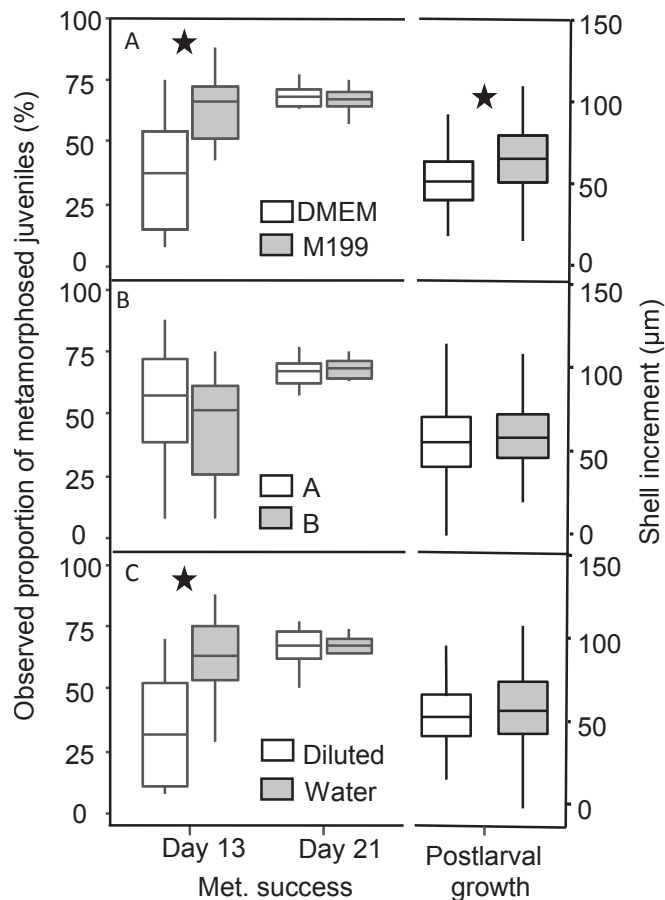


Fig. 1. The effects of medium type (A), antibiotic concentration (B) and dilution approach (C) on the *in vitro* metamorphosis success rate assessment of glochidia from *A. anatina* determined at two different time points (days 13 and 21). The postlarval growth increment at particular treatments is displayed on the right side of each plot. Median, interquartile range and min/max are displayed. The stars denote significant differences between levels ($p < .001$).

$52.79 \pm 29.50 \mu\text{m}$ and there was no significant difference in growth increment between juveniles originating from *in vivo* and *in vitro* culture techniques (LME: $t = -1.00$, $p = .32$). The mortality of juveniles during juvenile culture phase of *in vivo* varied between 0.0% and 13.0%.

4. Discussion

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 and 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 and Barnhart, 1999; Lima et al., 2006; Taskinen et al., 2011), while in others, a postponed quantification taken at 24 to 48 h in water is 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 h 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 et al., 2001), while in others, the juveniles were immediately transferred to water after metamorphosis was completed and then counted (Roberts and Barnhart, 1999; Fox, 2014; Kern, 2017). On the other hand the use of a unique partial dilution near the end of incubation has been also reported (Areekijserree et al., 2006; Kovitvadhi et al., 2006; Kovitvadhi and Kovitvadhi, 2013; Supannapong et al., 2008; Uthaiwan et al., 2002) with varying proportion of water added to the medium, ranging from 10% (Supannapong et al., 2008) to 40% (Kovitvadhi et al., 2006; Kovitvadhi and Kovitvadhi, 2013). Finally, Some studies use gradual dilution (Lima and 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-Staszczek 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 a 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 and Dimock, 2006; Tankersley, 2000; Wächtler et al., 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 is critical for the conservation of freshwater mussel species, producing juveniles for research, population reinforcements or commercial purposes.

Appendix A

Table A1

Full factorial design of the assessment of metamorphosis experiment, concentration in added solutions A = 100 U/mL penicillin, 100 µg/mL streptomycin, 200 µg/mL neomycin and 5 µg/mL amphotericin B; and B = 25 U/mL penicillin, 25 µg/mL streptomycin, 50 µg/mL neomycin and 1.25 µg/mL amphotericin B. Dilution (postmetamorphosis) treatment 1 = diluted medium in CO₂ 5% atmosphere, 2 = only water at atmospheric CO₂.

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

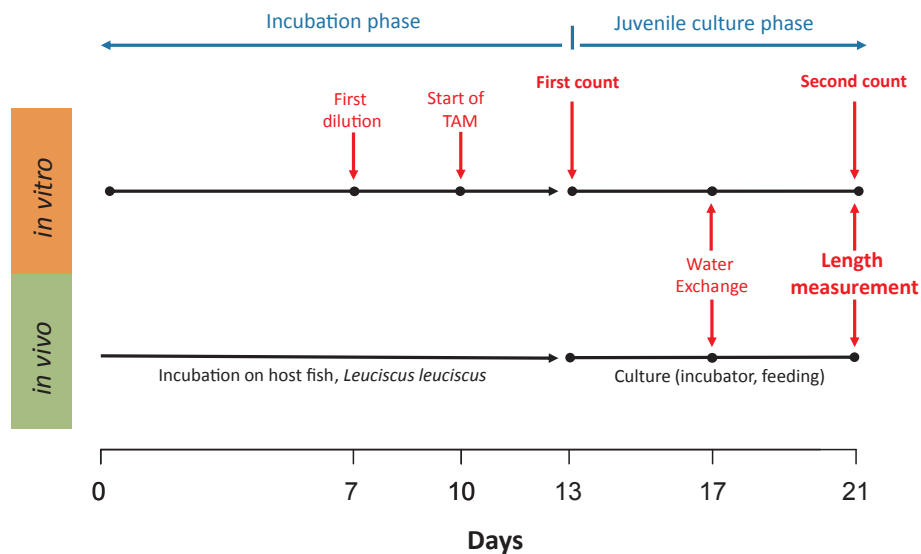


Fig. A1. Explanatory diagram of the experiment. During *in vitro* incubation phase, three factors were tested: medium type, antibiotic concentration, and treatment after metamorphosis (TAM), see text for details on the levels of each factor. The two counts were performed on day 13 and 21. The water exchange and the length measurements on days 17 and 21 were performed for both *in vitro* and *in vivo* juveniles.

Appendix B

Table B1

GLMM's Coefficient estimate, standard error of the estimate and *p*-value for the effect of medium type, antibiotics concentration and treatment after metamorphosis on the observed metamorphosis rate for both counts without interactions.

Variable	Estimate	Std. error	z -value	p
First count (day 13)				
Medium(M199)	1.25	0.31	4.09	4.36E-5
Antb(B)	-0.42	0.31	-1.37	0.17
TAM(Water)	1.48	0.31	4.84	1.27E-6

(continued on next page)

Table B1 (continued)

Variable	Estimate	Std. error	z -value	p
Second count (day 21)				
Medium(M199)	-0.11	0.21	-0.50	0.62
Antb(B)	0.17	0.21	0.80	0.43
TAM(Water)	0.12	0.21	0.56	0.58

Table B2

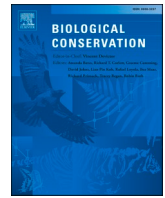
GLMM's Coefficient estimate, standard error of the estimate and p-value for the effect of medium type, antibiotics concentration and treatment after metamorphosis on the observed metamorphosis rate for both counts including interactions.

Variable or Interaction	Estimate	Std. error	z -value	p
First count (day 13)				
Medium(M199)	1.19	0.56	2.14	0.03
Antb(B)	-0.80	0.56	-1.44	0.15
TAM(Water)	1.54	0.55	2.80	0.01
Medium(M199):Antb(B)	0.96	0.78	1.23	0.22
TAM(Water):Antb(B)	0.74	0.78	0.94	0.35
TAM(Water):Medium(M199)	0.11	0.78	0.14	0.89
TAM(Water):Medium(M199):Antb(B)	-1.89	1.10	-1.71	0.09
Second count (day 21)				
Medium(M199)	-0.83	0.34	-2.41	0.02
Antb(B)	0.13	0.34	0.39	0.70
TAM(Water)	0.22	0.34	0.64	0.52
Medium(M199):Antb(B)	0.97	0.48	2.01	0.04
TAM(Water):Antb(B)	-0.66	0.48	-1.37	0.17
TAM(Water):Medium(M199)	0.69	0.48	1.44	0.15
TAM(Water):Medium(M199):Antb(B)	-0.46	0.68	-0.67	0.50

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Effects of in vitro metamorphosis on survival, growth, and reproductive success of freshwater mussels

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ABSTRACT

Captive breeding is an effective conservation strategy, but it has risks, especially when a life history stage of an organism is bypassed. Freshwater mussels (Unionida) are critically imperiled, and their larvae are parasites on fishes. Traditional mussel captive breeding involves artificially infesting fishes with larvae (in vivo), but increasingly used in vitro methods allow larval metamorphosis in culture media, bypassing the parasitic stage. We provide the first comparisons of mussel performance between in vitro and in vivo methods in the wild and throughout the mussel life cycle using two mussel species. In six streams, survival and growth did not differ between in vitro- and in vivo-produced *Lampsilis cardium*. Metamorphosis of *Sinanodonta woodiana* differed sharply between two in vitro protocols (methods 1 and 2), but metamorphosis for method 2 was twice as high as in vivo. Survival and growth after eight days was lower for in vitro method 1 than method 2 and in vivo, showing that suboptimal in vitro protocols can have lingering effects on juvenile performance. However, survival and growth did not differ among methods by the end of the first and second growing seasons. Most importantly, in vitro-produced mussels survived, grew to maturity, and produced F₂ juveniles naturally on fishes, all at rates that did not differ from in vivo-produced mussels. We detected no strong side effects of bypassing the mussel host-fish stage, but this study illustrates the importance of assessing consequences of captive breeding methods for any organism in a variety of environmental and life history contexts.

1. Introduction

Captive breeding techniques are an integral part of many conservation programs (Ebenhard, 1995; Seddon et al., 2007), but they may produce individuals poorly adapted to the wild, and releasing those individuals can compromise the fitness of wild populations (Araki et al., 2007; Davis et al., 2020; Snyder et al., 1996). Furthermore, multiple captive breeding methods exist for some organisms, but the fitness of

individuals produced by these methods may differ (Davis et al., 2020). Captive breeding and its effects are well-studied for some groups, including mammals (Pinder and Barkham, 1978), fishes (Attard et al., 2016; Fraser, 2008), and amphibians (Griffiths and Pavajeau, 2008). Consequences of captive breeding are less well-studied for invertebrates (Witzenberger and Hochkirch, 2011), but some evidence suggests that these groups also may experience negative outcomes related to this technique (Davis et al., 2020; Lewis and Thomas, 2001; Pearce-Kelly

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et al., 1998).

Freshwater mussels (order Unionida) are among the most endangered animal groups worldwide (Ferreira-Rodríguez et al., 2019; Lydeard et al., 2004). Many populations are extirpated, and many surviving populations are small, fragmented, and show little natural recruitment (Haag, 2012; Lopes-Lima et al., 2018). Consequently, release of captive-bred individuals is a frequently employed method for restoring or augmenting wild populations (McMurray and Roe, 2017; Patterson et al., 2018).

Mussels are unique among bivalves in having larvae (glochidia) that require a brief period as parasites on fishes, during which glochidia encapsulate in the host tissue and metamorphose into juveniles (Modesto et al., 2018). Mussels have been captive-bred for over 100 years by artificially infesting fishes with glochidia and harvesting metamorphosed juveniles, and this remains the most common method for contemporary conservation programs (Patterson et al., 2018). Mussels thus represent a unique group of parasites that have long been the subject of conservation efforts, which is only now being increasingly addressed in other parasitic animals (Carlson et al., 2020). The in vivo mussel breeding methods (using host fish) have the advantage of closely mimicking the natural process, but juvenile yields can be low and fish hosts are unknown for many species. In vitro techniques offer an alternative in which glochidia metamorphose in a nutrient-rich culture medium instead of on fishes. In vitro techniques offer the benefits of potentially higher juvenile yields, eliminating the need to maintain fishes in captivity alongside greater cost-effectiveness, and they may be the only option for captive breeding when host fishes are unknown (Gąsienica-Staszczek et al., 2018; Lima et al., 2012; Patterson et al., 2018; Taskinen et al., 2011; Uthaiwan et al., 2002).

Despite the advantages of in vitro methods, they represent a major modification of the natural process of metamorphosis. Furthermore, in vitro methods require antibiotic treatment to control bacterial growth in the media and a CO₂-enriched atmosphere to regulate pH (Roberts and Barnhart, 1999). Consequently, in vitro methods have the potential to produce mussels that differ substantially from those produced in the wild or in vivo. In 96-hour laboratory toxicity tests, in vitro juvenile mussels were slightly more sensitive to several toxicants than in vivo juveniles, but the magnitude of the differences was within normal toxicity test variation, suggesting that juveniles produced by both methods responded similarly (Popp et al., 2018). However, no studies have evaluated differences between in vitro and in vivo mussel performance in the wild or throughout the mussel life cycle. In particular, the ability of in vitro-metamorphosed mussels to produce glochidia that can successfully attach, encapsulate, and metamorphose on fishes is not known. In vitro methods are increasingly widely used, and at least 60 species have been produced using these methods (Lima et al., 2012; Patterson et al., 2018; Kovitvadhii and Kovitvadhii, 2012). A better understanding of differences in performance between in vitro- and in vivo-produced mussels is necessary to evaluate whether in vitro production is appropriate for widespread conservation use.

We evaluated differences between in vitro- and in vivo-produced mussels concerning traits that directly affect the success of conservation efforts. First, we compared growth of in vitro and in vivo juvenile *Lampsilis cardium* after rearing to five months of age in a hatchery and subsequent survival and growth in the wild during three-month exposures in six streams. Second, we compared metamorphosis success, survival, and early juvenile growth among *Sinanodonta woodiana* produced by two different in vitro methods and in vivo on two host fish species. Third, we raised *S. woodiana* produced in vitro and in vivo to sexual maturity (18 months) in mesocosms and compared their survival, growth, and reproductive success, including natural recruitment of F₂ juveniles from fishes. Our results provide a comprehensive comparison of in vitro and in vivo mussel performance in a variety of contexts and throughout the mussel life cycle, which will allow a more informed assessment of conservation approaches for these imperiled animals.

2. Materials and methods

2.1. Juvenile survival and growth in the hatchery and in the wild

We produced juvenile *L. cardium* at the Center for Mollusk Conservation, Kentucky Department of Fish and Wildlife Resources, Frankfort, Kentucky, USA. *Lampsilis cardium* is native to all of our USA study streams. We collected four gravid female *L. cardium* from the Licking River, Nicholas County, Kentucky. On November 3, 2014, we extracted glochidia from the gill marsupia by flushing them with a 20 mL syringe filled with sterile water, and we combined glochidia from all four females. We used about half of these glochidia for in vivo production and half for in vitro production. We produced juvenile mussels in vivo by pipetting few hundreds of glochidia onto the gills of anaesthetized host fishes (*Micropterus salmoides*, hatchery-reared). We held the infested fishes in a recirculating aquarium system at 19 to 23 °C, and peak metamorphosis occurred on day 23 post-infestation.

We produced juvenile *L. cardium* in vitro in 15 × 100 mm Petri dishes with 13.0 mL of a culture medium containing 3:1 parts by volume M199 cell culture medium (Sigma Aldrich M4530) and rabbit serum (Sigma Aldrich R4505), respectively. The medium was supplemented with 100 µg/mL each of rifampicin (Sigma Aldrich R7282), carbenicillin (Sigma Aldrich C3416), and gentamicin (Sigma Aldrich G1264); 1 µg/mL antimycotic amphotericin B (Sigma Aldrich A2411); and 10 µL/mL menhaden oil (Sigma Aldrich F8020). After flushing glochidia from marsupia, we cleaned them by rinsing with Eagles' minimum essential medium (Sigma Aldrich M5650), placed them in dishes, and incubated dishes at 1% CO₂ and 24 °C. On day 23 after start of the culture, we added sterile water at 1:4 by volume for 20 min then fully diluted dishes until they were clear.

We reared juveniles produced by both methods separately at 24 to 26 °C in 4-L trays within a recirculating aquaculture system with biological and mechanical filtration. Juveniles were fed a mixture of commercially available marine algae, represented by *Nannochloropsis*, Nanno 3600, TP 1800, and Shellfish Diet 1800 (all from Reed Mariculture, USA), and cultured freshwater algae, *Chlorella sorokiniana*. We reared mussels to about five months of age and measured shell length and mass (blotted wet mass, including shell) of all individuals on May 15, 2015.

We deployed mussels in streams from May 22 to June 10, 2015. We chose six study streams in Kentucky, USA, representing different ecological and conservation contexts. Elkhorn and Russell creeks are warm, well-buffered, productive streams in the Interior Low Plateaus physiographic province (drainage areas at the study sites = 1270 km² and 677 km², respectively; mean summer water temperature = 24.4 °C for both streams). The Green (4471 km², 23.2 °C) and Nolin (961 km², 21.3 °C) rivers are cooler, well-buffered, and moderately productive Interior Low Plateaus streams that are heavily influenced by karst. Horse Lick Creek (159 km², 20.1 °C) and the Rockcastle River (1564 km², 22.9 °C) are cool, moderately well-buffered, and less productive streams in the Appalachian Plateaus physiographic province. The conservation status of the mussel fauna varies widely among the streams from relatively healthy with ≤15% species loss (Elkhorn Creek, Green River) to essentially defaunated and >85% species loss (Horse Lick Creek, Nolin River; Haag et al., 2019).

We deployed mussels in concrete silos with a central holding chamber constructed of PVC pipe and covered with 1-mm mesh screen (Haag et al., 2019). Silos create an upwelling current through the central chamber, which delivers food and oxygen and carries away waste. We placed 16 mussels in each silo and ten silos at each site, five each containing in vitro and in vivo individuals. We retrieved silos from September 1–8, 2015. Exposure time varied among streams from 90 to 102 days. Upon retrieval we recorded the number of live mussels in each silo, returned live mussels to the laboratory on ice, froze them at −18 °C, and measured length and mass of all individuals within three months.

We calculated proportional survival as the number of live individuals

in each silo at the end of the experiment/the number of individuals placed initially in each silo. We expressed growth as instantaneous growth [$d: \ln(\text{final mass in g}/\text{initial mass in g})/\text{deployment period in days}$] based on the mean mass of all live individuals in each silo. We tested for differences in survival and growth between in vitro and in vivo individuals using paired *t*-tests because growth responses are highly dependent on stream conditions (Haag et al., 2019).

2.2. Metamorphosis success and early juvenile performance

We used *S. woodiana* for this and the following experiment because it is easily propagated in captivity and it can reach sexual maturity within 2 years (Chen et al., 2015). *Sinanodonta* (*Anodonta*) *woodiana* is of Southeast Asian origin and is an invasive species in Europe (Konečný et al., 2018). We collected gravid female *S. woodiana* from the Morava River, Czech Republic (48°41'13"N, 16°59'19"E) on May 15, 2018, and moved them to aerated 10 L tanks in a laboratory at the Czech University of Life Sciences Prague, Czech Republic. We used glochidia from six mussels (labeled A to F) to produce juvenile mussels by the methods described subsequently.

2.2.1. In vitro methods

We produced juvenile *S. woodiana* mussels in vitro in 15 × 90 mm Petri dishes with a culture medium containing 4:2:1 parts by volume of M199 cell culture medium (Sigma Aldrich M4530) previously successful for in vitro culture of mussels of the subfamily Anodontinae (Escobar-Calderón and Douda, 2019), horse serum (Sigma Aldrich H1270), and an antibiotic mixture containing 100 U/mL penicillin, 100 µg/mL streptomycin, 200 µg/mL neomycin (PSN mixture, Sigma Aldrich P4083), and 5 µg/mL antimycotic amphotericin B (Sigma Aldrich A9528). The medium was supplemented with 14.2 µL/mL cod liver oil (Sigma Aldrich 74380).

We produced juvenile mussels using two different in vitro methods that differed in the volume of the culture medium, the initial number of glochidia, and maternal individuals used. In method 1, dishes received 17.5 mL culture medium and 872 ± 189 glochidia (mean ± SD). In method 2, dishes received 10.5 mL culture medium and 338 ± 147 glochidia (mean ± SD); these two methods represented similar numbers of glochidia/mL. We used glochidia from females A–C for method 1 and from females D–F for method 2, and we used 12 dishes for each female for a total of 36 dishes for each method. For both methods, we incubated all dishes at 5% CO₂ under UV light for 1 h prior to adding glochidia. We rinsed glochidia with sterile water to remove any remaining marsupial tissue fragments and placed them in dishes with sterile Pasteur pipettes.

We incubated all dishes at 5% CO₂ and 24 °C for 6 days after which metamorphosis was complete. On day six, we added sterile water to all dishes at 1:1 by volume. On day seven, we completely replaced culture media with sterile water and returned the CO₂ level to atmospheric. On day eight, we examined the contents of each dish under a stereomicroscope and quantified metamorphosis success as the number of active juveniles/the initial number of glochidia added to the dish.

2.2.2. In vivo methods

We produced juvenile mussels by infesting two known host fish species for *S. woodiana*: *Rhodeus amarus* and *Gobio gobio* (Douda et al., 2012). We collected *R. amarus* and *G. gobio* from the Kyjovka (48°45'4"N, 16°59'32"E) and Lužnice (49°18'54"N, 14°30'1"E) rivers, Czech Republic, respectively, and acclimatized them in the laboratory for two weeks. We infested six individuals of each fish species with glochidia from each of the six maternal mussels by placing them in a common 6-L bath containing 4223 glochidia/L ± 1095 SD for 15 min (total 36 *R. amarus* and 36 *G. gobio* infested). We held infested fishes individually in 18-L recirculating aquaria with 3-mm meshes on the bottom to prevent fish predation on juveniles. We held fishes at 25.3 °C ± 0.7 SD and maintained them on commercial fish flakes. Fish mortality was <5% (3 fish) during the experiment.

We collected glochidia and juvenile mussels daily from each tank for 12 days by examining 139 µm filter screens through which recirculated water from the tanks flowed continuously. We examined filtered material from each tank under a stereomicroscope and classified each individual as either 1) live juveniles displaying foot or valve movement, or 2) dead glochidia or dead juveniles displaying tightly closed or permanently open valves, no sign of movement, or decomposing tissue. We quantified metamorphosis success from each fish as the sum of live juveniles/the sum of dead glochidia and dead juveniles observed over the 12 day examination period.

We examined differences in metamorphosis success among production methods (*Rhodeus*, *Gobio*, IV1, IV2) and female mussels based on Type III sums of squares from a two-factor ANOVA with arcsine-transformed metamorphosis success as the response variable, followed by Tukey's HSD to examine pairwise differences among production methods ($\alpha = 0.05$). Our response variable was metamorphosis success in each dish (in vitro methods) or on individual fishes (in vivo methods).

2.2.3. Early juvenile survival and growth

After metamorphosis was complete, we haphazardly selected a subsample of live juvenile mussels from each production method/female mussel combination and monitored survival and growth for eight days. We placed 2–20 juveniles each in three glass trays for each production method/female mussel combination for a total of 54 trays (9 trays from three females for each in vitro method, and 18 trays from six females each for *Rhodeus* and *Gobio*). Trays contained 250 mL dechlorinated tap water, and we maintained mussels at 24 °C and added 150 µL of commercial unicellular algae mixture (Plankto Marine P, Grotech; cell density ~ 25×10^6 /mL) to each tray daily. After eight days, we counted the number of surviving juveniles and preserved a subsample from each tray in ethanol then later measured their length as the greatest dimension parallel to the hinge. We examined differences in survival (arcsine-transformed) and final juvenile size (log-transformed) among production methods and female mussels with two separate two-factor ANOVAs (based on Type III sums of squares), followed by Tukey's HSD to examine pairwise differences among production methods ($\alpha = 0.05$). For these analyses, our response variable was mean survival or size across all trays in each production method/female treatment combination ($N = 18$ treatment combinations).

2.3. Survival to sexual maturity, growth, and reproductive success

We haphazardly selected from each of the four production methods juvenile mussels not used in the early growth experiment and transferred them to an outdoor mussel culture facility near the Lužnice River (49°18'25" N, 14°30'15" E). We distributed about 1500 juveniles each from *Gobio* and *Rhodeus* among six 5.3–9.3 L aerated tanks with river sand (grain size 0.5–2 mm) in a layer of 3–5 mm (~250 juveniles in each of six tanks for each fish species, total = 12 tanks). We placed about 250 juveniles each from in vitro methods 1 and 2 in three tanks (~250 juveniles in each of three tanks for each method, total = 6 tanks). Tanks were supplied with food by replacing tank water with filtered river water (100 µm) twice daily. We maintained mussels in these tanks at ambient river temperatures for the remainder of their first growing season and during winter (May 2018 to March 2019; mean temperature ± SD, May–November = 17.0 ± 6.4 °C, December–March = 6.2 ± 2.7 °C).

At the beginning of the second growing season (30 March 2019, 290–308 days post-metamorphosis), we haphazardly selected 8 individuals from each of the 18 tanks, measured their length (greatest anterior-posterior dimension) and individually marked them, and placed them in twelve, 700-L fiberglass mesocosm pools filled with river water and sediment. For this component of the experiment, we combined individuals produced from in vitro methods 1 and 2 because length did not differ among production methods at that time (see Section 3.3). We stocked twelve mussels from one of the three propagation

methods (in vitro; in vivo - *Rhodeus*; in vivo - *Gobio*) into each pool, and each propagation method had four replicate pools. To eliminate potential effects of female mussel, each pool received two individuals from each of the six original broodstock females, which ensured that each pool had a similarly mixed maternal origin. We added to each pool 14, one-year-old individuals of host fish *Scardinius erythrophthalmus*. An average of 10 (± 3.5 SD) *S. erythrophthalmus* in each pool survived to the end of the season. Pools were aerated continuously and fresh river water was pumped into the tanks 12 times daily (75% water change/day). River water passed through a ~ 100 μm filter, which allowed passage of mussel food from the river but prevented entry of wild mussel glochidia. The filter could have allowed mussel sperm to enter the pools; *S. woodiana* is reported from the Lužnice river basin (Beran, 2019), but densities are low and it has not been found within 5 km upstream of the study location (K. Douda, unpublished data). Mean water temperature in the pools during the second growing season (April–November 2019) was 17.3 $^{\circ}\text{C} \pm 6.0$ (SD).

At 237 days after the start of the second growing season (22 November 2019, 527–545 days post-metamorphosis), we emptied the pools, recorded mortality, measured all mussels, and examined the sediment in the pools on a 2 mm sieve for F_2 juvenile mussels. We counted and measured shell length of all juveniles retrieved from each pool.

We examined differences in juvenile size among production methods at the end of the first growing season and winter with a single-factor ANOVA, with log-transformed mean length within each mesocosm pool as the response variable. At the end of the second growing season, we expressed growth as instantaneous growth as described previously and based on initial size at the end of the first growing season. We examined differences in growth among production methods during the second growing season with a single-factor ANOVA, with mean instantaneous growth within each mesocosm pool as the response variable. We examined differences in survival among production methods at the end of the second growing season with a single-factor ANOVA with arcsine-transformed survival in each pool as the response variable. We expressed recruitment as finite population growth rate (λ/yr) based on the final adult population size and the number of F_2 recruits produced in each pool. We examined differences in λ and recruit size among production

methods with two separate single-factor ANOVAs with λ or log-transformed mean recruit size in each pool as the response variables. Statistical analyses were performed in R (v. 4.0.2, R Core Team, 2020).

3. Results

3.1. Juvenile survival and growth in the hatchery and in the wild

After five months of rearing in the hatchery, the mean length of *L. cardium* did not differ between in vitro- and in vivo-produced mussels (mean length, in vitro = 6.37 $\text{mm} \pm 0.35$ SD, in vivo = 6.43 $\text{mm} \pm 0.39$, $F_{1,58} = 0.41$, $P = 0.526$, based on mean length of 30 batches of 16 mussels for each method; Fig. 1A). Mean mass was significantly lower for in vitro mussels than in vivo mussels, but the magnitude of the difference was small and 95% confidence intervals overlapped widely between both groups (mean mass, in vitro = 0.037 $\text{g} \pm 0.005$ SD, in vivo, mean = 0.042 $\text{g} \pm 0.008$, $F_{1,58} = 7.09$, $P = 0.010$; Fig. 1B).

We recovered 57% of silos (34 out of 60), and the remainder were lost to flooding or vandalism. Survival was highly variable among silos at a stream (Fig. 1C), but mean survival did not differ significantly between in vitro and in vivo silos, and variability in mean survival was similar for both methods (paired t -test, $t_{0.05, 5} = -0.47$, $P = 0.657$, arcsine-transformed survival; mean survival, in vitro = 0.847 ± 0.189 SD, in vivo = 0.799 ± 0.187). Growth was highly variable among streams (range = 0.009 – $0.038/\text{d}$, as g), but growth responses were nearly identical between in vitro and in vivo silos (Fig. 1D; paired t -test, $t_{0.05, 5} = -0.56$, $P = 0.597$, mean difference in vivo growth – in vitro growth = -0.00049).

3.2. Metamorphosis success and early juvenile performance

Live juveniles of *S. woodiana* were produced in all treatment combinations, but metamorphosis success differed significantly among production methods ($F_{3,132} = 21.95$, $P < 0.0001$; Fig. 2A) and females ($F_{5,132} = 7.13$, $P < 0.0001$). The production method \times female interaction was not significant ($F_{9,132} = 1.10$, $P = 0.365$), and sums of squares indicated that there was substantially more variability among production methods than among females. Metamorphosis success was higher

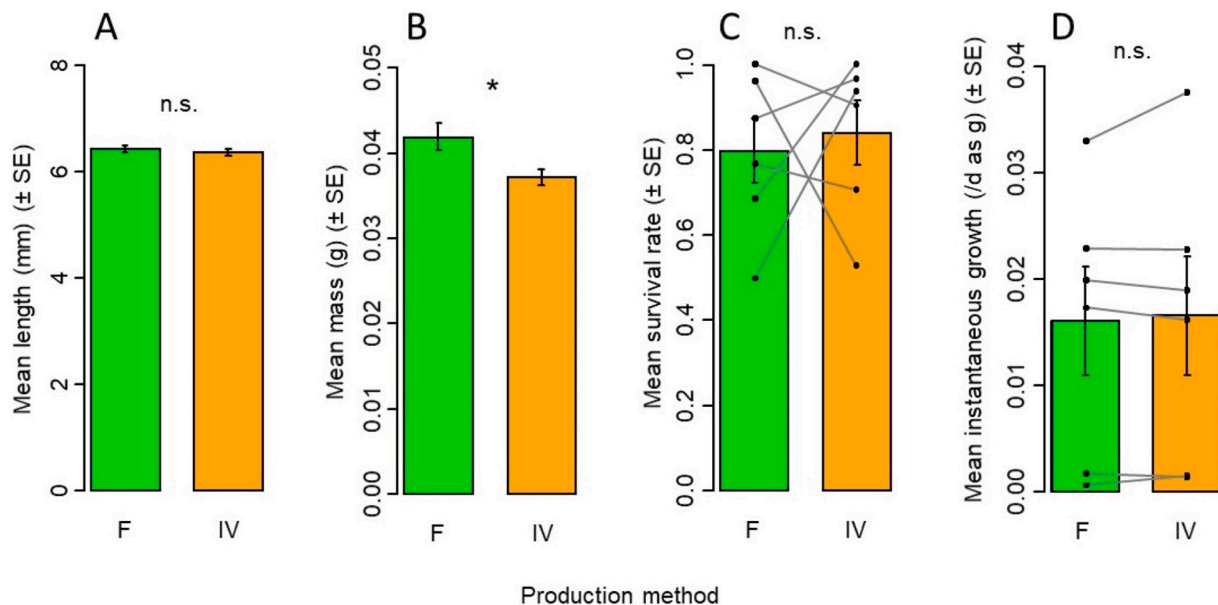


Fig. 1. Performance of in vivo-produced (host fish = *Micropterus salmoides* – F, green) and in vitro-produced (IV, orange) mussels (*Lampsilis cardium*). (A and B) Mean juvenile length and mass after five months rearing in hatchery conditions. (C and D) Mean survival and instantaneous growth during 90–120 day exposures in six streams. Asterisks indicate significant differences, n.s. = not significant (A, B: ANOVA; C, D: paired t -test; $\alpha = 0.05$). Grey lines connect mean values (black dots) obtained at the same stream. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

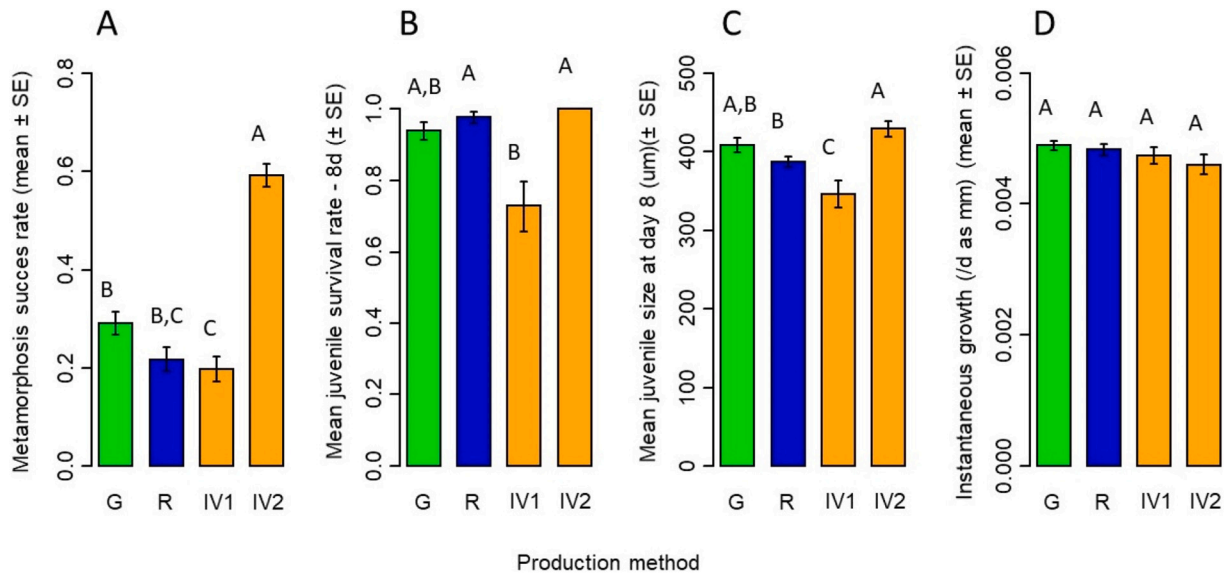


Fig. 2. Performance of in vivo-produced (host fishes = *Gobio gobio* – G, green; *Rhodeus amarus* – R, blue) and in vitro-produced (method 1 = IV1, method 2 = IV2 – orange) mussels (*Sinanodonta woodiana*). A) Proportion of metamorphosed larvae. B) Mean juvenile survival during 8 days post-metamorphosis. C) Mean juvenile size at 8 days post-metamorphosis. D) Instantaneous growth during the second growing season (March–November) (/d as mm). Different letters indicate significant differences among production methods (ANOVA, Tukey's HSD test, $\alpha = 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

overall for females used for in vitro method 2 (females D–F) than method 1 (females A–C), but metamorphosis success was higher from these females for all production methods, including on both fish species. After accounting for differences among females, metamorphosis success (proportion of successfully developed glochidia) was about twice as high for in vitro method 2 (back-transformed least square mean = 0.508) than in vitro method 1 (0.251) and on both fish species (*Gobio* = 0.282, *Rhodeus* = 0.197). Metamorphosis success for in vitro method 2 differed significantly from all other methods. Among the other three methods, metamorphosis success differed only between in vitro method 1 and *Gobio*, but the magnitude of the difference was small.

Mean juvenile survival after eight days across all production methods and females was 0.867, and 80% of values were >0.833. Four out of 54 trays had 0.000 survival, two from in vitro method 1 from female C, and one each from *Rhodeus*, females A and B. We considered these observations outliers and excluded them from analysis because they were far outside the range of other observations and did not appear to be consistently associated with any production method or female mussel. After excluding these values, juvenile survival differed significantly among production methods ($F_{3,9} = 4.77$, $P = 0.030$; Fig. 2B) but not among females ($F_{5,9} = 0.31$, $P = 0.894$). Juvenile survival was significantly lower for in vitro method 1 (back-transformed least square mean = 0.781) than in vitro method 2 (0.999) and *Rhodeus* (0.996), but it did not differ significantly from *Gobio* (0.976); survival did not differ significantly between in vitro method 2, *Rhodeus*, and *Gobio*.

Juvenile size after eight days differed significantly among females ($F_{5,9} = 4.15$, $P = 0.031$), but all pairwise comparisons of mean size among females were non-significant and sums of squares indicated that females accounted for little of the variation in size. Size differed significantly among production methods ($F_{3,9} = 21.28$, $P = 0.0002$). Juvenile size was significantly lower for in vitro method 1 (back-transformed least square mean = $332.6 \mu\text{m} \pm 29.8 \text{ SD}$) than for all other production methods. Size differed significantly between in vitro method 2 ($444.9 \mu\text{m} \pm 18.1$) and *Rhodeus* ($386.9 \mu\text{m} \pm 17.6$), but all other pairwise comparisons were non-significant (*Gobio* mean size = $407.8 \mu\text{m} \pm 23.1$; Fig. 2C).

3.3. Survival to sexual maturity, growth, and reproductive success

At the end of the first growing season and winter, size did not differ among production methods ($F_{3,12} = 2.45$, $P = 0.114$, grand mean size = $24.5 \text{ mm} \pm 2.2 \text{ SD}$). Mussel survival during the second growing season was high overall and did not differ among production methods ($F_{3,12} = 0.55$, $P = 0.655$, grand mean back-transformed survival = $0.979 \pm 0.043 \text{ SD}$); only seven of 144 mussels died (one each from in vitro method 2 and *Gobio*, three from in vitro method 1, and two from *Rhodeus*). Instantaneous growth during the second growing season did not differ among production methods ($F_{3,12} = 0.91$, $P = 0.463$, grand mean instantaneous growth = $0.0048/\text{d}$, as $\text{mm} \pm 0.0006 \text{ SD}$; grand mean final size = $76.9 \text{ mm} \pm 9.9 \text{ SD}$; Fig. 2D).

F_2 juvenile recruitment was observed in all 12 mesocosm pools. Population growth rate (λ) and number of recruits varied widely within production methods [in vitro, mean λ/pool (range), mean number recruits/pool (range) = $3.17/\text{yr}$ (1.33–5.58), 27 (5–58); *Gobio* = $5.08/\text{yr}$ (2.33–7.75), 49 (19–81); *Rhodeus* = $4.04/\text{yr}$ (1.58–9.17), 37 (8–98)]. However, λ did not differ significantly among production methods ($F_{2,9} = 0.50$, $P = 0.625$; Fig. 3A). Recruit size also varied within production methods [in vitro, mean length/pool (range) = 8.0 mm (4.5–10.3); *Gobio* = 9.7 mm (8.9–10.6); *Rhodeus* = 10.3 mm (6.1–16.3), 37 (8–98)] but was not significantly different among methods ($F_{2,9} = 0.57$, $P = 0.583$; Fig. 3B).

4. Discussion

There were few differences in the performance of juvenile *L. cardium* produced by in vitro and in vivo methods. After 5 months of growth in the hatchery, shell length did not differ between the methods, but the lower mass of in vitro juveniles suggests potentially poorer body condition for those individuals. However, in vitro juveniles were only 12% lighter on average than in vivo individuals, and the mass of individuals from both methods overlapped widely. Juveniles produced by both methods were reared in similar conditions, but small differences in food delivery to the trays or other factors may also explain the difference in mean mass. Even if the difference in mass is attributable to production method, this difference was not manifested in performance in the wild. Juvenile survival and growth in the wild were nearly identical for both

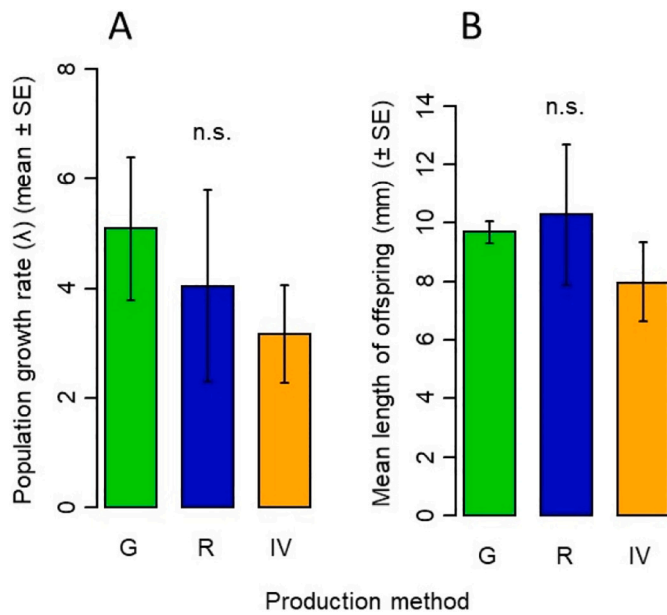


Fig. 3. Population growth rate (A) and mean lengths of F_2 juveniles (B) produced naturally on host fishes (*Scardinius erythrophthalmus*) by F_1 captive-bred mussels in mesocosms. F_1 mussels were produced by in vivo methods (*Gobio gobio*–G, green; *Rhodeus amarus*–R, blue) and in vitro (IV, orange). There were no significant differences among production methods (ANOVA, $\alpha = 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

methods. Juvenile mussel growth varies widely among our study streams with water temperature, nutrients, and perhaps anthropogenic factors (Haag et al., 2019), but none of this variation was attributable to differences between juveniles cultured by in vitro and in vivo methods.

We found important differences between in vitro and in vivo-produced juveniles of *S. woodiana*, but these differences were dependent on the in vitro protocol. The sharply lower metamorphosis success and early survival and growth of in vitro method 1 compared with method 2 may have been due to the greater depth of the media in method 1 (~3.4 mm in method 1 vs. 2.0 mm in method 2), which may inhibit gas exchange in the dishes during incubation (M. McGregor, unpublished data); or due to an unstudied physiological or genetical feature of the glochidia used. The greater number of glochidia/dish in method 1 also may have been a factor, but the number of glochidia/mL was similar in both methods; we were unable to assess which factor was responsible for the observed differences. Our protocol for method 2 for *S. woodiana* was similar to our protocol for *L. cardium* concerning depth of the media. Regardless of the mechanism, our results show that slight modifications of in vitro protocols or glochidia quality can have major effects on metamorphosis success and early juvenile performance. Interestingly, these differences disappeared by the end of the first growing season, by which time survival and growth did not differ between the two in vitro protocols. However, lower metamorphosis success and early survival partially negate the important benefits of in vitro production.

One of the most important benefits of in vitro production is that it can produce a substantially higher yield of juvenile mussels than traditional in vivo methods (Lima et al., 2012). Our results support this benefit: metamorphosis success from in vitro method 2 was about twice as high as from in vivo methods. Such differences in production can dramatically improve production efficiency for any species, but they are especially important for imperiled species for which broodstock may be difficult to obtain.

When an appropriate in vitro protocol is used, we found no important differences in post-metamorphosis performance between in vitro

and in vivo-produced juveniles of *S. woodiana*. The only significant difference we found between these methods was the smaller juvenile size for *Rhodeus*-produced juveniles compared with in vitro-produced juveniles, but this size difference disappeared by the end of the first growing season and winter. Most importantly, we showed that in vitro-produced mussels are capable of surviving and growing to maturity and producing F_2 juveniles naturally on fishes, all at rates that did not differ from in vivo-produced mussels. Sexual maturation of in vitro-produced mussels has been reported previously (Owen, 2009), but ours is the first study to compare in vitro- and in vivo-produced mussels throughout their life cycle.

Previous studies provide conflicting results about the performance of in vitro vs. in vivo mussel performance. Growth of in vitro and in vivo-produced *Anodonta anatina* and survival and growth of *Pyganodon grandis* did not differ after eight days and five months of hatchery culture, respectively (Escobar-Calderón and Douda, 2019; Kern, 2017), which is similar to our results for *S. woodiana* and *L. cardium*. Similarly, in vitro- and in vivo-produced juveniles did not differ substantially in their responses in toxicological trials (Popp et al., 2018; March et al., 2007). In contrast, survival and growth of in vitro-produced *Utterbackia imbecillis* and *Lampsilis fasciola* were significantly lower than in vivo-produced juveniles after 14 and 80 days of laboratory culture, respectively (Fisher and Dimock, 2006; Fox, 2014). It is possible that differences in in vitro protocols explain the lower survival and growth of in vitro-produced juveniles seen in these latter two studies, similar to the lower survival and growth we saw from our in vitro method 1, but we were unable to evaluate how those studies' methods may have affected their results. Optimization and standardization of in vitro methods for glochidia culture is needed to allow better comparisons between studies and to warrant optimal larval development.

Although we found no differences between mussels produced by appropriate in vitro methods and those produced in vivo, we were unable to compare the performance of captive-produced mussels (in vitro or in vivo) with that of mussels produced in natural habitats. Growth of *S. woodiana* in our study was similar to a wild population in China (Zheng and Wei, 1999). However, our growth results differed in some ways from in vivo-produced *S. woodiana* cultured for a comparable period in China (Chen et al., 2015): size was similar after eight days (mean length = 0.425 mm in our study versus 0.468), much lower in our study after 308 days (24.5 mm vs. 57.2), but higher after 434 days (76.9 mm vs. 58.2). Few estimates of population growth rate are available for mussels, and estimates from the wild are difficult to compare with our mesocosm environment. However, the mean population growth rate we saw for in vitro-produced mussels ($\lambda = 3.17/\text{yr}$) was similar to λ for populations produced from wild mussels in hatchery ponds at similar host abundance to our study (mean $\lambda = 2.0/\text{yr}$ at 1 fish/mussel), and recruitment was highly variable among ponds similar to our study (Haag and Stoeckel, 2015). More study is needed to compare the performance of captive-produced mussels with those produced in the wild, although such studies will be challenging due to the difficulty of collecting wild juveniles.

Our results provide information about two other aspects of captive mussel production. First, it is well known that metamorphosis success and other aspects of early juvenile performance can differ substantially even among suitable host fish species (e.g., Douda et al., 2017). However, we found no differences in performance between *S. woodiana* produced on *G. gobio* and *R. amarus* at adult stage. This result may be related to the fact that *S. woodiana* is a host generalist, able to metamorphose on many fish species, and it would therefore be desirable to perform similar tests on host-specialist mussel species. Second, we found substantial differences in metamorphosis success among different female mussels, and these differences were unrelated to production method. All of our female *S. woodiana* came from the same source population and we cannot explain these differences. These results emphasize the need to consider potential differences among females, including physiological status and genetic factors, in future evaluation

of production methods.

We found no major differences between the performance of in vitro and in vivo-produced juvenile mussels when an appropriate in vitro protocol was used, and our study is the first to compare these methods in a variety of contexts and throughout the mussel life cycle. In addition, our study species represent two widely divergent phylogenetic lineages, life histories, and conservation situations. *Lampsilis cardium* is in the tribe Lampsilini, which is endemic to North America, and it is a host-specialist and has a relatively lengthy parasitic period on fishes (about 23 days). It remains widespread and common over much of its range, but it has been extirpated from many streams, showing its sensitivity to some types of anthropogenic factors (Haag et al., 2019). *Sinanodonta woodiana* is in the tribe Anodontini, which is Holarctic in distribution, and it is a host generalist with a short parasitic period (about 6 days). It is invasive in Europe and elsewhere in the world, but it is declining rapidly in its native range (Liu, 2007). Studies of additional mussel species using this or similar approaches are needed for developing emergency conservation actions for freshwater mussels, particularly those that are difficult to culture in vitro (e.g., *Potamilus*, Wen et al., 2018). Nevertheless, our study represents a proof-of-concept for the equivalency of in vitro and in vivo mussel culture in a variety of contexts and based on two model species that represent a cross-section of mussel diversity.

Verification of the performance of populations of artificially bred invertebrates after release into the wild, as well as the success of their F1 generation, is not yet common, but will be increasingly important for the success of conservation programs. When other concerns of captive mussel culture are considered (e.g., genetic variation, Patterson et al., 2018), our results support in vitro culture as an appropriate conservation tool that has many benefits for the restoration of mussel populations. This study highlights the importance of assessing consequences of captive breeding methods for any organism in a variety of environmental and life history contexts to better guide the implementation of those methods in conservation.

CRediT authorship contribution statement

Karel Douda: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original Draft, Visualization. Wendell R. Haag: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original Draft. Felipe Escobar-Calderón: Methodology, Investigation, Writing - Review & Editing. Barbora Vodáková: Investigation. Martin Reichard: Validation, Writing - Review & Editing. Xiubao Chen: Writing - Review & Editing. Monte McGregor: Investigation. Jian Yang: Validation, Writing - Review & Editing. Manuel Lopes-Lima: Validation, Writing - Review & Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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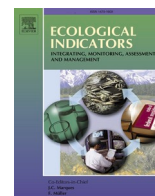
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Objective 2:



High-frequency video analysis extends beyond the capabilities of valvometry in acute behavioral disturbance detection in bivalves

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ABSTRACT

Bivalve filtering behavior has been extensively used as a sensitive *in vivo* indicator of water environment changes. However, greater use of this technique is hindered by the usually complex, invasive, and laborious technical approaches (glued-on sensors) needed to obtain high-resolution data on the movement of bivalve shells. Here, we introduce and test a high-frequency (5 frames per second) video analysis method for studying the potential for acute behavioral disturbance detection in bivalves. The method was tested by monitoring the behavioral response of the freshwater mussel (*Anodonta anatina*) to a reference toxicant, and the results were compared to data obtained through traditional valvometric evaluation using magnetic Hall sensors. Both methods showed high levels of sensitivity (Video: 0.97, 95% CI = 0.87–1; Valvometry = 0.97, 95% CI = 0.17–0.97) and specificity (Video: 0.97, 95% CI = 0.82–1; Valvometry: 0.92, 95% CI = 0.82–1) with no significant differences between the methods. Additionally, both methods performed equally well according to most binary classification metrics, such as accuracy (Video = 97.5, Valvometry = 95), positive predictive value (Video = 97.6, Valvometry = 93), negative predictive value (Video = 97.4, Valvometry = 97.3) and the area under the ROC curve (Video = 0.99, Valvometry = 0.96). A comparison between reaction times in response to stimuli of two reference toxicant concentrations (250 mg/L and 500 mg/L of nitrate-nitrogen) showed that reaction time measured from video data was significantly shorter (mean difference in reaction times = 1.56 ± 0.89 s, paired *t*-test₄; *p* = 0.01) in the 250 mg/L group due to the siphons closing first when exposed to the toxicant. We believe that approaches like the one presented here will allow future studies based on video data collection and analysis with a higher resolution than previously possible, complementing traditional gaping frequency measures and increasing our acute behavioral disturbance monitoring capabilities in bivalves.

1. Introduction

Behavioral studies in aquatic toxicology have been performed with great success in recent years (Chmist-Sikorska et al., 2020; Clements et al., 2021; Hong and Zha, 2019; Hu et al., 2020; Li et al., 2019; Parker, 2016; Pullaguri et al., 2020). The main advantage of assessing behavioral responses for ecotoxicological studies is integrating the internal physiological changes with the environment's external conditions (Pyle and Ford, 2017). Additionally, behavioral studies in aquatic toxicology have been found to have relatively high sensitivity and power and can be carried out in short periods (Melvin and Wilson, 2013).

In the case of bivalves, many studies have concentrated on the study of valve movement, also known as “valvometry” tests. Some of the first studies extracted the information about valve gaping behavior by the

use of monofilaments attached to the edge of the valve and connected to a displacement transducer (Byrne et al., 1990), muscle transducers (Doherty et al., 1987), strain gauges attached to analogous chart recorders (Djangmah et al., 1979; Higgins, 1980; Shumway and Cucci, 1987), and later, by the use of electromagnetic induction-based systems (Jenner et al., 1989). These methods were complicated to implement and required the bivalve to be fixed by one valve to a stable surface while measuring the free valve's movement, limiting the studies to highly artificial laboratory settings. This changed with the development of light-weight sensors, based on either impedance (Tran et al., 2003) or Hall effect (Nagai et al., 2006; Wilson et al., 2005) that do not require the animals to be fixed. The small and less invasive sensors have been used to allow the experimental individual to remain in free, more natural settings in the laboratory or directly by installing them into bivalves

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in the field. More recently, the coupling of the sensors with electronic units has extended the system's capabilities by permitting remote data acquisition and transfer to the internet (Schmitt et al., 2011; Sow et al., 2011). The valvometry approach has been used successfully to evaluate the response of bivalves to trace elements (Curtis et al., 2000; Doherty et al., 1987; Kádár et al., 2001; Tran et al., 2003), parasites (Chambon et al., 2007), toxic algal blooms (Shumway and Cucci, 1987), dispersed crude oil (Redmond et al., 2017), as well as a water quality bio-monitoring system (Borcherding, 2006; Chen et al., 2012; Sluyts et al., 1996).

In toxicological studies based on valvometry, valve behavior is used as a proxy of the changes in filtering induced by a stressor (Hopkins, 1933; Riisgård, 2001; Robson et al., 2009). The main idea is that pollutants disrupt the bivalve filtering behavior, causing changes in the observed gaping activity (Riisgård, 2001). However, despite its importance, valvometry-based assessments ignore the mussel soft tissue behavior involved in the filtration process. In particular, the inhalant and exhalant siphons are highly mobile and sensitive structures, furnished with receptors capable of detecting changes in water quality (Davenport, 1984); and can be protruded or retracted to control filtration rate (Riisgård, 2001). In fact, some authors consider siphon activity the best proxy for the bivalve pumping rate (Foster-Smith, 1976).

There is an increasing interest in the use of image analysis or computer vision in the field of ecology (Weinstein, 2018). The image analysis approach has the advantage of being highly efficient and, low on cost, allowing to gather high volumes of data with relative ease, especially for experimental laboratory systems (Pennkamp and Schtickzelle, 2013). Moreover, the breadth of open-source libraries and software available has made the adoption of image analysis techniques easier for ecologists worldwide. However, despite some previous studies, the image analysis approach in bivalve behavior has been limited mainly to manual analysis of images taken with low frequency (Carroll and Clements, 2019; Maire et al., 2007; Rodland et al., 2006) or used as an alternative to magnetic sensors to extract valvometry data (Dzierżyńska-Białończyk et al., 2019) with almost no attention paid to the potential of this method to study the movement of soft parts. Additionally, to our knowledge, there has not been an attempt before to quantify the difference in responses between the traditional valvometry approach and the method based on image analysis.

In this paper, we developed a new method based on video recordings and image analysis techniques to detect changes in bivalve behavior using variation of the siphon and mantle edge area as a proxy. The new method was used alongside traditional valvometry measures to assess and compare the response under different nitrate contamination levels. The main goals of our experiment were to evaluate the sensitivity and specificity of the new method to detect siphon movements and to compare these data with the data obtained from valve gaping behavior to characterize the response of bivalves to environmental stress according to siphon- and valve-based behavioral indicators simultaneously.

2. Methodology

2.1. Study species and collection site

We selected *Anodonta anatina* as our study species because it is a widespread species that can be easily maintained under laboratory conditions (Donrovich et al., 2017; Douda, 2010; Escobar-Calderón and Douda, 2019), inhabits a wide range of environments, and is resistant to various stressors (Froufe et al. 2014; Douda, 2010). A total of 30 mussels (Average measures \pm SD: Width = 2.62 ± 0.46 , Length = 7.85 ± 0.68 , Height = 4.58 ± 0.53 all in cm, Fresh weight = 40.83 ± 12.23 g) were collected by hand from the Luznice River ($49^{\circ}18'16.6''\text{N}$, $14^{\circ}30'11.9''\text{E}$, Czech Republic) a habitat of a rich freshwater mussel community with low average levels of nitrogen pollution ($\text{N-NO}_3 = 0.5 \text{ mg l}^{-1}$, $\text{pH} = 7.5$, Conductivity = $337 \mu\text{S cm}^{-1}$), and transferred to the laboratory in river

water, there the mussels were placed in plastic trays with ~ 5 cm of sand (grain size 1–3 mm); each mussel was kept separately in an acrylic glass aquarium ($25 \times 15 \times 11$ cm) with 2.5 L of river water. The mussels were then allowed to acclimate for ten days to tap water (Prague, Czech Republic, $\text{N-NO}_3 = 3.8 \text{ mg L}^{-1}$, $\text{pH} = 7.82$, Conductivity = $345 \mu\text{S cm}^{-1}$) and 20°C , because this is optimal temperature for this species with natural gaping activity observed in previous studies (Lurman et al., 2014). During this period, 50% of the water was extracted daily from each aquarium and replaced with dechlorinated tap water. Additionally, each mussel was fed by adding 0.5 mL of commercial algae mix (PlanktoMarine P, GroTech, Germany) once a day. During this period, the tanks were continuously aerated using air stones, and the mussels were subjected to a controlled light/dark regime (12 h:12 h).

2.2. Valvometry and video set up

For the valvometry data, we used Hall sensors (Continuous-time radiometric linear Hall effect sensor 02 K-A1302, Allegro MicroSystems, USA) connected to data loggers with flexible wires. Hall sensors were attached to the valves of each mussel using polyethylene tubes ($\text{Ø } 9.52 \text{ mm} \times 15 \text{ mm}$) glued to the left valve of the individuals. A Neodymium magnet ($10 \times 7 \times 2 \text{ mm}$) was placed on the right valve. The tube and magnet were placed using the following procedure. Each mussel was carefully extracted from its aquariums and had its valves thoroughly cleaned from algae and sediment. The periostracum of a small area ($\sim 25 \text{ mm}^2$) approximately 1 cm away from the shell margin was coarsened using sandpaper. The magnet and tube were then fixed to the right and left valve respectively by cyanoacrylate adhesive (Hartmann et al., 2016). After attaching the magnet and the tube, the mussels were returned to the original aquarium in 30–60 s. After the fixation of Hall sensor holders and magnets was completed, the mussels were allowed additional two days of acclimatization before the start of the tests. For the test, the Hall sensors and its wire were then introduced in the tube and kept steady using a plastic wedge, allowing us to maintain the sensor in a stable position and easily change it between individuals with minimal manipulation. Five data points of voltage output per second (proportional to the strength of the magnetic field, i.e., distance from the magnet on the second shell) were extracted and used to calculate valve positions.

For the videos, we fixed waterproof endoscopic USB cameras (USB-LED 8 mm Sunnysoft, Czech Republic) into custom-made holders designed to keep the cameras steady and fixed to the aquarium walls. The cameras were connected to a PC, and the open-source video surveillance software iSpy (www.ispyconnect.com) was used to control them simultaneously. The cameras were configured to record color videos at 5 frames per second at 320×240 px resolution. We ensured that the cameras always pointed towards the mussels during the trials and that the siphons were always visible.

2.3. Experimental set up

To induce a stress response and movement 23 mussels were individually exposed in 2.5L aerated test aquariums to different concentrations of N-NO_3 (final concentration in test aquarium and number of replications per treatment: $50 \text{ mg/L} = 3$, $100 \text{ mg/L} = 3$, $250 \text{ mg/L} = 8$, $500 \text{ mg/L} = 9$). A stock solution for each concentration was prepared using dechlorinated tap water and NaNO_3 (product code 106537, Merck), and the concentration was verified spectrophotometrically in 20 mL samples by the cadmium reduction method, following the specifications of the manufacturer (HI-96728C Nitrate-Nitrogen photometer, Hanna Instruments). Before testing, we inspected each tank to check if the mussels were active and filtering; completely closed individuals were excluded from the test. In each test, the mussel was equipped with a Hall sensor and a camera was installed in its holding tank. First, the mussel's behavior was recorded for 60 min. Afterward, 100 mL of nitrate stock solution was added to the aquariums, and the mussels were

recorded for an additional 60 min. The solution was added slowly for one minute to avoid overstimulation of the mussel from direct contact with the nitrate concentrate. Finally, at the end of the test, the mussels were stimulated to completely close to provide a reference value for the Hall sensor data. After the test, the mussels were transferred to new tanks with dechlorinated tap water, and the sediment in the trays was renovated.

2.4. Image analysis

The mussel's outer edge in each frame was obtained by applying a Gaussian-blur followed by automatic thresholding using the Otsu algorithm (Otsu, 1979). This process was performed in a user-selected region of interest of the video, and the shape of the resulting binarized image was analyzed using basic boundary-based geometrical descriptors. Four different descriptors were pretested (Eccentricity, Perimeter, Circularity, and Area), but only the Area was selected for further analysis. The Area in each frame was calculated using the zeroth-order spatial moment. A pretest using a sample video (116675 frames) showed a processing speed of 643.75 frames per second. A graphical description of the process can be found in Appendix A. The image analysis algorithm was developed in python v. 3.7.4 using the library OpenCV (Bradski, 2000).

2.5. Sensitivity and specificity test: valvometry vs. video analysis

We used videos obtained during the toxicant reactivity test from six randomly selected individuals to evaluate the sensitivity and specificity of the image analysis compared to the valvometry approach. We haphazardly selected 80 different time points and visually inspected the associated record in the search for instances of valve and siphon movement or sections with no visible movement. We used videos from both before and after nitrate addition to ensure the analysis could detect both the natural periodical movements without stimulus and the stronger movements induced due to the toxicant addition. The sections where no movement of the valves or the siphon was observed were identified as "negatives", while videos where at least one event of movement was present, were identified as "positives". The resulting video sections were 12–47 s long, 39 corresponded to "negatives" and 41 to "positives". We then extracted the corresponding data on the image descriptor area and valvometry signal for all 80 sections.

Sections with strong mussel movement generate signals of higher variability due to peaks and strong oscillations. As a result, we studied the signal variation (in the form of the coefficient of variation, CV, of each video section) as the main binary classifier to differentiate signals with and without movement. The classification obtained was then compared with the video's known categories to obtain the sensitivity and specificity. Because of the use of a quantitative measure the resultant binary classification of the positives and negatives depends heavily on the threshold selected for the CV. Thus, the data obtained for both methods were analyzed using receiver operating characteristic (ROC) curves. ROC curves permit the study of specificity variation in the specificity (horizontal axis) and sensitivity (vertical axis) of a classifier across all its possible thresholds, allowing comparison between different methods and classifiers since a good classifier has a larger area under the curve (AUC). Additionally, the curve allows one to find the best possible threshold for each classifier, which is the threshold value that generates the highest sensitivity and specificity.

2.6. Reaction time to nitrate-nitrogen addition

We used valvometry and image analysis data to assess the time until the first response after the addition of the nitrate solution. For this, we used 12 haphazardly selected video sections (5 at 250 mg/L, 7 at 500 mg/L) with a visible behavioral reaction to the addition of nitrates. Each video section started at the point of nitrate addition to the aquarium and

continued for three minutes. We also inspected the data for the concentration levels 50 and 100 mg/L, but since there was no reaction visible by either method, we do not present the data here. Selected videos were then analyzed using the algorithm presented above. The time until the first reaction was determined by finding the points of change in the mean of the series using the Pruned Exact Linear Time method in the function `cpt.mean` from the package `changept` (Killick and Eckley, 2014). The time until the first reaction was defined as the time until the first detected point of change in the series. To be able to detect small changes in the observed area of siphons and valves in the videos, the penalty used for the change point detection algorithm was relatively low (0.015), and the change points obtained were visually verified. The same approach was used for the corresponding valvometry series.

2.7. Data analysis

For the sensitivity and specificity test, we used the package `pROC` (Robin et al., 2011) to fit a ROC curve for each method. In each curve the class (either "negative" or "positive") was used as the response variable with the CV used as predictor. The threshold of maximum sensitivity–specificity for each method was determined by finding the point on the ROC curve closest to the top left corner of the plot (this corner denotes a classifier with perfect sensitivity–specificity). Additionally we also determined additional metrics of classifier performance: true positives (tp), false positives (fp), true negatives (tn), false negatives (fn) accuracy $([tp + tn]/\text{total number of videos})$, negative predictive value $(tn/[tn + fn])$ and positive predictive value or precision $(tp/[tp + fp])$, 95% confidence intervals were calculated for all metrics using the percentile method and stratified bootstrapping with 10,000 replicates (Carpenter and Bithell, 2000; Robin et al., 2011). Additionally, a p-value was calculated by converting this proportion back to counts and performing a McNemar exact test. We use the DeLong test to check if there was a statistically significant difference between AUCs (DeLong et al., 1988). For testing differences between the sensitivities and specificities of both methods, we used the method of Pepe (Pepe et al., 2009). Both the DeLong and Pepe tests are available in the package `pROC`. Finally, for the comparison between reaction times according to valvometry and image analysis data, two paired t-tests were used (one for each test concentration), after checking the normality of data by visual inspection and Shapiro Wilk test.

3. Results

3.1. Sensitivity and specificity test

The total area under the curve for the video method was greater (0.99) than that for the valvometry (0.96). Although the difference was not significant ($Z = -1.4187$, $p\text{-value} = 0.078$), the video method showed a higher average capacity to separate instances of positive movement from instances of negative movement detection. On the same line, the video method showed a maximum specificity of 0.97 (95% CI = 0.82–1) alongside a sensitivity of 0.97 (95% CI = 0.87–1). For the valvometry data, the maximum specificity was 0.92 (95% CI = 0.82–1) with a sensitivity of 0.97 (95% CI = 0.17–0.97) (Fig. 1). There were no significant differences between the sensitivity ($D = 0$, $p\text{-value} = 0.5$) and specificity ($D = -0.18766$, $p\text{-value} = 0.4256$). For a video comparison of the image analysis method against valvometry data, see Appendix B.

The performance metrics were all higher in the video analysis than their valvometry counterparts, although the differences were non-significant. The video method showed higher accuracy (difference: 2.5%) and positive predictive value (difference: 4.6%), but approximately the same negative predictive value (difference: 0.1%). A total of 4 and 2 videos were misclassified in the valvometry and video method, respectively (Table 1).

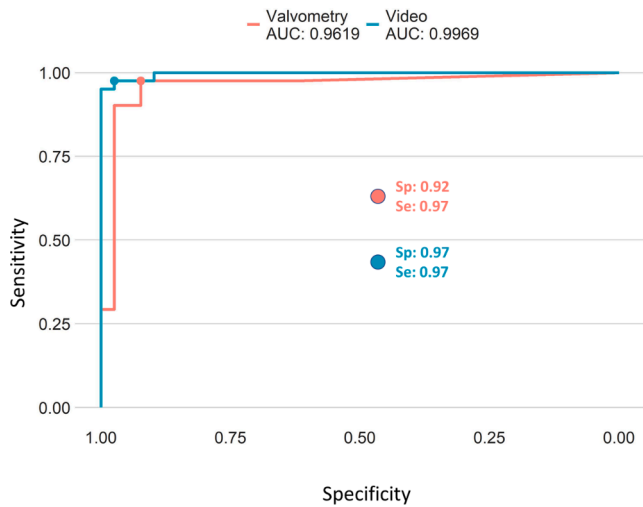


Fig. 1. ROC curves for the video and valvometry method. The points indicate the best sensitivity–specificity point of each curve. Sp = specificity, Se = sensitivity, AUC = Area under the curve.

Table 1
Comparison of classifier metrics for the video and valvometry methods.

Accuracy	Video (CI)		Valvometry (CI)		p-value
	97.5	95–100	95.0	91.25–98.75	
Negative predictive value	97.4	90.70–100	97.3	87.48–100	0.5
Positive predictive value	97.6	95.24–100	93.0	88.89–100	1
true positives	40	37–41	40	36–41	–
true negatives	38	37–39	36	34–39	–
false positives	1	0–2	3	0–5	–
false negatives	1	0–4	1	0–5	–

3.2. Time till first reaction

The average response time for the valvometry method was 56.5 ± 11.7 s, while for the video methods, it was 55.35 ± 12 s. Discriminating by concentrations, for the 250 mg/L treatment, the first reaction detected using the image analysis method happened on average 1.56 ± 0.89 s before the first detected reaction from valvometry data, and this difference was significant (paired t-test₄: $p = 0.02$, CI = 0.46–2.66), the range of difference in time of reaction for this concentration was 0.6 to 2.6 s. For the 500 mg/L treatment, on average, the image analysis method detected a reaction 1.2 ± 2.13 s earlier than the valvometry. However, this difference was not significant (paired t-test₄: $p = 0.186$, CI = -0.77 –3.17), and the range of differences in reaction time for this

concentration varied from 0.2 to 6 s (Fig. 2 and Appendix C).

4. Discussion and conclusion

The method proposed here to analyze bivalve behavior using image analysis of the changes in the parameter Area was able to successfully differentiate instances of calmness and movement with high specificity and sensitivity. Additionally, the method had a performance comparable to valvometry and, although the differences were not statistically significant, the video method showed higher specificity, positive predictive value, and accuracy. The AUC analysis for the ROC curves also showed an equivalent AUC for the video method and the valvometry. Furthermore, the video analysis showed an ability for earlier detection of mussel reaction to the addition of nitrate-nitrogen at 250 mg/L. In contrast, for the mussels treated with the nitrate-nitrogen solution 500 mg/L, the reaction time between the video method and the traditional valvometry was indistinguishable. The causes for this difference are likely to be related to the fact the video analysis method can detect not only changes in the aperture of the valves, but also the instances where the siphon is contracting. This means, that for the lower concentration the reaction of the mussels to stress involved first a closure or contraction of the siphon (up to 2.6 s before any movement of the valves) followed by the closure of the valves, while for the 500 mg/l treatment, except for one case, these stages happened almost simultaneously (mean difference in reaction between the valves and the siphon = 0.40 ± 0.21 s). The general area approach here described can track different instances of mussel behavior, including changes in mantle edge shape, siphon contraction, and valve opening, all of this has been linked in the past with changes in the filtration rate of bivalves (Famme et al., 1986; Thorin et al., 2001).

Behavioral stress reactions can be more complex than valve movements. For example, Davenport (1979) found that the first behavioral response of *Mytilus edulis* to salinity decrease was the closing of the exhalant siphon, thus effectively stopping water flow into the gills and halting filtration activity. Furthermore, the author also found that gaping behavior continued even with the exhalant siphon completely shut. It has been suggested that siphon area and valve gaping behavior are not controlled by the same processes, with the former being more influenced by the hydrodynamics of the surrounding waters or by physiological responses (Maire et al., 2007; Newell et al., 2001). Siphon activity is more likely to be intimately linked with filtration and metabolism than valve movement. However, a complete characterization of the siphon behavior and its dynamics at a high temporal resolution over longer periods is required to verify the extent of the siphon movement’s independence.

Despite its benefits, the method proposed here has some limitations inherent to using images for the collection of biological data. The automatic thresholding function worked very well for most cases, but it can fail to correctly select the edge of the mussel in cases where the

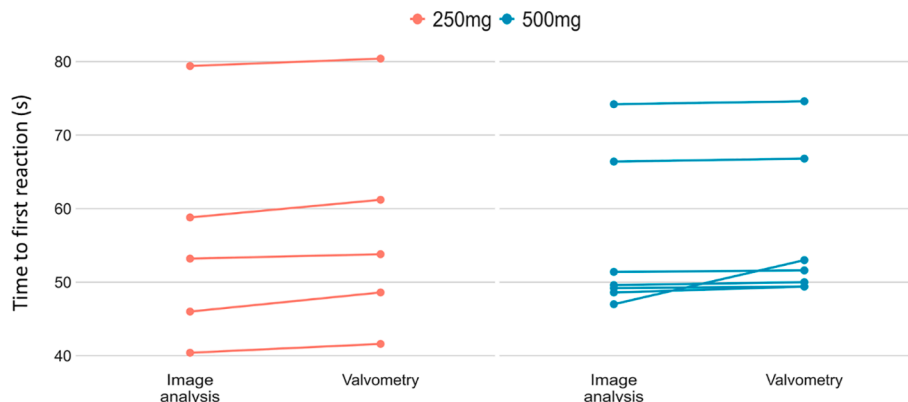


Fig. 2. Differences in time until the first reaction for the two test concentrations using image analysis and valvometry.

contrast between the mussel and the background is low. Additionally, with the mussels being able to move freely, there can be an effect of the angle and distance to the camera in the data. This limitation can be surpassed by improving the standardization of the video acquisition techniques (for example, by fixing the mussel into a stable position during observation). The method presented here is also an excellent complement to traditional valvometry and can be used alongside it. The use of multi-data sampling can help to overcome limitations in the calibration of bio-logging methods and can simplify the identification of real events (Ropert-Coudert and Wilson, 2004). Finally, during this study, because we were interested in rapid and strong behavioral responses, we used high concentrations of the nitrate stimulus, however the concentrations used here are much higher than the ones encountered by the mussels in the wild even in rivers with increased nitrogen contamination. Therefore, follow up tests of the method are recommended to characterize the difference in activity between the siphons and the valves under weaker stimuli.

This is to our knowledge, the first time that image analysis has been used to automatically retrieve records for mussel behavior with data volumes comparable to the valvometry approaches. Additionally, we present the first account of differences between the detection capacities of both methods. The use of the OpenCV library allows fast processing even with videos with tens of thousands of frames, which permits continuous record of the mussel behavior in contrast to images taken at fixed intervals, primarily used in previous studies (see below). Sequences of digital images have been used to measure siphon activity in several studies in the past because it provides a useful understanding of their long-term patterns of undisturbed feeding behavior in situ, especially in situations when there is no filtering but the valves are open (Newell et al., 2001). For example, Thorin et al. (2001) used video records of the siphon area to test the effect of water-current direction and velocity on the siphon aperture of *Mya arenaria*. A similar approach was used by Newell et al. (2001) and Riisgård et al. (2003) to study the effect of algal concentration and velocity in both valve opening and siphon aperture. Finally, Sénéchal et al. (2008) used a custom-made program to measure valve aperture and siphon area using high definition pictures to study the effects of mussel density in culture systems for *Mytilus edulis*. In these studies, the measurement of the siphon area was done manually in a set of frames taken at specified intervals, limiting the amount of data available for analysis. However, it demonstrates the potential and benefits of image-analysis of bivalve siphons activity consistent with this study.

An automatic analysis of mussel video records was used by Rodland et al. (2006) using underwater USB cameras. Instead of using changes in shape, the authors tracked the changes in brightness (defined as changes in values of gray-scale pixels) of a region of interest to extract points of change of valve and siphon movement on sequences of images obtained every 20 s. The authors also reported that, because the method is based on brightness level, it required either constant lighting or user calibrations specific to each video. The authors found consistent periodical patterns in three different species of marine bivalves living under different environmental settings, suggesting that valve and siphon activity are physiologically rather than environmentally controlled. Other studies (Maire et al., 2007) have attempted to adapt software used for behavioral studies in other groups of organisms to bivalve behavior. In this case, the authors were able to automatically extract the area of the exhalant siphon and the aperture of the valves in *Mytilus galloprovincialis* using image sequences obtained at 15 s intervals. More recently, Dzierżyńska-Białończyk et al. (2019) developed a method for automatic processing of videos with a larger number of frames per second (1 fps) applying plasticine color marks in *Dreissena polymorpha* shells and tracking gaping behavior using the Noldus Ethovision software (Noldus et al., 2001). However, the automatic method developed by the authors was aimed primarily to study movements of the valves, with siphon movements being studied only by visual inspection. Interestingly, this visual assessment of the videos revealed that no siphon extension was

observed when valve closure was superior to 80%. Furthermore, this period of incomplete valve closure and inactive siphons were more common in the individuals subjected to alarm cues, suggesting stress events might not induce a complete valve closure, and that siphon activity is a more sensitive way of studying bivalve behavioral responses to environmental stressors.

Except for the latter, most of the methods discussed previously depended on pictures taken at intervals that vary from 15 s to 1 min, limiting the capacity of detecting complex or rapid responses. Selecting the sampling frequency is critical because it can have substantial effects on the observed behavior. Moreover, the use of long intervals influences the number and duration of events detected (Robson et al., 2009; Ropert-Coudert and Wilson, 2004). The method presented here takes advantage of the inherent speed of computer vision platforms to overcome this issue, allowing rapid analysis of high-frequency data (5 frames per second for our case). Furthermore, this method can be adapted to be used with live-stream video instead of stored files, and therefore, it can monitor the response in real-time, which has so far been possible only in the case of valvometry (Schmitt et al., 2011; Sow et al., 2011; Witbaard and Fischer, 2019), but not image analysis.

In conclusion, the approach used here successfully detected behavioral changes using videos with very heterogeneous characteristics of mussel position and lighting. The method is capable of high-speed processing and allows high-frequency data to be recorded. Additionally, the image analysis method showed comparable performance in terms of specificity and accuracy in the classification setting. The mussel reaction time was faster when considering the siphon movement than valvometry data relying only on gaping behavior; this observation was related to the model toxicant concentration. Further improvements using the native features of various high-speed video processing software (like automatic edge detection algorithms and live stream video analysis) can improve the applicability and quality of data obtained through image recording. We believe that implementing methods like the one proposed here can increase the reliability of behavioral studies in bivalves and permit the high-frequency siphon dynamics of these important ecological indicator species to be recorded, something that was only partially achieved with other approaches.

CRediT authorship contribution statement

Juan Felipe Escobar-Calderón: Methodology, Investigation, Software, Formal analysis, Visualization. **Joanna Chmista-Sikorska:** Investigation, Methodology. **Karel Douša:** Conceptualization, Supervision, Methodology, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendices A–C. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ecolind.2022.108656>.

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Objective 3:

In situ and low-cost monitoring of particles falling from freshwater animals: from microplastics to parasites

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A simple and low-cost method of monitoring and collecting particulate matter detaching from (or interacting with) aquatic animals is described using a novel device based on an airlift pump principle applied to floating cages. The efficiency of the technique in particle collection is demonstrated using polyethylene microspheres interacting with a cyprinid fish (*Carassius carassius*) and a temporarily parasitic stage (glochidia) of an endangered freshwater mussel (*Margaritifera margaritifera*) dropping from experimentally infested host fish (*Salmo trutta*). The technique enables the monitoring of temporal dynamics of particle detachment and their continuous collection both in the laboratory and *in situ*, allowing the experimental animals to be kept under natural water quality regimes and reducing the need for handling and transport. The technique can improve the representativeness of current experimental methods used in the fields of environmental parasitology, animal feeding ecology and microplastic pathway studies in aquatic environments. In particular, it makes it accessible to study the physiological compatibility of glochidia and their hosts, which is an essential but understudied autecological feature in mussel conservation programs worldwide. Field placement of the technique can also aid in outreach programs with pay-offs in the increase of scientific literacy of citizens concerning neglected issues such as the importance of fish hosts for the conservation of freshwater mussels.

Key words: Aquatic animals, drop-off, fish, freshwater mussels, glochidia, host–parasite relationships, microparticles, microplastics

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Introduction

The inherent complexity of various ecological processes warrants the efficient combination of laboratory and field experiments. However, despite the rapid development of tools

designed to enhance field data collection (e.g. remote electronic control systems; Burnett *et al.*, 2013; Wilson *et al.*, 2014; Kubizňák *et al.*, 2019) there still exist many research areas where no field-based, low-cost technical solutions are available for primary data collection. This situation is espe-

cially true for aquatic organisms, which may restrict the collection of data for many ecological and physiological processes to laboratories or during short-term invasive sampling campaigns (e.g. Barber *et al.*, 2008; Hart *et al.*, 2018).

Diverse research fields such as ecological parasitology, ecotoxicology, aquatic animal nutrition and reproductive biology require techniques to collect objects detaching from live aquatic animals. Laboratory methods exist for collecting parasite stages (Dodd *et al.*, 2005; Marchiori *et al.*, 2013), faecal pellets (Shomorin *et al.*, 2019) or eggs (Gonsar *et al.*, 2012) in recirculating systems on screens. These methods make the following possible: the study of the time course of particle detachment at the individual level, the evaluation of the daily feed intake of animals in aquaculture facilities and the collection of particles over extended periods of time. The collection of fallen particles has also proven essential for understanding various aspects of aquatic animal physiology such as digestibility analyses (Da Mota *et al.*, 2015; Dvergedal *et al.*, 2019) and host–parasite compatibility (e.g. Rogers-Lowery *et al.*, 2007; Dodd *et al.*, 2005; Donrovich *et al.*, 2017). However, laboratory approaches have the disadvantage of being limited to a range of model organisms for which long-term holding under artificial conditions has been mastered (Levy and Currie, 2015; Russell *et al.*, 2017). Consequently, a lack of data persists for most animal species in which the laboratory approach is not feasible because it can inadvertently affect their behaviour, biological rhythms and physiology (Calisi and Bentley, 2009). This, coupled with the high operational costs and labor requirements of the research facilities needed, makes the laboratory approach unsuitable in many areas of ecology and conservation physiology research.

Here, and as a proof of concept, we describe a new technique that can be used in the field to collect objects detaching from (or interacting with) aquatic organisms using a flow-through cage system. For this, we assessed the technique's efficiency to collect (i) microplastics (polyethylene microspheres) and (ii) juveniles (post-parasitic stage) of an endangered species. We choose these two cases because in one hand microplastics have gain traction as a recent relevant research topic due to the possible deleterious effects on consumption, growth, reproduction and survival of aquatic animals (Foley *et al.*, 2018). However, the level of knowledge in freshwater ecosystems lags behind what has been explored in marine ecosystems (Erkes-Medrano *et al.*, 2015), and the interaction between microplastics and freshwater organisms is particularly understudied for wildlife compared to laboratory models (de Sá *et al.*, 2018). On the other hand, and given their complex life cycle, we used one species of freshwater mussels (*Bivalvia*: Unionida), one of the most threatened faunal groups in the planet, which in the past decades has been highly studied and subjected to several conservation management plans including captive breeding programs (Lopes-Lima *et al.*, 2017; Ferreira-Rodríguez *et al.*, 2019). This group of bivalves has a temporarily parasitic larval stage (glochidium; size,

50–400 µm) that must attach to the body surface of a suitable fish and become encapsulated in the epithelial layer to metamorphose into a juvenile mussel (Kat, 1984; Modesto *et al.*, 2018), then it ruptures the capsule and detaches from the host. Freshwater mussel–fish relationships have become useful models for addressing questions in fish ecology (Gopko *et al.*, 2018; Horký *et al.*, 2019; Methling *et al.*, 2019), toxicology (Defo *et al.*, 2019; Douda *et al.*, 2019) and the conservation biology of host–affiliate relationships (Tremblay *et al.*, 2016; Schneider *et al.*, 2017).

Various laboratory methods have been established for the study of the metamorphosis success rate of glochidia using adapted multi-unit laboratory fish-holding recirculation systems (Dodd *et al.*, 2005; Hazelton *et al.*, 2013; Douda *et al.*, 2018; Dudding *et al.*, 2019), sets of aquaria adapted for periodical or continuous siphoning (Reis *et al.*, 2014; Douda *et al.*, 2014; Reichard *et al.*, 2015; Donrovich *et al.*, 2017) or other custom-made fish holding tanks (Taeubert *et al.*, 2013; Eybe *et al.*, 2015; Huber and Geist, 2017; Soler *et al.*, 2018). However, some of these methods can be problematic (especially when used for fish collected in the field), leading often to high fish mortality during experiments (Taeubert *et al.*, 2013; Huber and Geist, 2017; Soler *et al.*, 2018), reducing the representativeness of the results. The fact that there is currently no available method for the collection of mussel juveniles falling from the fish host under field conditions strongly limits our ability to test new potential hosts in species where transport to the laboratory is problematic, or in areas without suitable laboratory infrastructure. Such limitation is one of the main reasons for the insufficient knowledge of the host sources of freshwater mussels (Modesto *et al.*, 2018) and for the need to look for new methods that are feasible without a laboratory (Hart *et al.*, 2018).

Given the above-described background and the need to develop simple methods that increase information about basic autecological processes, the main aim of this study was to describe a low-cost technique that may be employed in several ecological topics related to conservation physiology of aquatic animals (from simple assessment of animal–microplastics interactions to more complicated analysis of host–parasite relationships). We also discussed the use of this technique in other topics, including outreach programs.

Methods

To demonstrate the utility of the technique in real-world ecological problems, we present two examples that can be performed with this device, whether it is in a laboratory or a field. The first quantifies the interaction time and capture efficiency of the device for externally added standard particles in the laboratory with potential use in the study of animal–microplastics interactions. The second illustrates a breakthrough advance in field-based fish–glochidia interaction studies by addressing questions previously tractable only under laboratory conditions.

Principle and construction of the device

Floating board and cages

The floating drop-off particle collector (FDPC) unit operates on a free-floating board (width: 50 mm; polystyrene) weighed down from the upper and bottom side by protective sheets (thickness: 5–10 mm; polypropylene). Five animal holding tanks are suspended below the floating board, each positioned within five different divisions (Figs 1 and 2). The divisions are created by heat welding 5–10 mm polypropylene sheets perpendicular to the main floating board at regular intervals. The bottom of the tank lies on a single sheet (thickness: 5 mm) to which the perpendicular sheets of the divisions are heat welded. In each division, an experimental tank is placed to form a cage for the fish. Commercially available boxes with a smooth and undiversified internal surface can be used. Here, polypropylene fish tanks (volume: 20 L, length x width x height: 34 x 22 x 28 cm; T-Box S, Keter Italia S.p.A., Italy) were used. To firmly fit the tanks into each division and allow passage of water from the exterior, a gap between the floating board and the tanks in each division was created by inserting two silicone blocks (height: 12 mm) with smooth edges to prevent injury to the fish. The dimensions of the silicone blocks need to be adjusted to the size of the organisms tested.

Air and water flow

The FDPC device operates using the principle of airlift pumping. Each tank is equipped with its own riser pipe (diameter: 20 mm; PVC pipe), the pressured air required by the units during operation is provided by land-positioned compressors. The air is injected into the bottom part of the riser pipes in each holding tank, and because the mixture of air and water is less dense than the surrounding water, it rises to the top aperture, sucking water and solids from the bottom of the tank and transporting them to the collection net positioned above the main floating board. The riser pipe outlet in the top of the FDPC is connected to a 90-degree bend, ending 130 mm above the water surface level (80 mm above the floating board surface), just above a collecting filter cylinder. The main air supply line starts with an electrical air compressor to which a hose (inner diameter: 135 mm) is connected. The other end of the hose is attached to one end of the FDPC device on top of the floating board. From there, a manifold air divider valve distributes the air to the different riser pipes (or is left open to stabilize the airflow if needed—see below) through 4 mm (inner diameter) silicone tubes. Each tube is equipped with a two-way air control valve. A single air compressor can feed several FDPC units; here, one 100-W compressor (airflow: 110 L min⁻¹; air pressure: 0.035 MPa, 102 W; Hailea ACO-009, China) was successfully used to feed 2–3 FDPC units.

Because the flow rate determines the entrapping effectivity of the pump, it is necessary to measure the water flow through each filter and standardize it among tanks. The water flow through the outlets can be measured by a graduated collection

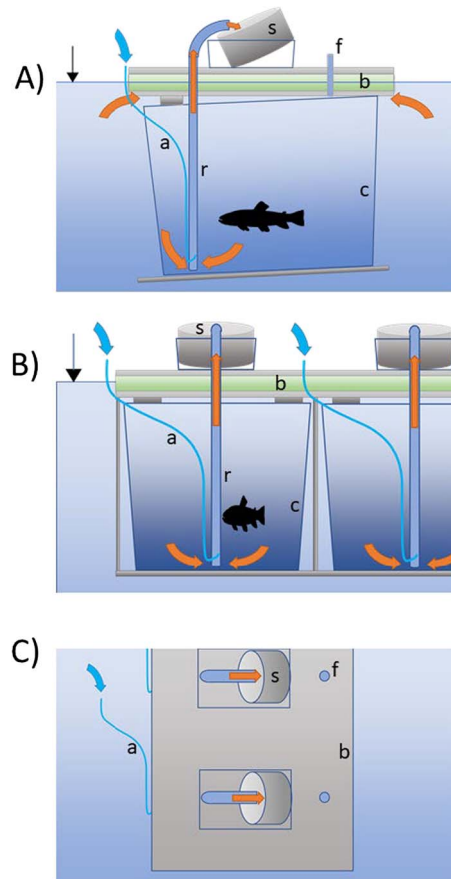


Figure 1: Side (A), front (B) and top (C) schematic view of FDPC. r, riser pipe; s, filter cylinder; f, feeding and calibration port; b, floating board; c, polypropylene cage; a, air delivery hose; red arrows, water flow; blue arrow, air flow.

vessel placed where the filter cylinders are usually located and adjusted by changing the amount of air being pumped to the riser pipes using the two-way air valves connected to the air tubes for each tank. The mean \pm SD water flow through the individual tanks under the above-described settings during both experiments was 45.6 ± 9.6 mL s⁻¹.

Collecting cylinders

The collecting filter cylinders (Fig. 2C) are made from PVC pipe (diameter: 115 mm; height: 65 mm) with a nylon screen of specific mesh adjusted to the size of the monitored particles attached (here, we used a loop size of 139 μ m). The filter cylinders are placed into PVC positioning box fixed on top of the FDPC, which stabilizes the position of the filter at the desired angle against the riser pipe outlet (we used 45 degrees as the optimal angle). The height of the openings in the positioning box determines the water level around the cylinder and allows the presence of a pool of water above the bottom part of the screen. This pool keeps the particles under water after recovery if needed. Alternatively, other type of

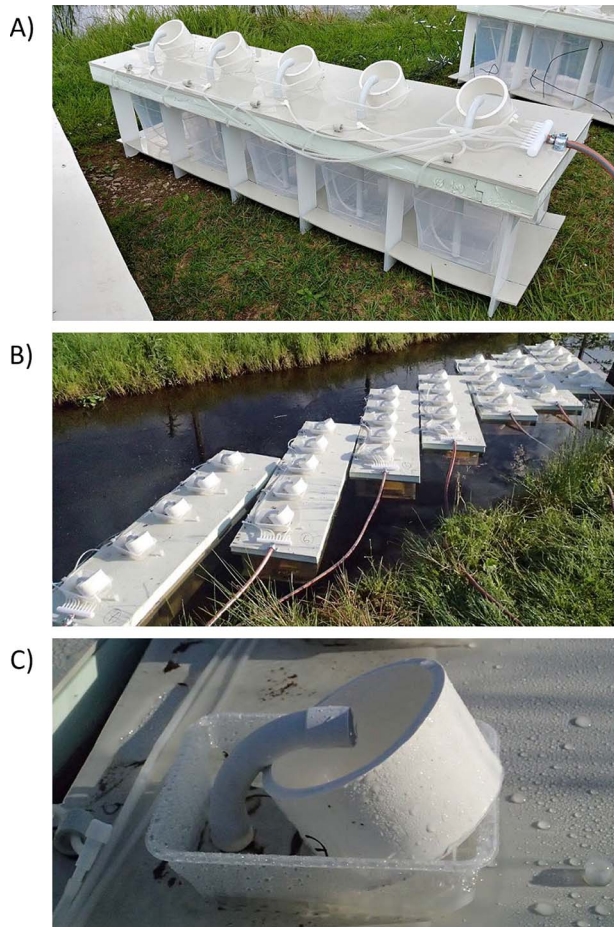


Figure 2: Example use of the FDPCs for the sampling of *Margaritifera margaritifera* juveniles dropping from host fish (*Salmo trutta*): (A) polypropylene structure arrangement for a 5-cage system, (B) field deployment of 7 systems with 34 x 22 x 28 cm cages and (C) detail of the collection cylinder.

screens, such as wedge wire screens, can be used, if necessary, to keep the recovered material out of the water (not tested here, see Shomorin *et al.*, 2019 for details).

Feeding and calibration port

The FDPC is equipped with a set of additional ports located in each section opposite to the main riser pipe. A silicone tube (inner diameter: 10 mm) is positioned in each opening (ending 5 mm above the floating board). The function of these apertures (hereinafter feeding and calibration ports) is to allow the introduction of external food items during the experiment (if needed) or a known number of particles of interest for exposure or calibration purposes.

The cost of the system described for all experiments was approximately \$1105 per 35 tanks distributed among 7 FDPC units (see Table S1 for a detailed description). The system can

be easily built using an electric saw, plastic welding heat gun (with compatible polypropylene rods), electric screwdriver and drill bit and moved to any water body with available electricity on the bank. While we used plug-in compressors and a 230-volt power connection, solar or battery sources alongside voltage converters can be used to make the system more portable. The device does not require any construction of solid structures or racks and adapts to possible fluctuations in water level.

Proof of concept

Example 1: polyethylene microspheres

Cyprinid fish *Carassius carassius* (Linnaeus, 1758) individuals (mean total length: 127 mm; mean body mass: 34 g) obtained from a laboratory breeding population at the Czech University of Life Sciences Prague (Czech Republic) were kept in a 250-L aquarium at 15°C, and a light–dark regime of 12:12 h before the start of the experiment. Fish were fed daily with commercial fish pellets (Pond Pellet, 5–6 mm; Tetra, Germany) before and during the experiment. A FDPC unit was installed in a 200 x 100 x 100 cm (length x width x height) laboratory tank with dechlorinated tap water (1800 L) under identical temperature and photoperiod conditions as described above. On the day of the start of the experiment, five randomly selected fish were extracted from the aquarium and placed into each of the tanks of the FDPC.

The microplastics, Red Polyethylene Microspheres (1.12 g cc⁻¹, 500–600 μm), were purchased from Cospheric (Santa Barbara, CA, USA). To prevent the particles from floating or creating clumps, an organic food-grade surfactant (Tween 80 Biocompatible Surfactant, Cospheric, CA, USA) was used. The microplastics (106–114 particles) were introduced into the respective tanks in the FDPC with the help of a syringe attached to a silicone tube. The assessment of flushed particles was performed at 1, 6, 24, 48, 72, 96, 120, 144 and 168 h after the start of the experiment. At each time, the five collecting cylinders of the FDPC were replaced with new clean filters, and the used filters were then observed under a microscope to assess the number and status of particles recovered.

Example 2: parasitism success in an endangered species

A second experiment applied the FDPCs to monitor parasitism success and to collect juveniles of the freshwater mussel *Margaritifera margaritifera* (Linnaeus, 1758) detaching from its fish host, *Salmo trutta* Linnaeus, 1758. It should be noted that previous field studies have been restricted to evaluation of glochidia attachment intensity observed on wild fish (Salonen *et al.*, 2017; Dias *et al.*, 2020), whereas evaluation of metamorphosis success has been limited to laboratory studies (e.g. Douda *et al.*, 2017; Schneider *et al.*, 2017). The success of *M. margaritifera* parasitization was tested using

larvae from two different source populations (with different qualities of glochidia) experimentally infesting host fish from two different populations.

For this, the experimental *S. trutta* were caught by electrofishing (650 V, 4 A, pulsed D.C.) in two streams (population Fish-A, Živný potok stream, 49°2'39"N, 14°1'32"E; population Fish-B, Častá stream, 48°55'4"N, 13°40'27"E) within the Vltava River basin (Czech Republic) with no current *M. margaritifera* populations. The fish were anaesthetized with 2-phenoxy-ethanol (0.2 mL L⁻¹; Merck KGaA, Germany), measured (total length: mean 164 mm, range 95–216 mm), weighed (body mass: mean 40 g, range 6–90 g) and individually marked 6–13 days before the infestations. Passive integrated transponders (PITs; Trovan ID100, 0.1 g in air, 12 × 2.1 mm; EID Aalten B.V., Aalten, the Netherlands) were inserted into the dorsal muscle using a syringe. After marking, the fish were kept in side-arm of the Vltavský potok stream (48°59'0.5"N, 13°39'38"E) in the Šumava National Park hatchery before infestation with glochidia.

Parasitic glochidia of *M. margaritifera* were obtained from female mussels sampled from two different populations in the Vltava River basin (Czech Republic) (population Gloch-A, Blanice River, 48°55'34"N, 13°58'12"E; population Gloch-B, Malše River, 48°39'01.5"N 14°28'00.3" E). To obtain glochidia of *M. margaritifera*, several mussel individuals were monitored in the field and when glochidia release was observed, the individuals were collected and placed into a shallow 5-L vessel to stimulate further glochidia release. The clumps of glochidia released were extracted with the help of a pipette and observed under a microscope to assess viability. Then, the glochidia were transferred to 5-L containers with river water. Two separate mixtures of glochidia obtained from 35 and 3 female mussels from populations Gloch-A and Gloch-B, respectively, was used (August 2018, 7 days between the two infestation events). After the glochidia were extracted, the females were returned to the same collection location. The containers with glochidia were transported immediately in cooling boxes to the Vltavský potok stream, where the infestations were performed in the same day.

Fish were infested with glochidia in August 2018 in a common bath suspension with densities of 15 400 ± 3666 and 11 200 ± 3516 (mean ± SD) glochidia L⁻¹ for populations Gloch-A and Gloch-B, respectively. Density was assessed by counting ten 1-mL subsamples. The viability of the glochidia was tested by evaluating their snapping action in a NaCl solution immediately before infestation (Roberts and Barnhart, 1999). The average percentage of viable (reacting) glochidia in the inoculation bath was 31% in Gloch-A and 74% in Gloch-B. The infestation procedure lasted 15 min, and the density of fish in the glochidia suspension was 1 fish L⁻¹. Individuals from both fish populations were infested in a common bath. The control (uninfested) fish were treated with the same handling procedures (i.e. transfer

between baths). After infection, the fish were released into a seminatural side-arm of the Vltavský potok stream with a natural gravel/sand bottom (length: 47 m; width: 2–3 m; depth: 0.1–0.6 m) and an adjacent earth pond (area: 139 m²; max. depth: 1.5 m).

The monitoring of falling juvenile mussels using FDPCs was initiated upon reaching the sum of temperatures reported as usual for the start of juvenile mussels dropping from host fish (Hruška, 1992), which occurred in June 2019 (total number of days from infestation: Gloch-A, 310 and Gloch-B, 317). The average daily temperature during the whole period ranged between 0.2 and 16.1°C, and the total sum of daily degrees until placement in the FDPCs ranged between 1573 and 1783. Seven FDPC units (total: 35 holding tanks) were placed directly at the site where the fish had spent the previous part of the parasitic period. The fish were caught as described above and were gradually placed in the FDPCs, where they spent 6–8 days at average daily temperature during monitoring 13.2 ± 1.0°C (range: 12.0–15.1°C). The relative body weights (condition factors) of 12 randomly selected fish individuals were determined using the equation $K = 100 \times \text{somatic weight (g)} / (\text{standard length [cm]})^3$ before the placement and after the removal of the FDPCs. We have verified the functionality of the feeding and calibration ports for live feeds but did not add food items on a regular basis because the presence of live aquatic invertebrates (mayfly larvae, benthic crustaceans) was regularly detected on the filters, indicating natural food being supplied to the tanks in this experiment. The FDPC collecting cylinders were exchanged at 1–2-day intervals and inspected at 10–40x magnification under the microscope. Juvenile mussels falling from the hosts were classified as live if valve or foot movement was observed. The average rate of parasite detachment from fish (number of juvenile mussels day⁻¹ g⁻¹ of fish body weight) was determined together with the success rate of metamorphosis during the monitored period (the percentage of dead and live juveniles falling from the fish). Fish individuals were returned to their site of capture after the experiment.

To verify the temperature conditions in the FDPCs, a datalogger (temperature accuracy: 0.1°C; Hobo, Onset, USA) was placed inside and outside the device, recording data every 15 min for 7 days. For the field flushing efficiency test, uninfested control fish were placed in 3 tanks of an FDPC, and 36–74 mussel juveniles were then placed inside the unit using the feeding port. For the next 96 hours, monitoring was performed as described above to determine the success of recapture.

We used paired Wilcoxon rank-sum tests to determine whether the detachment rate of juveniles and metamorphosis success (arcsine-transformed proportion of viable juveniles) differed between the different host–parasite population combinations. Paired t-tests were used to compare fish condition factor and temperature differences. All analyses were performed in R 3.5.2 (R Core Team, 2019).

Results and discussion

Example 1: polyethylene microspheres

Mortality of *C. carassius* during the experiment was zero and there were no signs of skin or fin injuries. The capture efficiency using polyethylene microspheres showed a mean (\pm SD) particle flushing efficiency of $95.9 \pm 4.5\%$. Most of the particles ($91.3 \pm 5.1\%$) were flushed in the first 24 h, and the last particles were recovered 120–144 h after insertion (see Table S2 for details). Microspheres recovered in the later stages of the experiment (72–144 h) showed that they had been mechanically damaged and small particle fragments were also present. Although it was not specifically studied here, the relatively long residence time and physical damage of these particles indicated that they had passed through the digestive tract of the fish and were harmed by the pharyngeal teeth.

By capturing particles leaving the enclosure space, the device allows determining the time and concentration of exposure to particles while being held under ambient environmental conditions. The availability of well-defined (colour, size, relative density, shape) plastic particles for experimental purposes enables this to be done effectively and offers new experimental possibilities. In addition, water flow through the system can be regulated to adjust the residence time.

Example 2: parasitism success in an endangered species

Host fish (*S. trutta*) mortality was zero, there were no signs of skin or fin injuries, and the condition factor of fish did not change ($P > 0.05$) during the experiment. The flushing efficiency of the *M. margaritifera* juveniles in the field (recapture rate of added juveniles) ranged between 88.1% and 100.0%, and 90.4% to 98.6% of juveniles were recovered within the first 24 hours. There was no difference in the temperature recorded inside and outside the devices ($P > 0.05$; mean difference \pm SD: $0.05 \pm 0.08^\circ\text{C}$).

The estimated average *M. margaritifera* juvenile detachment rate across all fish was 0.16 ± 0.47 juveniles $\text{day}^{-1} \text{g}^{-1}$, and the average percentage of successfully metamorphosed glochidia was $74.0 \pm 30.2\%$. In terms of the detachment rate, there were significant differences between the fish infested with different mussel populations (Fig. 3A). Fishes infested with Gloch-A had a significantly ($P < 0.001$) lower juvenile detachment rate (0.01 ± 0.02 juveniles $\text{day}^{-1} \text{g}^{-1}$) than fish infested with Gloch-B (0.68 ± 0.79 juveniles $\text{day}^{-1} \text{g}^{-1}$); but there were no detectable differences in the juvenile detachment rate between host fish populations ($P > 0.05$).

In terms of juvenile mussel metamorphosis success, a slightly higher percentage of live juveniles was associated with the fish infested with Gloch-B (78.6%, versus 70.4% for Gloch-A, Fig. 3B), which corresponds with the higher detachment rate in this fish population, but no significant

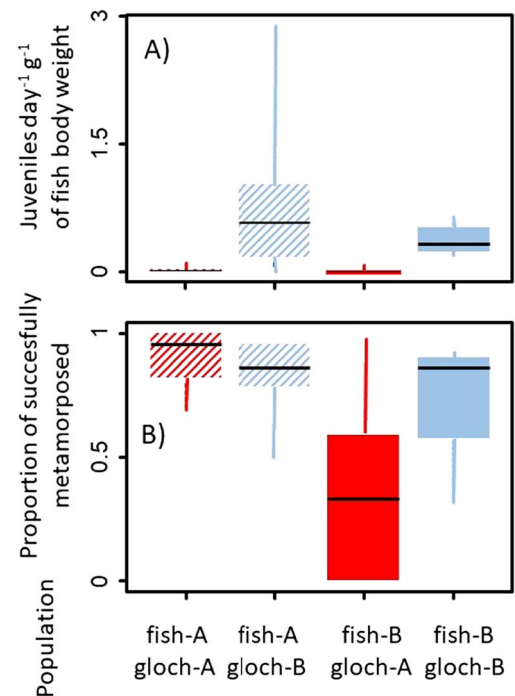


Figure 3: (A) The rate of *Margaritifera margaritifera* juvenile detachment per gram of fish body weight (pairwise Wilcoxon test, differences between mussel populations— $P < 0.001$, $n = 4-18$) and (B) the proportion of successfully metamorphosed glochidia during the 14-day monitoring period (1573–1783 degree days from infestation; pairwise Wilcoxon test, all $P > 0.5$, $n = 4-18$) as detected by the FDPC. The median, interquartile range and min/max for different combinations of source populations of parasites (red/blue) and hosts (hatched/unhatched) are displayed.

differences were detected (all $P > 0.05$). A total of 2377 detached *M. margaritifera* juveniles were sampled.

These results show that FDPC is able to detect differences in the physiological compatibility of different combinations of source glochidia and host populations. In our case, the results demonstrate a greater efficiency in the use of *S. trutta* hosts by the glochidia from population B possibly due to immunological mechanisms (Rogers-Lowery *et al.*, 2007), or due to a lower quality of glochidia produced by population A (indicated also by the initial viability analysis, see above), a common problem in freshwater mussel propagation activities (Patterson *et al.*, 2018). In terms of conservation application, it shows us which mussel population provides a more efficient source of glochidia for possible rescue or bioindication breeding. On the other hand, the results do not indicate a different ability of the two fish strains to host *M. margaritifera* due to local adaptation as recorded by previous studies (e.g. Douda *et al.*, 2017; Schneider *et al.*, 2017). Although a more complex study design would be needed to take into account the effect of glochidia viability, and test the effects of recorded lower metamorphosis success rates in the combination of Fish-B and Gloch-A populations, both populations can be considered

physiologically compatible hosts. Therefore, the FDPC allows addressing the geometry of local adaptations between mussels and fish by studying metamorphosis success directly in the field (in remote geographical locations, under natural temperature and photoperiod regimes and water quality conditions), which to our knowledge has not been possible before.

General discussion and way forward

This study described the construction of a field-deployable floating device for the continuous monitoring of detachment or interaction regimes of particles associated with aquatic animals. This novel approach is cheap and mobile, and can be used in other type of environmental studies (e.g. faeces-based molecular diet analyses and ingested microplastic quantification) (Nelms *et al.*, 2019) using fishes and other aquatic animals (e.g. crayfish and other macroinvertebrates, amphibians).

The use of nonlethal methods to collect fish faeces from animals exposed to microplastics can prove to be a valuable addition to this type of study (Hoang and Felix-Kim, 2019; Kazour *et al.*, 2018), allowing us to record the dynamics of microplastic excretion. The device can be especially useful when a long transport distance would be necessary and risky, or when the acclimatization to the available laboratory conditions is problematic (Calisi and Bentley, 2009). It should be highlighted that this method cannot be easily used for non-specific monitoring of plastics in the field due to their possible source from the surrounding environment and the device itself (Löder and Gerdts, 2015; Li *et al.*, 2018). On the contrary, the proposed use tested here as proof of concept consists on the controlled exposure of organisms to plastics of specific properties and detectability (Shim *et al.*, 2017; Heinrich *et al.*, 2020) either before or during (as showed here) the placement into the system and monitoring the regime of particles-animal interaction under natural water quality and temperature and photoperiod regimes.

Although we showed that the FDPC is ideal for collecting particles dropping from or interacting with fish in laboratory and oligotrophic habitats, slight alterations to the presented system can further increase its range of applications. The use of wedge screens can be suitable for the collection of faeces more effectively (Dvergedal *et al.*, 2019; Shomorin *et al.*, 2019), and the system can be surrounded by protective nets to prevent input of other prey items (when providing food items manually) or other types of potential interference (e.g. in the case of filter-feeders). Another possibility to extend the usability of the system is the implementation of technological accessories to record and report online the behavioural activity of the objects studied, environmental conditions and system malfunctions, which has not been possible without continuous operator presence until recently (Kubizňák *et al.*, 2019; Sheehan *et al.*, 2020).

Another promising opportunity for the FDPC application is the conservation biology of freshwater mussels, which are

declining worldwide (Lopes-Lima *et al.*, 2014, 2018). The use of FDPCs in mussel conservation can involve two main activities. First, as demonstrated here using *M. margaritifera*, the FDPC represents a cheap, reliable, and deployable mean of testing the glochidia metamorphosis success rate—a critical knowledge for the determination of conservation units and host resource management (Modesto *et al.*, 2018). Second, the FDPC can be a powerful tool for the recovery of both larvae and juveniles from endangered freshwater mussels. The use of this (or similar) techniques to increase our knowledge about basic autecological features of freshwater mussels is highly welcome, because it has been shown that adult mussels held in the laboratory conditions over long terms exhibit lower growth, altered metabolism and higher mortality (Patterson *et al.*, 2018; Roznere *et al.*, 2014). Although a great increase in the number of studies addressing ecological and conservation issues of freshwater mussels can be found in the past decades, the reality is that basic information on key autecological (e.g. distribution, density, population size structures) features are still lacking (Lopes-Lima *et al.*, 2020) especially in some areas where equipped laboratories or personal are not available. In fact, one key information gap is their reproduction and the metamorphosis of glochidia to juveniles. The device and methodology described here can overcome some of the bias (water quality, feeding and temperature differences) already described in the usual laboratory procedures and can help to expand this type of research into new geographical areas.

The device also has good potential for use in other biotic interactions. For example, Trematoda parasites produce in their intermediate (molluscan) host free-living larvae (cercariae), which swim actively or float passively in the water to find and infect the next host. An important branch of aquatic parasitology is the estimation of cercarial production. This is challenging in field conditions, because so far, the only way to estimate cercariae production has been to place the mollusc in a container for a period of time to be able to count the larvae (e.g. Taskinen 1998). The FDPC system described here can be an important innovation in this type of research. In addition, the possibility of placing the system in a freely accessible (compared to a remote and quarantined laboratory) location in the field can be beneficial for educational purposes. In the case of our field site near the fish hatchery of Šumava National Park, there were many opportunities to demonstrate the device to students and other visitors and thus communicate the fish-mussel host-parasitic system and their importance for conservation research programs.

Despite the possible advantages, it is important to take into consideration that although the device can be located in a river or a lake, it is not a physically natural habitat but an enclosure. Thus, it brings an effective advantage in some fundamental parameters (temperature and light regimes, and water quality), but on the contrary, it does not allow a number of natural behaviours (e.g. movements of animals to foraging areas or an interaction with substrate). Therefore, in particular cases, it will be necessary to determine whether

the caging can affect the studied parameter. In the same vein, and although the device can eliminate the need of organisms transport over long distances and reduce the risks of disease transfer to or from laboratories or among catchments, as a field-deployable device, the FDPC itself could contribute to the movement of diseases and species. Because of this, we strictly recommend that all parts of the FDPC in contact with water must be disinfected and allowed to dry completely before being transported to another location.

In conclusion, collecting particles dropping from aquatic animals directly in the field not only provides opportunities to greatly increase the volume and type of data that can be collected in environmental parasitology or animal feeding ecology, but also enables the acquisition of new types of data in emerging research fields, such as microplastic pathway studies. Further research is needed to test FDPCs in other water systems and in association with other research topics. This system has excellent prerequisites for interconnection with remote electronic monitoring systems. Continued technological advances will make field-deployed floating systems an increasingly viable and versatile option without needing a sophisticated laboratory for holding organisms originating in the wild with the associated long-distance transport. The simple and low-cost design, field accessibility and easy operation also allow its use in outreach programs, increasing the scientific literacy of citizens in very specific topics such as the importance of fish to conserve critically endangered freshwater mussels.

Supplementary material

Supplementary material is available at Conservation Physiology online.

Author contributions

K.D. conceived the idea and designed the hardware. F.E.-C., B.V. and K.D. performed the calibration, laboratory and field experiments. P.H., O. S. and K.D. collected the fish hosts and deployed the field units. All authors provided critical feedback, participated in manuscript writing and approved the final manuscript.

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Conclusion

The findings of the current thesis helped to clarify some important aspects related to the conservation of freshwater mussels, with an emphasis on the parasitic stage. Most of the work presented here revolved around my developments of *in vitro* culture methods, specifically we found that the way and time in which metamorphosis success is measured can greatly affect the observed metamorphosis success of *in vitro* protocols. The influences also extend to the observed effects of the treatments of the experiments making some treatments seem influential when this is only an effect of the late pedal feeding activation of some juveniles. We recommend keeping the metamorphosed juveniles for a period after the incubation phase to follow their survival and growth, this approach will enhance the evaluation of *in vitro* protocols and make conclusions more reliable. This is important because according to our long term *in vitro* experiment, the main factor affecting juvenile survival was the variation of the *in vitro* protocol, baring this factor, *in vitro* is not only more efficient in producing juveniles (with metamorphosis success rates that are higher than the ones observed when using fish host) but also there is no observable difference in growth, survival or capacity to infect new host between *in vitro* produced juveniles and juveniles produces using fish host. However, the conclusions of our study are limited by the great adaptability and generalist behaviour of the model species used and further studies should focus on *in vitro* and its effects in endangered or rare species. An example of this is the extremely variable response on *M. margaritifera* early survival during experiment number two.

Furthermore, the results of our automatic analysis methods showed that new technologies and techniques can be combined with traditionally used methods to enhance our understanding of mussel behaviour and reactions to stress, something that can prove vital for conservation efforts as well as in applied scenarios. The automatic image analysis method was able to correctly identify instances of mussel activity and reaction to the nitrate pollution, even achieving higher sensitivity and specificity than the use of magnetic valvometry sensors (although the difference was not significant), moreover the image analysis method detected an initial soft tissue reaction of the animals subjected to 250 mg/L of nitrate nitrogen that was later followed by closure of the valves, pointing to different levels of bivalve behaviour in response to stress.

Another output, which we expect could have practical application in research and conservation of freshwater mussels in near future is pontoon system (Study 5). Its broader use could, for example, contribute to the protection of certain species in Southern Europe (such as *Microcondylaea bonellii*).

To all of this we have to add the remaining results not published yet and not included in this thesis. The initial results of these experiments are promising, and I believe that once the analysis is completed they will shed further light on aspects of host-parasite relationships of freshwater mussels like the juvenile vitality variation between host populations, the host selection process in females and glochidia and the effects of co-occurrence history.

Description of my exact contributions

My contribution for each one of the main experiments presented is described in this section, for most experiments the initial conceptualization and main aim was established by my supervisor and my own contribution develop from there, I was involved in the further refinement of the conceptualization of the papers as explained below.

Experiment	Contribution	Manuscript	State
<i>In vitro</i> A. <i>anatina</i> and the effect of time of counting in observed metamorphosis	Development of in vitro protocol, experiment preparation and execution, data collection and analysis, manuscript writing.	Escobar-Calderón, F., & Douda, K. (2019). Variable performance of metamorphosis success indicators in an in vitro culture of freshwater mussel glochidia. <i>Aquaculture</i> , 513, 734404. https://doi.org/10.1016/j.aquaculture.2019.734404	Published
Early survival <i>In vitro</i> of <i>M. margaritifera</i>	Development of in vitro protocol, experiment preparation and execution, data collection and analysis, manuscript writing.	Escobar-Calderón, F., Vodáková, B., & Douda, K. (2019). Early responses of in vitro cultured fish-growing glochidia: The effects of taurine, lipids and sera on <i>Margaritifera margaritifera</i> . <i>Aquaculture Research</i> , 51(3). https://doi.org/10.1111/are.14453	Published

Effects of <i>in vitro</i> in the long-term survival and reproduction of <i>S. woodiana</i>	Development of <i>in vitro</i> protocol, help with data collection, help with manuscript writing	Douda, K., Haag, W. R., Escobar-Calderón, F., Vodáková, B., Reichard, M., Chen, X., McGregor, M., Yang, J., & Lopes-Lima, M. (2021). Effects of <i>in vitro</i> metamorphosis on survival, growth, and reproductive success of freshwater mussels. <i>Biological Conservation</i> , 254, 108964. https://doi.org/10.1016/j.biocon.2021.108964 (Douda et al., 2021)	Published
Image analysis of mussel behaviour and comparison with valvometry	Data collection, video processing, development of Image analysis algorithm, analysis of data, manuscript writing.	Escobar-Calderón, J. F., Chmist-Sikorska, J., & Douda, K. (2022). High-frequency video analysis extends beyond the capabilities of valvometry in acute behavioral disturbance detection in bivalves. <i>Ecological Indicators</i> , 136, 108656. https://doi.org/10.1016/j.ecolind.2022.108656	Published
Intrapopulation variability of <i>M. margaritifera</i> host affinity, developing a <i>in situ</i> method to the study of mussel-host interactions	Data collection, assistance on the construction, testing and deployment of the new device (FDPC). Analysis of test data for the FDPC device. Manuscript writing	Douda, K., Escobar-Calderón, F., Vodáková, B., Horký, P., Slavík, O., & Sousa, R. (2020). In situ and low-cost monitoring of particles falling from freshwater animals: From microplastics to parasites. <i>Conservation Physiology</i> , 8(1). https://doi.org/10.1093/conphys/coaa088	Published

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