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Host-parasite relationships of freshwater mussels and fish – the importance for species dispersal and larval development

Doctoral Dissertation Thesis

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I hereby confirm that doctoral dissertation thesis “Host-parasite relationships of freshwater mussels and fish – the importance for species dispersal and larval development” 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.
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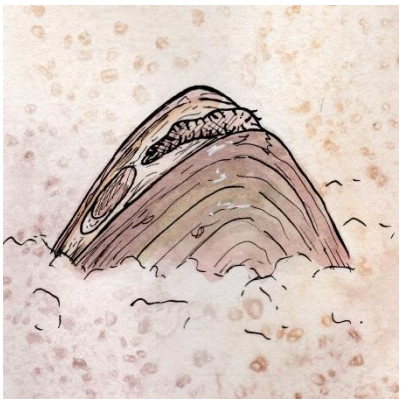
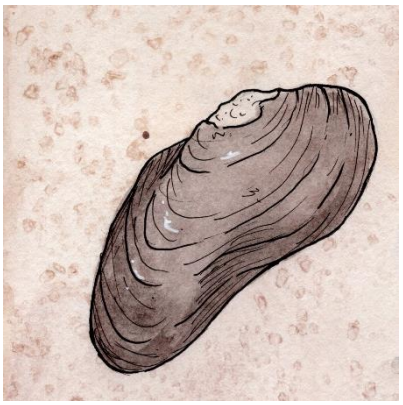
Your efforts will never betray you. All your efforts will pay off.

Lee Taeyong





I would like to give special thanks to my colleagues and coauthors of papers presented in this thesis. This thesis wouldn’t be possible without their contribution. My family and friends also played a large role in the process of my PhD studies by their endless support and advice in time of need.

Host-parasite relationships of freshwater mussels and fish – the importance for species dispersal and larval development

Summary

This thesis focuses on the life cycle of Unionida (Stoliczka 1871), especially on the host-parasite relationship between fish and glochidium. In addition, the research presented in this thesis is tied together also by the area of central Europe. The introduction provides a summary of the host-parasite problematics of Unionida, their conservation with focus on *M. margaritifera* and use of in vitro rearing of glochidia as a promising conservational tool. The introduction also points out important findings about the effect of pollutants on the host-parasite relationship and sums up the biological invasion of *S. woodiana* – unionid mussels that influence the native mussels and their interactions with hosts.

This thesis presents seven studies (two of them unpublished). Two studies focus on easily analysed biomarkers – glycogen level in soft tissue of adult mussels and lipid reserves in juvenile mussels. Samples for the lipid quantification were collected via monitoring system allowing in situ collection of juvenile mussels detaching from the individual hosts. This system, that we developed, was shown as suitable for the target purpose without causing any harm to the host fish. The assessment of the lipid reserves in juvenile *M. margaritifera* was performed in collaboration with National Park Šumava and will have a direct impact on conservation program and future research in the target area.

The conservation topic is covered also by presented in vitro studies. In vitro is a promising conservation tool for artificial rearing of unionids. We studied the effect of medium composition on *M. margaritifera* glochidia at the start of the cultivation and our results are a step forward in development of in vitro culture methodology for endangered species which is not possible to propagate by in vitro approach nowadays. In addition, research comparing performance of in vivo and in vitro reared juvenile mussels and parameters of F2 generation shown in vitro as a usable tool for Unionida conservations.

Last published study presented in this thesis is research of an influence of tramadol and methamphetamine on the host-parasite relationship between fish and mussels. Our results can be used as argument in debate about risks of pharmaceuticals in sewage treated waters. Last but not least, the overview of research focused on *Sinanodonta* spp. invasion is presented in this study. Invasion of *S. woodiana* can be one of reasons for Unionida declining populations and due to its widespread and biology it can be used as easily accessible model species for research.

Keywords: Unionida, host-parasite relationship, freshwater ecology, conservation, biological invasion, glochidia

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1 Introduction

Mussels are an important part of freshwater ecosystems. They act as ecosystem engineers due to several attributes and provide a number of ecosystem services (Gutiérrez et al. 2003; Sousa et al. 2009; Vaughn 2018; Zieritz et al. 2022). Some of these services are filtration activity and bioaccumulation, they can also be used for bioindication, and mussel shells provide shelter for other benthic organisms and base that sedentary organisms can grow on (Spooner & Vaughn 2006; Strayer & Malcom 2007; Bódis et al. 2014). Largest diversity of Unionids is in North America (Nearctic ecoregion), inhabited by 33% of all unionid species (Lopes-Lima et al. 2018) (fig. 1). Meanwhile, mussel populations are declining on a global scale (Lydeard et al. 2004; Haag 2019). This thesis is focused on the central European area where a number of mussel species is endangered or even extinct in some regions of their native range (Cuttelod et al. 2011). There are many reasons for this trend such as environmental changes, worsened water and habitat quality, habitat loss and fragmentation, anthropogenic stress, dependence on the host and possibly also spread of invasive species such as Chinese pond mussel *Sinanodonta woodiana* (Lea 1834), zebra mussel *Dreissena polymorpha* (Pallas 1771), Asian clam *Corbicula fluminalis* (Müller 1774) and Asian basket clam *Corbicula fluminea* (Müller 1774) (Aldridge et al. 2004; Haag 2012; Crespo et al. 2015; Bielen et al. 2016; Lopes-Lima et al. 2017, 2018; Denic & Geist 2017; Chowdhury et al. 2018; Modesto et al. 2018; Urbańska et al. 2021).

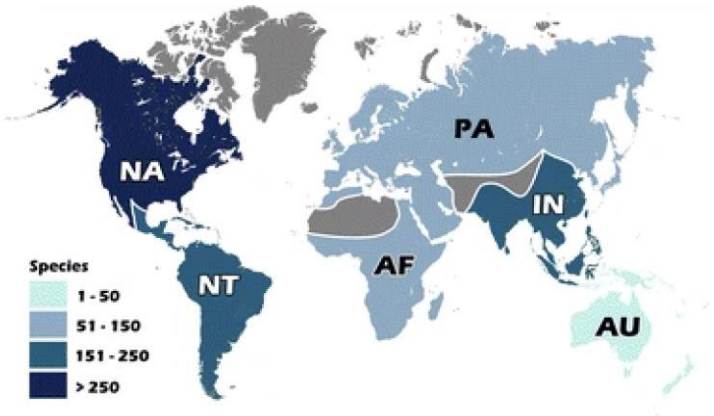

Fig. 1: Diversity of Unionida by ecoregions. NA Nearctic, NT Neotropical, AF Afrotropical, IN Indological, AU Australian. Glaciated and desert areas void of mussels are in grey. Adapted from Lopes-Lima et al. (2018).

This thesis focuses on the order Unionida (Stoliczka, 1871) and several key aspects of their larval stage in their life cycle. Deepening the knowledge and new findings about their reproduction are an important part of conservation of the endangered species and for ensuring

success of the reintroduction projects and captive conservation efforts. The next pages outline the problematics of complex host-parasite interactions between Unionida and their fish hosts.

Powerful emerging conservation tool is also ex-situ propagation of the animals for strengthening of their wild populations (Geist et al. 2023). In Unionida research, this tool can be represented among other methods by in vitro rearing of the larvae – e.g., on Petri dish in cultivation medium instead of using artificial infestation of a live fish host. This approach can limit the possible bottleneck effect of host immunity response and results in a larger ratio of successfully transformed glochidia into juveniles than in vivo approach, and in addition it is also more economically efficient (Lima et al. 2012). On the other hand, there is a large risk of bacterial and fungal contamination of the dishes (Lima et al. 2012; Ryan et al. 2022) and there are still species without successful in vitro propagation methodology available. This thesis covers this topic by studying its possible effect on vitality of the in vitro reared population and its reproduction success (STUDY 2). I also present a study focused on development of in vitro medium culture for endangered species that isn't currently possible to rear without live fish hosts (STUDY 1).

One important part of the research on the host-parasite relationship is estimation of the success of glochidia parasitation on specific host. For this problem we developed an easy and low-cost system that can be used for in-situ observation and samples collection (e.g., collection of detached juveniles and glochidia) (STUDY 3). Another important aspect of any research is to estimate the health and vitality of used animals (in our case maternal mussels, juvenile mussels and glochidia). There is also a need for condition estimation in reared juveniles in breeding programs and research evaluating the compatibility of used host for larval metamorphosis into juvenile. I present possible use of fluorescence microscopy to quantify lipid reserves in juveniles (STUDY 4) to compare the host-parasite compatibility between specific mussel and fish populations. This thesis presents an updated nonlethal glycogen quantification analysis method in tissue of adult mussels (STUDY 5). This method is particularly useful in long term research and work with endangered species due to the fact that it is one of the few non-lethal analysis methods of physiological markers that can be used for vitality estimation in mussels.

Furthermore, the interspecies relationship described and studied in this thesis can be affected by a diverse range of pollutants (Morley 2009). Water environment is very sensitive to pollution (Amoatey & Baawain 2019) and the pollutants studied in this thesis are water borne pharmaceuticals. Mussels are often used as bioindicators, and this topic is important in their conservation. We can use the host-parasite relationship between fish and unionids as a model system for research of the effect of chemicals in the water environment at multispecies level.

The pharmaceuticals could affect host and glochidia separately and subsequently alter the host-parasite interaction in different ways. We used the interaction between glochidia and their host as an endpoint for research of effect of tramadol and methamphetamine on interspecies interaction of freshwater organisms (STUDY 6).

In case of invasive species with parasitic larvae such as *S. woodiana* it is also crucial to focus on its reproduction and life cycle for understanding how invasive species spread and affect the non-native ecosystems. This thesis provides an overview of current research focused on *Sinanodonta* spp. (STUDY 7) to pinpoint gaps in current research on this topic.

To sum it up, this thesis presents an overview of several papers and some unpublished research focusing on different aspects of host-parasite relationship between fish and Unionid mussels. The presented subtopics are tied together by this interspecies relationship and show the problematics from different points of view to obtain better understanding of various aspects that could possibly influence this interaction.

1.1 Larval parasitism and host-parasite relationship of fish and mussels

Significant part of the reproductive cycle of mussels of the order Unionida (Stoliczka, 1871) is an obligatory parasitic larval stage called glochidium (Keller & Zam 1990; Lima et al. 2012). The maternal individual keeps the glochidia in the modified gills (marsupia) until they are ripe and released into the water column (Kat 1984). The larvae must attach on the fish host from which they obtain nutrition (Denic et al. 2015). They parasite mostly on gills, fins and in some cases also on the skin of the host (Lima et al. 2012). The number of glochidia attached to the host correlates with the host size and age and with the glochidia concentration in the water (Hastie & Young 2001; Taeubert & Geist 2013; Marwaha et al. 2019). They attach to the host tissue and encapsulate, the length of the parasitic stage is species specific (Keller & Zam 1990; Barnhart et al. 2008; Douda et al. 2013). There are species that have a very short parasitic stage in a matter of days, and some species require even months for the larvae development (Reis et al. 2014; Eybe et al. 2015). After the end of the parasitic stage, larvae detach from the host and live freely (Kat 1984) (fig. 2).

The maternal mussels have multiple possible strategies to lure the host and to increase probability of attachment for the released glochidia. Such strategies are “lures” or “mantle display” – modification of the mantle tissue to simulate prey of the host, like small fish or insects (Haag & Warren Jr 1999; Haag et al. 1999; Corey et al. 2006; Rypel 2008). These displays are in line with the host feeding habits (imitated species, circadian rhythm) in many

cases (Haag et al. 1999; Corey et al. 2006; Rypel 2008; Sietman et al. 2012). This strategy not only increases the probability of attracting the host but also decreases the probability of encountering unsuitable fish species (Haag et al. 1999). The mantle display can be also motionless and without specific visuals to clearly mimic existing animals, probably luring fish hosts to unspecific food items (Sietman et al. 2012). Attracted fish can be even caught by the mussel between valves and held for the time needed for glochidia releasement (Barnhart et al. 2008). Furthermore, there can be modifications of the glochidia releasement - some species release them in conglutinates that are mimicking worms and insects and induce feeding behaviour in the host (Haag et al. 1995; Jones & Neves 2002; Haag & Warren 2003). Releasing the glochidia in conglutinates like that is an effective way to attract the host, and the mussel species with this strategy has to produce smaller amount of glochidia than species releasing larvae freely into water column (Haag & Staton 2003; Barnhart et al. 2008). The maternal mussel can also show specific locomotion behaviour. There are recorded cases of spurting behaviour in thick shelled river mussel *Unio crassus* (Philipsson 1788) when the adult mussel moves to the shore and spurts a trickle of water with glochidia on the water surface. That behaviour could possibly lure the host fish that is attracted by possible insects or other prey falling into the water. Another reason could be the deposition of glochidia into the mainstream directly and possible increase in distance of the glochidia dispersion (Vicentini 2005; Aldridge et al. 2023). The spurting behaviour was recorded only for *U. crassus* and can be overlooked in the field due to the short time duration of the act. This phenomenon is possibly not occurring at some localities at all (Aldridge et al. 2023).

1.1.1 Host and parasite compatibility

The suitable fish host species is specific for each mussel species and the host specificity in Unionida ranges from generalist (e.g., is able to successfully use a wide range of fish species as a host) to host specialist (Lima et al. 2012). Finding a suitable host is a critical point in the mussel's life cycle (Douda et al. 2012a) and it is one of the key problematic factors for the mussel production in captivity (Keller & Zam 1990). The host species matters also in case of mussels with a wide range of hosts (McNichols et al. 2011). The results of McNichols et al. (2011) have shown that the difference in proportion of attached glochidia reaching successful metamorphosis (e.g., metamorphosis rate) can be vast between host species. They examined 3 suitable fish host species for each of 2 tested North American unionid mussels, northern riffleshell *Epioblasma torulosa rangiana* (Rafinesque 1820) and wavy-rayed lampmussel *Lampsilis Fasciola* (Rafinesque 1820). The resulted metamorphosis rates were 44 ± 9%, 42 ±

6% and 10 ± 3% for used host species of the first mussel and 82 ± 2%, 63 ± 8% and 37 ± 7% for host species of the *L. fasciola*. The results were in line with species co-occurrence variability and host preferences of the mussels. Study by Douda et al. (2012a) presented 14 fish species that can be used by *U. crassus* as host for successful metamorphosis but the majority of attached glochidia metamorphosed on only 3 of these fish species: common rudd *Scardinius erythrophthalmus* (Linnaeus 1758), common minnow *Phoxinus phoxinus* (Linnaeus 1758), and European bullhead *Cottus gobio* (Linnaeus 1758). This finding shows some level of host preferences in mussel species with a wide range of possible host options and the fish management could have effect on the *U. crassus* decline even though there are cooccurring fish belonging in the wide range of possible hosts present in the water body. These findings are important for proper management especially in case of endangered affiliated species.

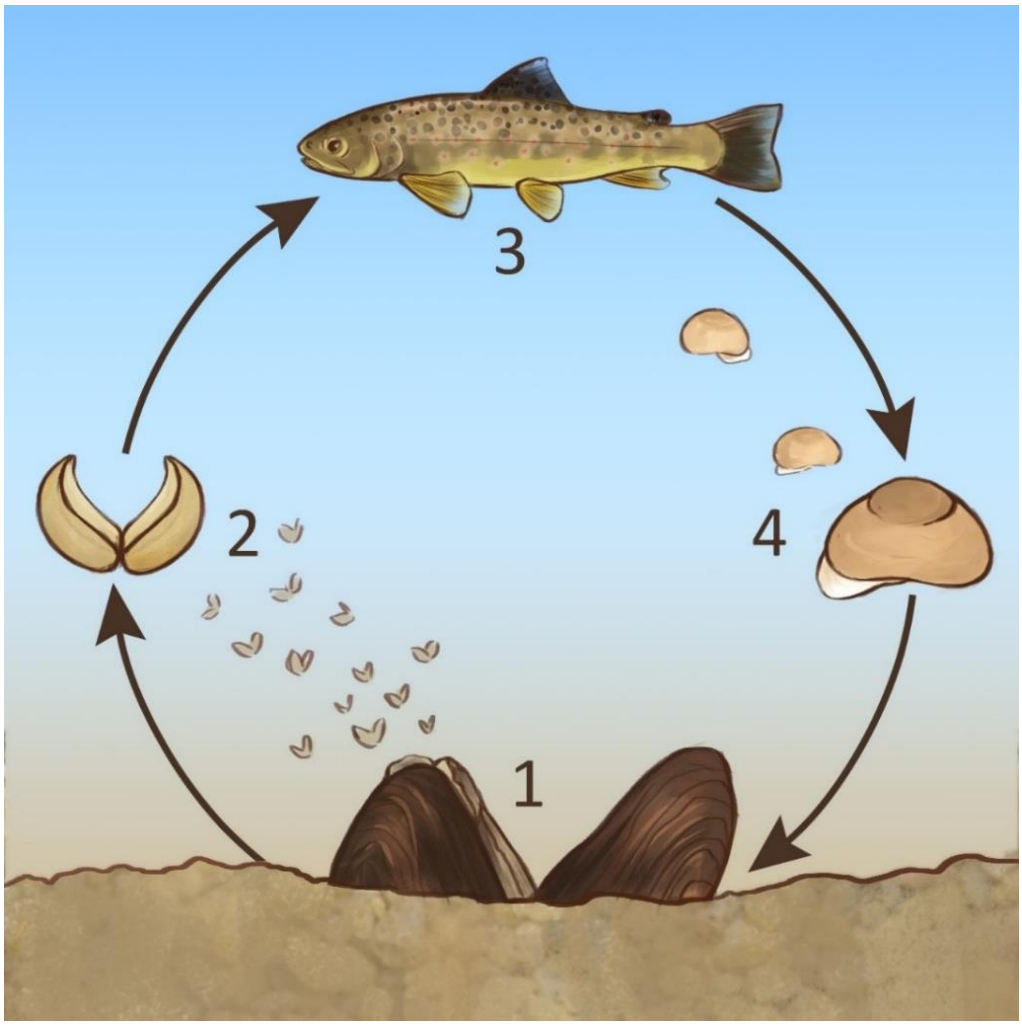

Fig. 2: Schematic illustration of the life cycle of Unionida portrayed on the example of *Margaritifera margaritifera* and *Salmo trutta*. 1 – Adult mussels buried in sediment, releasing glochidia; 2 – glochidium; 3 – host fish that glochidium attaches on; 4 – detaching juvenile mussel. Original illustration by the author of this thesis (Vodáková 2023).

The host-parasite relationship between fish and mussels plays an important role in the coevolution of hosts and mussel species, and it is shaped not only by the abundance of the host but also by the local adaptations and resistance of the host to the parasite (Haag & Stoeckel 2015; Douda et al. 2017a; Donrovich et al. 2017; Kitaichi et al. 2021; Taskinen & Salonen 2022). Finding the host of the right species isn't the only problem. The other factor of successful host infestation is withstanding the host immunity reaction triggered by the parasites. This factor is called host parasitic compatibility. The specific immunity response is developed after first glochidia infestation and is strengthened with repeated infestation (Dodd et al. 2005; Donrovich et al. 2017).

Local adaptation could also affect the host-parasite compatibility. It is particularly well shown in the case of *S. woodiana* and bitterlings (Douda et al. 2017a). Bitterlings lay eggs into the adult mussels and aren't suitable hosts for glochidia of sympatric species as a result of long-term coevolution and mussels have defence mechanisms as well (Mills & Reynolds 2003; Reichard et al. 2010; Huber & Geist 2017). *S. woodiana* is a host generalist that is invasive in a big part of Europe (for details of the invasiveness of this mussel see chapter 1.3). The ability of using bitterlings from native range by *S. woodiana* is low due to ancient sympatry. In contrast, *S. woodiana* is successfully and widely using bitterlings as hosts in non-native European areas of current spread. These fish are also sympatric but only for a limited time and they are naïve hosts from the long-term evolutionary perspective (Douda et al. 2017a). This finding demonstrated the importance of long-term sympatric coevolution in shaping the host-parasite relationship between mussels and fish hosts. The variability of host and mussel compatibility should be taken into account also in environmental management.

The differences in host-parasite compatibility aren't only on level of species but also on intraspecies level when it depends on the life history of the host and there are also differences in compatibility of specific lineage or populations of the host and mussel (Taeubert et al. 2010; Douda et al. 2014; Reichard et al. 2015). We could assume that the sympatric populations are more compatible with the specific cooccurring mussel populations due to possible long term coevolution but sometimes hosts from the allopatric population could be more suitable. This topic is particularly studied in case of endangered host specialists such as the freshwater pearl mussel *Margaritifera margaritifera* (Linnaeus 1758) and it was also described for *U. crassus* or Texas fatmucket *Lampsilis bracteate* (Gould 1855) (Österling & Larsen 2013; Douda et al. 2017a; Schneider 2017; Seagroves et al. 2019; Taskinen & Salonen 2022).

The suitability of the host can be evaluated by several methods. One of the commonly used methods is to evaluate glochidia metamorphosis success or glochidia attachment rate (Dodd et al. 2005; Taeubert et al. 2010; McNichols et al. 2011; Österling & Wengström 2015; Douda et al. 2017b; Huber & Geist 2019). There are also methods based on physiological biomarkers such as measuring lipid content in the juveniles (Douda 2015) (for more details about this topic see chapter 1.2.2).

1.1.2 Effect of the glochidia infestation on the host

Glochidia infestation can have multiple effects on the fish host. Chowdhury et al. (2021) found that brown trout *Salmo trutta* (Linnaeus 1758) infested by *M. margaritifera* glochidia has significantly lower growth rate than fish without parasitic mussel larvae in 10 months of glochidia parasitation and there was no significant effect on host mortality in the study. The negative effect on the weight of the host was also shown in data by Treasurer et al. (2006). The study has shown that the infected host fish had significantly lower weight than the control group 15 weeks after exposure to glochidia, on the other hand the difference was not significant after a year and there were no significant differences in fish condition index. The body mass reduction and lower condition index of the fish host can be also induced by *S. woodiana* glochidia infestation as well as changes in parameters of plasma of the host in the invasive range of this mussel (Douda et al. 2017b). Infestation by *S. woodiana* glochidia can increase cortisol levels (hormone serving as a stress indicator in fish) in plasma of European bitterling *Rhodeus amarus* (Bloch 1782) (Reichard et al. 2023). *S. trutta* has shown significant temporal enlargement of spleen in response to encystment of *M. margaritifera* glochidia (Thomas et al. 2013). The attached glochidia also have effect on the respiratory abilities (Crane et al. 2011) of the host since they parasite mainly on the gill tissue. The fish exposed to glochidia in the study by Thomas et al. (2013) have shown decreased ability to recover from hyperventilation. Crane et al. (2011) recorded increase in ventilation activity and weight loss in rainbow darters *Etheostoma caeruleum* (Storer 1845) parasitized by *Ptychobranchus occidentalis* (Conrad 1836) and *Venustaconcha pleasii* (Marsh 1891) glochidia. This result suggests a negative effect on the respiratory ability of the host. This hypothesis is supported by Slavík et al. (2017) who found increased Cl- concentration in blood samples of common carp *Cyprinus carpio* (Linnaeus 1758) parasitized by *S. woodiana* glochidia which indicates gill dysfunction. There was also an increase in aspartate and alanine aminotransferase levels which is a marker of liver injury. Possible kidney injury was also indicated by high concentrations of potassium in the blood samples. In addition, the glochidia can alter the reproduction of the host. It was recorded that

duck mussels *Anodonta anatina* (Linnaeus 1758) have an effect on male Eurasian minnows *Phoxinus phoxinus* (Linnaeus 1758) during spawning. The curvature of sperm trajectory was reduced as well as the quantity of breeding tubercles and sperm motility (Kekäläinen et al. 2014). Kekäläinen et al. (2014) also noted that the negative effect of the glochidia on the reproductive success of infected host probably doesn't create significant trade-off in sperm quality. On the other hand, the lower sperm quality of the infected host fish could be important in case of conditions with limited resources. Impact of the glochidia on the host is not only on physiological level, behavioural changes can be also detected in the infested host fish. Horký et al. (2014) found reduction in activity and upstream dispersion in hosts parasitized by *A. anatina*. Moreover, the glochidia infestation has an effect on the swim speed and mortality of the host as shown by Taeubert & Geist (2013) in a study with oversaturated infestation baths. These findings are important for wellbeing of the host during artificial infestation for propagation programs of endangered unionid species.

It is important to note that the survival of the glochidia is directly linked to the survival of the host therefore the negative effects of the glochidia parasitation cannot be lethal and shouldn't be harmful in large extent to the host in a way that it would be more likely to be predated in normal natural conditions.

There are positive effects of the cooccurrence of mussels and their host as well. Mussels can improve water quality by filtration activity which could be beneficial to the fish populations. Algae growing on shells of the mussels can lure invertebrates that can be further prey to the fish community (Vaughn 2018; Zieritz et al. 2022). Chowdhury et al. (2021b) studied joint effect of parasitation by *M. margaritifera* glochidia and exposure to fatal bacteria *Flavobacterium columnare* (Bernardet & Grimont 1989) on *S. trutta*. There were 3 groups of fish - one with ongoing glochidia infestation, one group 14 months post-glochidia infestation and a control group. There was significant difference in sensitivity to the bacteria between groups. Surprisingly, the time of survival after exposure to the bacteria was statistically prolonged by 1 hour in the groups with glochidia infestation (ongoing and also post-infestation) in comparison with the control group. The survival of the fish was also positively connected to the glochidia abundance. Ziuganov (2005) found that exposure to the glochidia could prolong host life span in a long-term perspective. The *M. margaritifera* glochidia infestation could prolong the life span of *S. trutta* host fish up to 13 years – in comparison with common approximation of 5 to 11 years (Jonsson et al. 1991). The principle could be that the exposure to the glochidia could stimulate resistance to nonspecific stress that could lead to deceleration of the senescence. The fish were more resistant to cutaneous mycoses and epithelioma which

could mean that the exposure to the parasite helps to reduce senile changes in regulatory cascade hypothalamus-pituitary-peripheral endocrine glands-hypothalamus at the host fish.

1.2 Conservation of Unionida

1.2.1 Conservation of Unionida in Europe with focus on *M. margaritifera*

Populations of Unionids are an endangered group worldwide (Lopes-Lima et al. 2018) with even enigmatic decline occurring (Haag 2019). The remaining populations of several unionid species can be segmented and scattered with low or no natural recruitment recorded (Lopes-Lima et al. 2017, 2018). The human alteration of the environment can shape the interaction between host and parasite in many ways. Some of them cause changes in two key factors of this relationship – the encounter frequency and species compatibility (Budria & Candolin 2014). The host density can be altered by habitat change, pollution or by fishing in case of fish species (Marcogliese et al. 1990; Mbora & McPeek 2009; Wood et al. 2010). On the other hand, the parasite density also can be changed by human interactions, for example by introducing non-native species (Koie 1991) which is very important in case of European Unionids as described in chapter 1.3. The effects of specific pollutants on host-parasite interaction of the fish and Unionids is described in detail in chapter 1.2.3 and STUDY 6.

One of target species of this thesis is freshwater pearl mussel *M. margaritifera*. It can be found in clean waters with cooccurring salmonid fish. The distribution of the species could be affected by the habitat preferences of the host (Hastie et al. 2000, 2003; Taeubert et al. 2010; Denic & Geist 2017; Taskinen & Salonen 2022). This mussel species was common across Europe (fig. 3) and eastern and central North America where it still can be found (Walker 1910; Geist 2010; Zanatta et al. 2018) but it faces dramatic global decline especially in its European range (Geist 2010), nowadays its population is extensively fragmented (Moorkens et al. 2017). *M. margaritifera is* critically endangered in most of European countries (Lopes-Lima et al. 2017) and probably extinct in Belarus, Poland, Denmark, and Lithuania (Cuttelod et al. 2011). The remaining European populations have significant problems with recruitment (Boon et al. 2019; Geist 2010; Österling et al. 2008; Simon et al. 2015; Taskinen et al. 2011) and furthermore, there is a problem with low genetic variability (Geist 2010). The low genetic variability is significant to the extent that some populations can be considered “functionally extinct” (Boon et al 2019; Geist 2010; Stoeckle et al. 2017).

Our research was conducted in the Czech Republic where this species is critically endangered (Hejda et al. 2017). The species faces the same problems in this area as the majority of the

European populations – the population is severely fragmented, with low genetic variability and with a large ratio of old individuals (Simon et al. 2015). The conservation program in the Czech Republic has focused on this species since 1983 almost continuously. Despite the conservation efforts also consisting of methods such as releasing trouts infested with glochidia or ex situ juvenile rearing for supporting the existing populations (Švanyga et al. 2013), there is still no recruitments in the wild populations. One of the obstacles for the juvenile margaritiferids in Czech Republic is unsuitable habitat quality which is reducing their chance for survival and success in maturing (Simon et al. 2015), even though there were programs focusing on habitat revitalisation in the area (Švanyga et al. 2013).

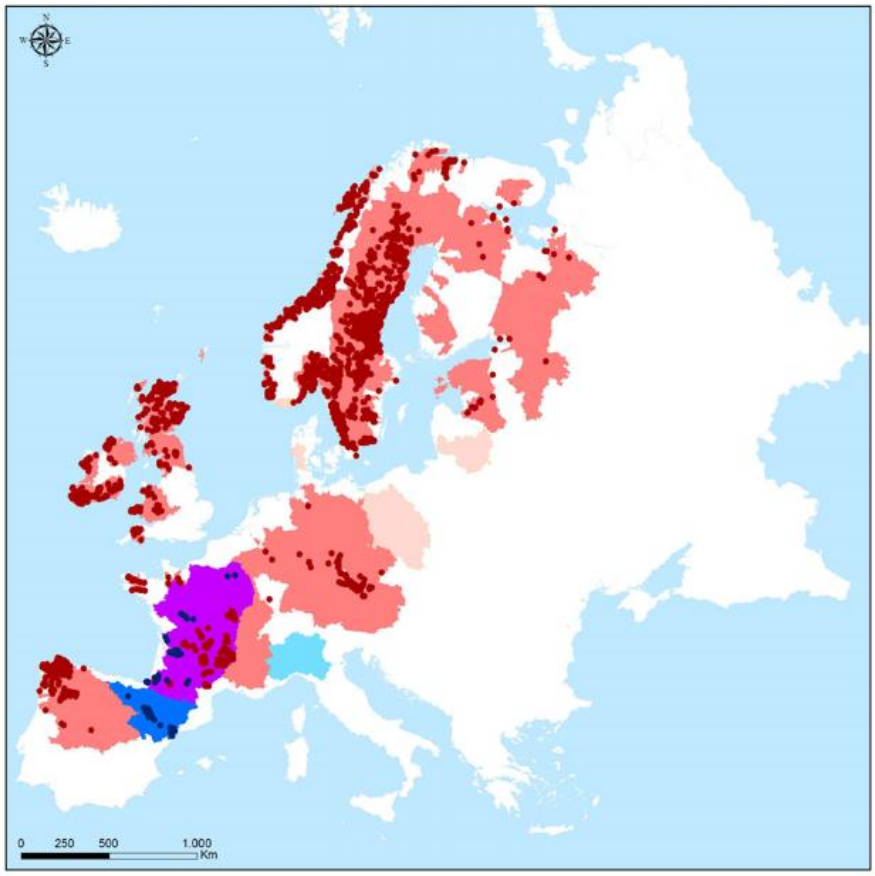

Fig. 3: Distribution of *Margaritiferidae* in Europe. Historical distribution in the hydrographical basin is marked with light shade of the colour (prior to 1992), present distribution in the hydrographical basin is marked by dark shade of the colour (after 1992), known populations are marked by dark dots (after 1992). Giant European freshwater pearl mussel *Margaritifera auricularia* (Spengler 1793) is represented by blue colour, on the other hand red colour represents *M. margaritifera* distribution. Purple colour marks overlap of these two species. Published at Lopes-Lima et al. (2017).

The focus has to be not only on the species itself during the conservation programs. Habitat conservation and restoration and conservation of affiliated species have to be considered as

well. *M. margaritifera* can play the role of an umbrella species in more complex conservation projects due to its ecosystem functions and interactions (Švanyga et al. 2013). The juvenile detached from the host buries itself in the substrate until it reaches sexual maturity (Geist 2010). The freshwater pearl mussel can be found in fine sediments mixed with larger types of substrates in shaded areas of running water from second to fifth stream order in central Europe (Hastie et al. 2000, 2003) and the habitat preferences can vary among populations (Jung et al. 2013). In general, the quality and suitability of the substrate are essential, juveniles prefer a habitat with sand of a specific size to burrow into (Hastie et al. 2000; Hyvärinen et al. 2021). In addition, the juvenile mussels in general are also sensitive to pollutants (Naimo 1995; Belamy et al. 2022). Studies by Belamy et al. (2022, 2020) present referential values of most commonly evaluated pollutants such as nitrates, cadmium, arsenic, or ortho-phosphates that influence *M. margaritifera* juveniles. Finding the right habitat conditions is one of the important factors for successful repatriation programs.

M. margaritifera glochidia can finish metamorphosis only on *S. trutta* or Atlantic salmon *Salmo salar* (Linnaeus, 1758) according to geographics. The *S. salar* is an important host in northern Europe and the relationship shifts to *S. trutta* towards the south and in central Europe it is the only suitable co-occurring host (Taeubert & Geist 2017; Wacker et al. 2019; Marwaha et al. 2021). *M. margaritifera* reproduction and its parasitical stage is heavily studied nowadays with the aim of conservational use of results of the studies. The freshwater pearl mussels glochidia are metamorphosing into the juvenile during parasitic period when they are attached to the salmonid fish. The metamorphosis from larvae into post-larvae stage with formation of a full set of organs can take 200 days (Castrillo et al. 2022) and the whole parasitic stage lasts up to eleven months (Hruška 1992; Hastie & Young 2001). Survival of the host for this whole long period is crucial for successful juvenile development.

It is important to stress the potential of local adaptations involved in *M. margaritifera* relationship with their host which is supported by a number of studies. Glochidia from populations using *S. trutta* as a host cannot successfully infest *S. salar* host in large numbers and vice versa as proven by experimental fish infestations (Wacker et al. 2019). This finding supports the role of local adaptation in host-parasite relationship as discussed in chapter 1.1. Even when the right host species is presented there can be further compatibility issues. Large differences in level of host-parasite physiological compatibility between *M. margaritifera* and its host depending on specific populations and strains can be found as well (Karlsson et al. 2014; Taskinen & Salonen 2022). The *M. margaritifera* populations preferring different host species also strongly differ genetically (Wacker et al. 2019). Results by Taskinen & Salonen

(2022) showed sympatric trouts as better hosts for *M. margaritifera* than allopatric ones. There are also contradictory studies finding no evidence supporting the local adaptation theory (Kitaichi et al. 2021). The role of allopatric fish as a better host for unionids is also supported by several studies (Österling & Larsen 2013; Douda et al. 2017a; Schneider 2017). Marwaha et al. (2021) studied the topic from the host's point of view and there was higher host mortality of the less suitable host (in this case it was brown trout) than of the more suitable host species for the used *M. margaritifera* population. It is necessary to further study this topic for optimalization of the rearing programs in conservation efforts of *M. margaritifera* and to fully understand mechanisms shaping the interaction between glochidia and host.

Nowadays, the main techniques in the conservation of Unionida species involve artificial infestation of the host in captivity (Patterson et al. 2018). The recommended concentration of the infection bath is 300 glochidia per one gram of the weight of the host fish to ensure highest possible juvenile yields without risking hosts welfare (Taeubert & Geist 2013). This step makes the whole process dependable on the selection of the right host population and individuals, as well on the selection of the maternal mussels. This selection possibly could bring unintentional and unwanted alteration to the genetic pool of the resulting mussels juvenile population.

The negative effect of the host immunity response can be excluded by using in vitro methods for the larvae rearing (Gąsienica-Staszeczek et al. 2018). Even though in vitro is a promising conservational tool and there is ongoing research (Popp et al. 2018; Escobar-Calderón et al. 2020; Douda et al. 2021; Ryan et al. 2022) and new successful in vitro protocol published recently (Thompson et al. 2022), this technique is overlooked and sometimes not mentioned in conservation reviews (Geist et al. 2023).

In vitro cultivation of glochidia is performed in an artificial cultivation medium on a Petri dish placed in a CO_2 incubator. Typically used mediums are M199 and DMEM in combination with fungicides, antibiotics, and animal serum (typically fish, calf or horse). An important part of the medium composition is also an antifungal component such as Amphotericin B (Lima et al. 2012). This commonly used substance could be toxic to glochidia in larger concentrations according to a recent study (Ryan et al. 2022). In vitro methodology is developed nowadays for more than 50 bivalve species with different rates of success (Lima & Avelar 2010; Lima et al. 2012; Kovitvadhi & Kovitvadhi 2012; Kern 2017; Gąsienica-Staszeczek et al. 2018; Ma et al. 2018; Thompson et al. 2022).

The successful methods are developed mainly for species with short length of the parasitic stage (Roberts & Barnhart 1999). There are still a number of species for which the in vitro rearing isn't possible these days. Species that are problematic are especially the ones with glochidia

that significantly grow during the parasitic stage. A freshwater pearl mussel *M. margaritifera* is one of such species (Barnhart et al. 2008) and the in vitro method for this species is still not developed.

One of the reasons why the in vitro protocols still fail in *M. margaritifera* case could be possible higher demands for nutrients of the glochidium (Taeubert et al. 2014; Taeubert & Geist 2017). Taskinen et al. (2011) had partial success with rearing glochidia that started the development on live fish hosts and then were transferred into cultivation medium. Their effort to produce live juveniles was not successful but the results of the experiments provided a step forward in the problematics. It shows the possible existence of so far not known factor that the host fish provides to the glochidium and is missing in the cultivation medium. This missing compound could be taurine (Wu et al. 2018) – non-protein amino acid (Welborn & Manahan 1995) that serves as osmolyte during osmoregulation (Yancey et al. 1982). Hypothesis about the importance of taurine is also supported by the fact that taurine was found in mussels in high concentrations (Allen 1961) and there is an assumption that it could play a big part during development and growth on marine larvae. Welborn & Manahan (1995) recorded an increase of taurine during development of veliger larvae of marine bivalve Pacific Oyster *Crassostrea gigas* (Thunberg 1793). The larvae grow by 360% and their final concentration of taurine has grown 43x in comparison with the start of the experiment.

The level of taurine can be a response to osmotic stress induced by rapid growth in a hyperosmotic marine environment. It can be assumed that taurine plays a significant role also in development of freshwater larvae growing during parasitic the stage. On the other hand, it is important to stress that osmotic stress is significantly larger in marine environment than in freshwater habitats.

Captive breeding of animals has its downsides as well. Captive breeding is part of a number of conservation programs (Seddon et al. 2007) but releasing these animals into the wild can be problematic. These individuals could have a poor ability to adapt in the wild and there is a significant possibility of strong negative effects on the fitness of the wild populations (Snyder et al. 1996; Araki et al. 2007; Davis et al. 2020). Captive breeding could also have an effect on the genetic adaptations of the reared populations (Williams & Hoffman 2009). Furthermore, the quality of the populations bred in captivity can differ method by method (Davis et al. 2020) which makes an assessment of potential risks even harder. The problematics is well studied for many animal groups (Williams & Hoffman 2009): mammals (Pinder & Barkham 1978), fishes (Fraser 2008; Attard et al. 2016) and amphibians (Griffiths & Pavajeau 2008). There are even some studies on this topic conducted on invertebrates (Witzenberger & Hochkirch 2011). The

in vitro rearing of glochidia is a big leap from their natural life cycle so we can assume that it could also affect their fitness in comparison with animals from the wild. Popp et al. (2018) found that in vitro reared juveniles are more sensitive to some toxicants. On the other hand, the difference didn't exceed the normal variation of toxicity tests. We aimed to compare the performance of in vitro and in vivo (by using live fish hosts) reared mussels in scope of the whole life cycle of the species. Evaluation of such parameters is crucial for informed decision making in conservation and repatriation programs.

1.2.2 Monitoring and evaluation of host-parasite compatibility and analysis of energetic reserves

There is a wide range of available methods for monitoring and observation of parasitic processes and sample collection (Dodd et al. 2005; Barber et al. 2008; Österling 2011; Marchiori et al. 2013; Hart et al. 2018). The methods range from in situ across seminatural condition to fully laboratory settings (Barber et al. 2008; Taskinen & Salonen 2022). The wide range is also in the technological complexity and financial cost of the methods and needed equipment. For each research case there is a different suitable type of method and there are still topics that are more suitable for simple and low-cost approaches.

The host-parasite compatibility of unionids and their hosts is mostly evaluated by parasitation success of the glochidia after artificial host infestation in a bath with known concentration of glochidia. The success of parasitation of the glochidia is evaluated based on several quantitative parameters of collected data such as rate of metamorphosis success of glochidia (proportion of glochidia from artificial infestation bath that successfully developed into detached juvenile mussels), glochidia attachment rate (proportion of attached glochidia from artificial infestation bath) or number of encysted glochidia on the host (Dodd et al. 2005; Barnhart et al. 2008; McNichols et al. 2011; Österling 2011; Huber & Geist 2019). The encysted glochidia can be counted on dead hosts during dissection (Huber & Geist 2019; Taskinen & Salonen 2022) or on live fish by non-destructive photo-method (Österling 2011). The non-lethal methods are particularly important in case of work with endangered species. It is also needed to evaluate the condition of host individuals involved in the research. This is typically based on fish condition factor (or index) and its changes during the experiment (Treasurer et al. 2006; Douda et al. 2017b). Condition index can also be used in evaluation of bivalves in long term studies (Irisarri et al. 2015).

The viability of glochidia used for experiments is evaluated before the experiments as well. The healthy glochidia placed in water are showing snapping behaviour (Gąsienica-Staszeczek et al.

2018). The viability of glochidia is typically tested by reaction to NaCl solution or by adding crystals of this chemical directly in the water with monitored glochidia. The reaction to NaCl exposure is evaluated under microscope and in some cases by using video record of the reaction (Markich 2017; Douda et al. 2019; Huber & Geist 2019; Benedict & Geist 2021). Glochidia that close or are snapping after exposure to the NaCl are marked as viable with healthy reaction (Zale & Neves 1982; Markich 2017; Reichard et al. 2023).

Monitoring of detachment of juvenile mussels from their hosts

As shown above, one important part of the research of Unionida host-parasite interactions is monitoring the falling of juveniles from fish hosts. Samples are mostly collected in laboratory setting using series of tanks with siphoning (Douda et al. 2014; Reis et al. 2014; Reichard et al. 2015; Donrovich et al. 2017) or other types of tanks with custom made alterations and addons (Taeubert & Geist 2013; Eybe et al. 2015; Huber & Geist 2017; Soler et al. 2018). Laboratory methods of the sample collection have a number of advantages such as almost complete control over the conditions (for example light and temperature regime, nutrition conditions and population density). On the other hand, there is a high risk of fish mortality (Taeubert & Geist 2013; Huber & Geist 2017; Soler et al. 2018), especially when fish from the field are used as the host in laboratory conditions. Unexpected mortality of experimental animals can put the whole experiment into jeopardy. However, using wild fish for monitoring of glochidia detachment might be problematic in laboratory settings, due to the fact that the host collection itself can be hard and the transport causes a lot of stress to the fish (Davis & Parker 1986; Tacchi et al. 2015; Hoseini et al. 2022). In addition, the transport and habitation in non-native conditions can affect the representative value of the data set because it can change animals' behaviour and biorhythms (Calisi & Bentley 2009). In contrast, for some types of host-parasite experiment it is crucial to use fish from captivity to have certainty about their parameters such as condition, life history or genetic origin. For these experiments the in-lab sample collecting systems are irreplaceable.

Low-cost and simple systems can be used in situ and in laboratories as well. Thus, the monitoring pontoon system presented in this thesis (STUDY 3) brings new research options to less well-equipped laboratories and teams with limited budgets. Although it is needed to stress that the in-situ systems have to be durable to withstand the environmental conditions for the needed period of time.

Thanks to methods such as the pontoon system presented in this thesis it is possible to monitor detachment or release of the monitored particles with clear information about time and

individual origin. Data from such systems can provide us valuable insight into physiology of aquatic animals (da Mota et al. 2015; Dvergedal et al. 2019) and host-parasite topics (Dodd et al. 2005; Rogers-Lowery et al. 2007; Donrovich et al. 2017).

This thesis presents a study focused on the development of a low-cost system that would allow in situ monitoring of detaching glochidia and other particles. The aim was for the resulting system to be easy to use and to construct and modify for specific use in other research fields.

Simple biomarkers for the monitoring of vitality in unionids

As was previously mentioned, the host-parasite compatibility of fish hosts and mussels is a widely studied topic and the frequently used approach to the evaluation of this compatibility are quantitative metrics. These methods are not used only to compare different species combinations but also to evaluate suitability of specific populations. Even though these methods were proven to be reliable, they don't provide any information about actual vitality of the produced juvenile mussels. Therefore, these methods cannot pinpoint if the host that produces a large number of juvenile mussels also produces juveniles with equal vitality as a host that seems less suitable according to this approach.

This possible problem can be solved by adding more methods into the experiment design. One such method is a long term monitoring of the juvenile mussels and even monitoring their reproduction success and vitality of their next generation (Douda et al. 2021). This approach gives us a strong robust data set but on the other hand it isn't suitable for every mussel species especially for long lived species such as *M. margaritifera*. Next disadvantage of this approach is a large time consumption, and it is also logistically demanding.

There are a number of simple methods for assessment of condition and health of the mussels in the experiments. The vitality, condition and health status of mussels is mostly evaluated indirectly from growth, mortality or condition index (Blaise et al. 2017). It can also be evaluated more directly by evaluation of various biomarkers such as glycogen concentration in adult soft tissues (Naimo et al. 1998; Patterson et al. 1999; Vodáková & Douda 2019), metabolic analysis of haemolymph (Putnam et al. 2023), lipid and fatty acid analysis in adults (Prato et al. 2010) or lipid and glucose quantification in juveniles (Tankersley 2000; Sim-Smith & Jeffs 2011). Easy biomarker for monitoring stress can be the observation of locomotion behaviour of juveniles (Belamy et al. 2023).

In case of research focused on host-parasite compatibility, it is suitable to use analysis of biomarkers in juveniles. On the other hand, the analysis of biomarkers in adults can be used in evaluation of vitality of the maternal mussels and in monitoring the effects of environmental

changes on mussel health. Physiological markers are crucial for monitoring responses of organisms to immediate changes of the conditions and quick detection of changes in their health status (Fritts et al. 2015b) in comparison with slowly responding changes in growth and mortality rate.

This thesis focuses on two easily analysed markers of condition status in mussels. These biomarkers are suitable for routine checks in more robust experimental design and have the potential to bring routine qualitative approach to the topic and could be also used for easy evaluations in rearing programs. The lipid reserves of juveniles are one of such biomarkers that this thesis focuses on. There were studies pointing out a relationship between lipid content in soft tissue during larval development and subsequent vitality of the juvenile mussels (Tankersley 2000). We can assume that the quantification of lipids in juveniles can be a usable marker of population condition and host suitability (Douda 2015). The quantification of lipid reserves in juvenile mussels based on Nile red staining and subsequent fluorescence microscopy with analysis of fluorescence microscopy photos of individual juveniles (Tankersley 2000; Douda 2015) provides us detailed data and even information about spatial distribution of the lipid reserves within juvenile mussels. Next advantage is the ability to analyse small samples – such as a single juvenile mussel. A study presented in this thesis aimed to investigate the possible use of lipid quantification in juvenile mussels with the use of fluorescence microscopy as a novel qualitative approach (analysis of energetic reserves) to the topic of host-parasite compatibility. The method was used in combination with standard approach of monitoring metamorphosis success rate and length of the juveniles for comparison of these two approaches and obtaining a stronger data set whose results were provided for local conservation management. The model species can also provide us with findings that can be applied generally in research of the host-parasite compatibility of mussels and their host and in mussel conservation programs.

The described method is a lethal analysis of the whole juvenile biomass. Evaluation of physiological markers in mussels is still mostly based on lethal whole-body analysis methods (Tankersley 2000; Gustafson et al. 2005; Sim-Smith & Jeffs 2011; Douda 2015). The development and optimalization of nonlethal methods for analysis of physiological biomarkers are also important and the need of nonlethal methods should be even more stressed out in case of endangered groups such as freshwater mussels. In this case the sacrifice of even a small number of individuals can be a threat to the whole species (Naimo et al. 1998; Haskell & Pan 2010). Other reason for focusing on nonlethal methods is the fact that the individual can be used

repeatedly in long term projects, and we can obtain more detailed and relevant data (Gustafson et al. 2005).

Spectrophotometric glycogen analysis of foot tissue samples is one non-lethal method that can be used for condition assessment of adult mussels. Glycogen is the primary energetic reserve in adult mussels (Ke & Li 2013; Cordeiro et al. 2017) and this reserve can break down into glucose that can be further transferred into tissues (Martínez-Pita et al. 2012). The glycogen level and fluctuation of the glycogen concentration reacts to many factors such as changes of environmental conditions, nutrition conditions, sexual and life cycle and different types of stress (Fisher & Dimock 2006; Dridi et al. 2007; Anacleto et al. 2013; Cordeiro et al. 2017). Therefore, glycogen concentration in mussel tissue can serve as an indicator for monitoring stress in different scenarios and even in ecotoxicological experiments (Yusufzai et al. 2010; Anacleto et al. 2013; Hazelton et al. 2014; Cordeiro et al. 2016, 2017).

The non-lethal method of glycogen analysis by Naimo et al. (1998) is based on spectrophotometry of phenol-sulphuric acid solution. This method isn't widely used nowadays in a research routine. The reason can be high workload and material consumption, and the spatial distribution and dynamics of glycogen in bivalve tissues isn't well studied. Aim of one study presented in this thesis was to simplify the methodology for glycogen analysis by Naimo et al. (1998) and its optimalization for modern laboratory equipment. We also aimed to lower the needed workload and volume of produced waste. Next aim was to compare glycogen concentration and variability in several types of tissues of *A. anatina* for optimalization of the sampling process for the subsequent analyses by the target method.

1.2.3 Effect of pollutants on host-parasite relationship of fish and unionids

Pollutants often have a major effect on the interspecies relationships (Coors & De Meester 2008; Brodin et al. 2014). The whole interspecies system can be exposed to the substance during research focused on this topic (Budria & Candolin 2014; Riedl et al. 2018; Cadmus et al. 2018). The nature of unionid relationships with their hosts gives us an opportunity to study the effects of chemicals on the complex system of affiliated species. The host-parasite relationship is affected by internal factors such as immunity response of the host or host-parasite compatibility – described in more detail in chapter 1.1. The external stressors affect individuals in this relationship as well and we can assume that it will also have an effect on their interaction. This ecological unit was used as an endpoint to the asymmetrical exposure to pharmaceuticals in a study presented in this thesis (STUDY 6). It was previously proven that the relationship between host and parasite can be altered by pollutants (Morley 2009; Hua et al. 2017) and that

there are records of changes in prevalence and intensity of parasitation in the host when the community is exposed to stress (Morley et al. 2006; Sures 2006; Milotic et al. 2017). We can assume that the relationship between unionids and fish hosts isn't an exception and can be altered by exposure to pollutants in water. The approach to the topic used in this thesis provides us with an opportunity to distinguish the influence of pharmaceuticals on individual sides of the host-parasite interaction and on the interaction itself as well.

There is a number of important pollutants in the freshwater systems such as heavy metals, pharmaceuticals or microplastics (Buřič et al. 2018; Amoatey & Baawain 2019; Santos et al. 2021; Yin & Zhao 2023; Khanjani et al. 2023). Pollution and decrease of water quality can affect physiology, anatomy and behaviour of water organism in many ways (Fitriawan 2011; Hazelton et al. 2014; Hedgespeth et al. 2014; Buřič et al. 2018; Santos et al. 2021). Other aspects that can be affected by pollutants are complex interspecies relationships and whole ecosystem interactions such as the effect on trophic levels (Daughton & Ternes 1999; Ginebreda et al. 2010) and transfer between them.

In Unionida, pollutants affect for example filtration activity of adults that can be monitored by valvometry (Vereycken & Aldridge 2023) and their avoidance behaviour could be used as a biomarker in ecotoxicological studies (Hartmann et al. 2016). Mussels also have the ability to accumulate heavy metals (Jing et al. 2019) and pollutants during chronic exposure to such an extent that it can alter their anatomy (Fitriawan 2011). It was proven that glochidia and juvenile mussels are sensitive to pesticides even in sublethal concentrations (Bringolf et al. 2007) and glochidia can also be affected by various metals (Markich 2017).

The tested substances were tramadol and methamphetamine. Tramadol is a widely used opioid, and methamphetamine stimulates the nervous system (Sulzer et al. 2005; Miotto et al. 2017). Both of these drugs are regarded to be environmentally important. They persist in the environment, are consumed in significant volumes (Fent et al. 2006; Baker & Kasprzyk-Hordern 2013) and can be found in treated sewage water and subsequently in water bodies such as rivers or streams (Boles & Wells 2010; Golovko et al. 2014; Santos et al. 2021). Their concentration in water can vary in time and space according to the input (Baker & Kasprzyk-Hordern 2013). Generally, the highest environmentally relevant concentrations are in values of hundreds of ng L^{-1} (Mackuľak et al. 2016; Grabicova et al. 2017). The designed concentrations that affect humans are higher than those found in waters but that doesn't rule out possible effect on the aquatic animals and other non-target organisms and possible effects of chronic exposure to them. Furthermore, the variation in concentration of the pollutants can result in unevenly exposed populations. There are studies proving effect of tramadol and methamphetamine on

the behaviour of aquatic animals such as crayfish (Buřič et al. 2018), dragonfly (Bláha et al. 2019) or fish (Liao et al. 2015) in small concentration of 1 μg L^{-1} or even less. Methamphetamine has an effect on the physiology of the animals such as the increase of heart rate in crayfish (Iqbal et al. 2023), several anatomical alterations and pathological changes in fish (Sancho Santos et al. 2020) after exposure to the same small concentration of 1 μg L^{-1}. There are results implying possible induction of addiction in fish that are chronically exposed to methamphetamine in water (Horký et al. 2021). Tramadol was proven to affect fish behaviour after a chronic exposure to low concentration of 1 μg L^{-1} (Santos et al. 2021).

Study presented in this thesis aimed to research possible effects of tramadol and methamphetamine on host-parasite relationship between unionid larvae and host fish in full factorial design with asymmetrical exposure of the species to separate the various effects of the chemicals. The research was conducted with the use of environmentally relevant concentrations of the used pharmaceuticals.

1.3 Invasive bivalve species in Europe with focus on *S. woodiana*

The host-parasite relationship described in this thesis is strongly affected by biological invasions (Donrovich et al. 2017). The topic of spread of non-native species is increasingly relevant and studied over the years. There is a number of non-native and invasive both freshwater fish and bivalve species in Europe that can be taking part in the host-parasite relationship and affecting it in various ways. Space for new experiments and studies of interactions between species that weren't cooccurring in the past was created by the biological invasions and can bring important new knowledge to the general understanding of mechanisms of these relationships. Non-native fish species can disturb this relationship by being a less compatible or even unsuitable host. This can decrease the probability of glochidia attachment on suitable host species (Douda et al. 2013).

Spread of the species

Important invasive species from the order Unionida is *S. woodiana*. Its original area is almost all of China, parts of Russia and it also natively occurs in Malaysia, Hong Kong, Japan, Taiwan, Cambodia and Korea (Watters 1997; Graf & Cummings 2007; Kraszewski 2007; Popa et al. 2007). In present day this species spread across almost all of Europe (Paunovic et al. 2006; Popa et al. 2007; Munjiu & Shubernetski 2008; Pou-Rovira et al. 2009; Lajtner & Crnčan 2011; Sîrbu et al. 2016; Donrovich et al. 2017; Bespalaya et al. 2018; Kondakov et al. 2018, 2020b,

2020a; Beran 2019). Its invasive area also spread to central America (Watters 1997) and some parts of Asia (Watters 1997; Bogan & Schilthuizen 2004; Bolotov et al. 2016; Vikhrev et al. 2017; Bogan et al. 2021). There is a record from one pond system in the USA (Bogan et al. 2011) where it was probably eradicated (Benson 2023). Recently there were first records of this species from Africa, specifically Algeria and Morocco (Mabrouki & Taybi 2022; Bensaad-Bendjedid et al. 2023).

S. woodiana was introduced into European waters in the sixties with imported fish from China's Yangtze River (Blue River). These fish were naturally infected with *S. woodiana* glochidia. Introduction with host fish such as grass carp *Ctenopharyngodon idella* (Valenciennes 1844), Chinese schemer *Hypophthalmichthys molitrix* (Valenciennes 1844) or bighead carp *Hypophthalmichthys nobilis* (Richardson 1845) (Kraszewski 2007; Soroka et al. 2014; Sîrbu et al. 2016; Kondakov et al. 2018) is probably almost exclusive pathway of *S. woodiana* into new areas. One exception is Italian Tuscany where *S. woodiana* was intentionally introduced for production of freshwater pearls (Clusa et al. 2017). This mussel can be found in almost the whole area of Czech Republic and it cooccurs with native species such as painter's mussel *Unio pictorum* (Linnaeus 1758), *U. crassus*, swollen river mussel *Unio tumidus* (Philipsson 1788), depressed river mussel *Pseudanodonta complanata* (Rossmässler 1835) and *A. anatina* (Douda & Čadková 2018).

Competition with native mussel species

S. woodiana has a number of advantages in the cross-species competition. Firstly, it is more resistant to worsened environmental conditions such as water eutrophication and pollution (Corsi et al. 2007; Douda et al. 2012b; Qu et al. 2016; Giari et al. 2017; Douda & Čadková 2018; Jing et al. 2019) having a higher tolerance to anthropogenic stress (Bielen et al. 2016). The competition between *S. woodiana* and native mussel species for hosts (Bauer et al. 1991; Dodd et al. 2005; Donrovich et al. 2017) is a significant topic in the *S. woodiana* invasion. As mentioned earlier, fish that never encounter glochidia (so called naïve host) are a better host because previous glochidia infestation triggers development of specific immunity response (O'Connell & Neves 1999; Dodd et al. 2005; Donrovich et al. 2017). Donrovich et al. (2017) have proven that if glochidia of *A. anatina* infest fish that previously encountered glochidia of *S. woodiana*, the metamorphosis success of *A. anatina* larvae is reduced by 45,4%. Benedict & Geist (2021) have shown that *S. woodiana* glochidia could have significantly higher thermal tolerance in comparison with cooccurring native mussels (specifically *U. crassus*) by testing glochidia viability during exposure to different water temperatures (5, 15, 17, 20, 25 °C) for a

relatively long period of time (up to 7 days). This difference in thermal tolerance means another advantage in competition for the host for *S. woodiana* and could be an even more significant advantage in future due to global climate change. *S. woodiana* populations at anthropocentrically heated localities can even show continuous reproduction and gravid females can be found there at any time of the year (Labecka & Domagala 2018; Labecka & Czarnoleski 2021). In addition, *S. woodiana* outperforms native species by reproductive traits such as the amount of offspring, metamorphosis success, and length of parasitic stage – glochidia of *S. woodiana* reach metamorphosis faster than swan mussel *Anodonta cygnea* (Linnaeus 1758) and *A. anatina* (Huber & Geist 2019).

Furthermore, this problem is deepened by the fact that *S. woodiana* is a host generalist (Douda et al. 2012b) and has a wider host range than some native species (Huber & Geist 2019). It can even use fish species that aren't suitable for native mussels in general such as *R. amarus*. (Reichard et al. 2012, 2015). This fact reverses the host parasitic dynamics between *R. amarus* and mussels and means one more advantage in competition between *S. woodiana* and native mussels.

Management

There is only a handful of articles about the eradication and prevention of spread of *S. woodiana*. Eradication of *S. woodiana* is almost impossible due to its benthic occurrence and high abundance. Sousa et al. (2014) provided summary of the possible methods for controlling invasive bivalves in the water bodies. These methods can be many forms of chemical treatment, gas impermeable benthic barriers or physical removal of individuals like harvesting mussels by hand picking or use of suction dredging. There is also the option of using desiccation, freezing, magnetic field, thermal shock and many more. The methods can be also divided into proactive and reactive groups. Reactive approaches target the adult bivalves meanwhile the proactive are focused on larval stages to prevent their settlement (Sousa et al. 2014). Every of these removal techniques has its pros and cons. It is important to evaluate the effect of other parts and organisms of the ecosystems in selecting the controlling approach. There are methods targeting the species specifically (for example physical removal of individuals), but most of the approaches affect a number of cooccurring species as well or even the whole habitat. We can learn from pathways of European spread in case of prevention of introduction of *S. woodiana* into new areas like North America or Africa (Mabrouki & Taybi 2022; Bensaad-Bendjedid et al. 2023).

The most cost-effective approach to the management of the invasive species tends to be prevention of its spread to new localities by increasing public awareness, legislative changes and monitoring programs (Finnoff et al. 2007).

Possible benefits

Eradication of *S. woodiana* in Europe is objectively impossible. On one side, there is a need for continuous research of its effects on native species and ecosystems. On the other side, we could look for benefits of this species to make the best from the situation. *S. woodiana* can live in areas with high anthropogenic stress where native species cannot thrive (Bielen et al. 2016) and acts as ecosystem engineers in the ways the native mussel would – contribute into dispersion of particles from sediment, purify water by filtration activity and their shells provide habitat for sedentary organism and shelter in the form of empty shells (Gutiérrez et al. 2003; Sousa et al. 2009; Vaughn 2018; Zieritz et al. 2022). Study by Yu et al. (2020) has shown that *S. woodiana* could decrease the negative effect of omnivorous fish – specifically bitterling *Acheilognathus macropterusas* (Bleeker 1871) – on water quality aspects such as nutrient concentration. The interaction between this mussel and fish could enhance growth of the periphytons. On the other hand, this interaction could have a negative effect on the recovery ability of submerged macrophytes.

There are attempts to use *S. woodiana* as fish feed (Konieczny et al. 2021) or in human diet (Stangierski et al. 2021). Their large spread, short parasitic period, fast growth, large amount of offspring and the fact that this species is a host generalist (Douda et al. 2012b; Konečný et al. 2018; Huber & Geist 2019) make them an easily accessible model species for Unionida research and for the study of variety of aspects that could have effect on host-parasite relationship in general in Unionids and for studies requiring production of multiple generations (Douda et al. 2021).

The overview of current research presented in this thesis aims to show trends in research areas and show possible topics that need to be more addressed in studies in the future. The paper that is in the submission process containing the overview of research papers among various topics aims to provide structural summarisation of current knowledge of *S. woodiana* research for better accessibility of the information to researchers.

2 Scientific Hypothesis and Objectives

2.1 Objectives

1: To test several important aspects of in vitro culture medium for rearing *M. margaritifera* glochidia for finding suitable culture medium for this mussel species. (STUDY 1)
2: To compare the vitality of mussels produced by in vivo (on live fish host) and by in vitro (on a Petri dish in culture medium) methods. (STUDY 2)
3: To develop a low-cost in-situ monitoring device for monitoring of detaching juvenile mussels under natural condition. (STUDY 3)
4: To test compatibility of specific populations of *M. margaritifera* and *S. trutta* by using fluorescence lipid quantification in juvenile mussels. (STUDY 4)
5a: To modernise glycogen quantification method in mussel soft tissues by Naimo et al. (1998) for modern laboratory equipment and reduce the material consumption of the method to enable its routine use in research for testing energetic reserves of adults used for experiments. (STUDY 5)
5b: To study variation of glycogen concentration among tissues of mussels for further optimalization of sampling for this analysis. (STUDY 5)
6: To study the effect of asymmetrical exposure to water borne pharmaceuticals (tramadol and methamphetamine) on host-parasite relationship of fish and mussels with use of environmental relevant concentrations of the used pollutants. (STUDY 6)
7: To make an overview of current research and knowledge of *S. woodiana* biological invasion for assessment of possible impacts and finding gaps in current knowledge. (STUDY 7)

2.2 Hypothesis

H1a: There is no significant difference in rate of developing glochidia of *M. margaritifera* in in vitro culture with and without added taurine to the medium mixture.
H1b: There is no significant difference in rate of developing glochidia of *M. margaritifera* in in vitro culture if a different culture medium mixture is used. Specifically with use of different types of serum (horse, calf) and lipid source (fish oil, ELM).
H2: There is no significant difference between the vitality of mussels produced by in vivo (on live fish host) and in vitro (in culture medium) rearing.
H3: It is possible to develop a simple and cheap system for in-situ monitoring of detaching juvenile mussels from fish hosts.

H4: There is no significant difference in lipid reserves of juvenile *M. margaritifera* based on population combinations of used fish host populations and populations of maternal mussels.
H5a: It is possible to modernise the method of glycogen content by Naimo et al. (1998) and make the method more efficient.
H5b: There is no significant difference of glycogen content among types of tissues of adult mussel.
H6: There is no significant difference in glochidia reactivity to NaCl, glochidia infection success, attachment rate and in spatial distribution of glochidia attached on host body between exposed and unexposed groups in asymmetrical exposure to tramadol and methamphetamine in fish and mussels.
H7: It is possible to identify gaps in current knowledge of *S. woodiana* global invasion and possible future impacts by making an overview of the current research focused on this topic.

2.3 Expected benefits of the research

Our finding about *M. margaritifera* were obtain for direct application in decision making in conservation program of this species in tested populations. We believe that our research contributes to an increase of effectiveness of local conservational program. Advancement in knowledge of in vitro rearing of Unionida also contributes to the conservation of these species as emerging tool to the propagation of mussels. Furthermore, possible effect on vitality of the juveniles produced without live fish host has to be taken in account in general scope of in vitro approach to Unionida propagation. Our research contributes into obtaining the needed knowledge for solving some of these problems and questions.
Development and optimalization of methods for study several aspects of host-parasite relationship between fish and mussels (biomarkers analysis and device for in-situ monitoring of the host and detaching juvenile mussels) have potential to bring attention to qualitative approaches to the evaluation of the host-parasite compatibility (e.g., simple biomarkers analysis methods presented in this theses). We believe that the development of in-situ monitoring low-cost device will be beneficial not only for research focusing on Unionida and their host but also for parasitologists, nutritional research and study of microplastics.
Our ecotoxicological study helps to better understand the influence of pollutants in freshwater environment and show us new possible effects of chemicals in treated sewage water. It can also provide us new insight into the problematics for future research and for prediction of possible effects of the pollutants. Our used interspecies relationship can be used as a model for a more

generalised approach. Results presented in this thesis can also be used as argument to show how important it is to develop new methods that properly remove pharmaceuticals from sewage water.

Last but not least, deeper knowledge and excessive research of invasive species such as *S. woodiana* could contribute to increasing the public awareness and results of such studies can be an argument for placing *S. woodiana* on List of Invasive Alien Species of Union concern (Union list) created by European commission. This legislative step would mean further regulation of this species in the European union.

3 Materials and Methods

3.1 Rearing of mussel juveniles under laboratory conditions

3.1.1 Effect of medium composition in an early stage of *M. margaritifera* in vitro cultivation (STUDY 1)

This chapter presents a short summary of methodology used in the study Escobar-Calderón et al. (2020). Please see the published study for detailed methodology.

3.1.1.1 Animals

The experiment was conducted by using *M. margaritifera* glochidia from the Vltava River basin, specifical Blanice river (48°55'34"N, 13°58'12"E) for trial 1 and Malše river (48°39'01.5"N 14°28'00.3"E) for trial 2. The maternal mussels were placed into a small tank with a shallow water level to stimulate glochidia release after natural glochidia releasement occurred in the monitored population. The viability of glochidia was evaluated immediately after extraction under a microscope and then they were placed into 5L vessels with fresh river water. The glochidia were immediately transferred into the laboratory (Prague University of Life Sciences, Czech Republic) where the in vitro cultivation took place.

Trial 1 was conducted with the use of a glochidia mixture collected from 35 maternal individuals, the mixture of glochidia collected from 3 females was used for trial 2. Females used for glochidia collection didn't spend more than 30 minutes outside the river and they were placed back into their original location within the river after the collection of glochidia.

Incubator with 4 °C temperature was used to store the glochidia in the laboratory before cultivation. The glochidia were rinsed with sterilized water to separate any clumps. The viability of glochidia was tested by a snapping reaction to NaCl (Roberts & Barnhart 1999). The glochidia used for trial 1 had lower viability according to this test than the glochidia cultivated in trial 2. The proportion of undeveloped glochidia was 50% and 5% in the mixture for trials 1 and 2 respectively. Subsamples of the mixture of glochidia were preserved in 70% ethanol for subsequent measurement of length.

3.1.1.2 Composition of cultivated medium

The study tested 3 factors of the cultivation medium using a full factorial design. The tested aspects were serum type, lipid source and taurine addition – each with two variants (levels).

There were 32 dishes in total for each trial – 8 treatments with 4 replications per treatment. Each treatment included 2mL of M199 medium (Sigma-Aldrich, product code M4530) per dish. The next component was 1 mL of serum per dish – horse serum (Sigma-Aldrich, product code H1270) or newborn calf serum (Sigma-Aldrich, product code 12133C). 50 µl of lipid source was added into each dish – fish liver oil (Sigma-Aldrich, product code F8020) or emulsified lipid mixture (ELM) (Sigma-Aldrich, product code L5146). The last tested parameter of the cultivation medium was an addition of taurine or no addition of this substance. 150 µg of powdered taurine (final concentration in dish = 42.86 µg/ ml, Sigma-Aldrich, product code T0625) was added to specific dishes. 0.5 mL of antibiotic and antimycotics mixture was also added into each dish to prevent infections. PSN mixture consisting of penicillin, streptomycin, and neomycin (Sigma-Aldrich, product code P4083) with added amphotericin B (Sigma-Aldrich, product code A9528) was used. The cultivation medium had 4:2:1 proportion of medium:serum:antibiotic mixture (Roberts & Barnhart 1999).

Prior to the placing of glochidia into Petri dishes with a cultivation medium, all dishes were placed into a CO_2 incubator (NB-203, N-Biotek, Korea) with UV light for one hour for sterilisation. The CO_2 concentration was 5% during the cultivation and the temperature was kept at 18 °C. The medium exchange was performed on the fifth day of the cultivation. The aim of the experiment was to keep the larvae in the medium minimally for 11 days since it is known that *M. margaritifera* glochidium can survive up to 10 days without a host (Jansen et al. 2001). It would not be sure if the survival rates observed during shorter cultivation than 11 days are a reaction to the medium composition or a non-tested factor.

3.1.1.3 Measuring the length of the larvae and evaluation of their reaction to the cultivation medium

Start of the metamorphosis was considered successful by observation of the closure of glochidia. If glochidia attach to live fish hosts in nature, they fully close due to exposure to the host tissue and remain closed afterwards (Jansen et al. 2001) and glochidia that close due to contact with an unsuitable surface open after a few minutes (Wood 1974). In case of death of the glochidia, the shell widely opens due to the relaxation of adductor muscles (Roberts & Barnhart 1999).

This criterion divided glochidia in our study into two categories – developing (closed) and non-developing (opened). The rate of developing and non-developing glochidia was quantified 3 times for each dish during each trial. These quantifications were performed on the second, fifth

and eleventh day of the cultivation. In each quantification, 50 – 60 glochidia were counted per dish and the rate of developing glochidia was calculated in each dish as the amount of closed glochidia over the total number of counted larvae in a specific dish. All remaining glochidia were used for the quantification in the case of dishes where a big portion of larvae was lost during cultivation. Length measurements of individuals were performed before and after the cultivation to obtain a more detailed data set. Subsamples of 8 – 25 juveniles per treatment were taken at the end of the experiment and placed into 70% ethanol. The length was measured from pictures taken by microscope at 40x magnification and then examined in ImangeJ software (Schneider et al. 2012). The length of glochidia was measured as the longest axis perpendicular to the hinge line of the animal.

3.1.1.4 Statistics

Statistical analysis was performed via generalised linear mixed models (GLMM) using a binomial distribution with the logit link function. Analysis was conducted in R version 3.6.1 (R Core Team 2019) with the package lme4 (Bates et al. 2015). Each trial was analysed separately because of a large difference in the variability of the data sets.

Fixed effects explanatory variables were the response lipid source and the presence or absence of taurine. The random effect variable was the dish for all analyses. A linear mixed-effects model was used for the analysis of the final length of the mussels. The linear mixed-effects model was used after testing the data set for normality with a quantile–quantile plot. Serum type, lipid source and taurine level were fixed effect factors, final length was the response variable, random factor was represented by the dish. Parameters were estimated by maximum likelihood in both models.

One-sample t-tests were used for each level and each factor for comparison of the difference between the initial and final length of the mussels and for assessment if there is a difference between the average change of the length from zero. A subsequent Bonferroni procedure (Holm 1979) was used to correct p-values obtained by the previous testing.

3.1.2 Assessment of the effect of in vitro rearing on subsequent vitality of the mussel population (STUDY 2)

This chapter presents a study focused on a comparison of the performance of in vitro (in culture medium) and in vivo (using alive fish host) reared mussels. Detailed methodology can be found at Douda et al. (2021).

3.1.2.1 Animals

The juveniles of *S. woodiana* were produced via in vitro cultivation for this experiment. There are several reasons why this species of mussel was chosen for this study. This mussel reaches sexual maturity in a short time (2 years) (Chen et al. 2015), the parasitic stage of their life cycle is relatively short (Huber & Geist 2019) and they can be easily cultivated in vitro.
Maternal individuals were collected in the Morava river (48°41'13"N, 16°59'19"E) 15th May 2018 and transferred to the laboratory of the Czech University of Life Sciences Prague (Czech Republic). Mussels were transported in 10 L aerated tanks with fresh river water. In the lab, glochidia were collected from six maternal individuals marked as A – F.

3.1.2.2 Rearing of juveniles

3.1.2.2.1 In vitro cultivation

The protocol for in vitro cultivation was based on a methodology used successfully in the past for rearing other species from the Anodontinae subfamily (Escobar-Calderón & Douda 2019). Cultivation medium consisted of M199 culture medium (Sigma Aldrich M4530), horse serum (Sigma Aldrich H1270) and a mixture of antibiotics (PSN mixture, Sigma Aldrich P4083) with antimycotic (amphotericin B, Sigma Aldrich A9528) in ratio 4:2:1 of volume, respectively. The medium was enhanced by 14.2 μL/mL of cod liver oil (Sigma Aldrich 74380).
The in vitro cultivation was performed in two different designs. The difference was in the volume of cultivation medium, the number of glochidia per dish and used maternal mussels. Method 1 had 17.5 mL of medium and 872 ± 189 glochidia (mean ± SD) per dish. The medium volume in method 2 was 10.5 mL per dish with glochidia counts 338 ± 147 glochidia (mean ± SD). The ratio of glochidia per medium volume was the same for each method. Method 1 cultivated glochidia from adults A, B, C and glochidia from maternal individuals D, E, F were cultivated by method 2. 12 dishes per maternal individual were used (total 36 dishes for each method). Dishes with medium were placed under UV into an incubator with 5% CO_2 for 1-hour prior to glochidia addition. Glochidia were rinsed in sterile water before being placed in the cultivation medium. Dishes were incubated in the CO_2 incubator with 5% CO_2 and a temperature of 24° C for 6 days. The juvenile metamorphosis was complete after this period. Then sterile water was added into each dish in a volume ratio 1:1 with cultivated medium. On the seventh day, the cultivation medium was completely replaced by water and the dishes were placed back into the incubator, but the CO_2 level was decreased to atmospheric concentration. Eighth day dishes were placed under stereomicroscope for quantification of active juveniles

and the metamorphosis success rate was determined as the rate of counted active juveniles/number of glochidia placed into the dish at the start of cultivation.

3.1.2.2.2 Juvenile rearing on live fish host

In vivo cultivation of *S. woodiana* glochidia was performed by using live fish host *R. amarus* and gudgeon *Gobio gobio* (Linnaeus 1758). *R. amarus* was collected at Kyjovka river (48°45'4"N, 16°59'32"E, Czech Republic), *G. gobio* at Lužnice river (49°18'54"N, 14°30'1"E, Czech Republic). The acclimatisation period in laboratory tanks was 2 weeks (between collection and glochidia infestation). Six individuals per species were infested per maternal mussel (36 *R. amarus* and 36 *G. gobio* in total). Fish infested by glochidia from the same maternal individual were placed into 6L of common glochidia bath for 15 minutes (4223 glochidia/L ± 1095 SD). After infestation, fish were held in individual 18L aquariums. The bottom of each aquarium was equipped with 3mm mesh to prevent predation of detached glochidia and juveniles by the host. Temperature was 25.3° C ± 0.7 SD and the fish feed was commercial fish flakes. Mortality of the host was less than 5% (3 individuals) during the experiment. Detached glochidia and juveniles were collected daily from each tank for 12 days. The recirculating system continuously flushed the juveniles and glochidia from the bottom of the tank into 139 μm filter mesh. Water flowed through the filter continuously to prevent the juveniles from drying out. The collected individual mussels were than evaluated under stereomicroscope and classified as live juvenile (with foot or valve movements) or dead (dead glochidia or dead juvenile – e.g., juvenile that is tightly closed with no signs of movement or permanently opened with no movement or even decomposing tissues). Metamorphosis success was quantified as the sum of live juveniles/sum of dead glochidia, and dead juveniles collected during 12 days of the observation period.

3.1.2.2.3 Statistical analysis of metamorphosis success

The differences in metamorphosis success among propagation methods (*Rhodeus*, *Gobio*, IV1, IV2) and used maternal mussels were tested. The evaluation was based on Type III sums of squares from a two-factor ANOVA with arcsine-transformed metamorphosis success as the response variable. Subsequent Tukey's HSD was performed for examination of pairwise differences among propagation methods ($\alpha = 0.05$). Metamorphosis success per dish and per individual host fish (according to propagation method) was used as a response variable.

3.1.2.3 Juvenile survival and growth

Random subsample of live juveniles from each rearing method was selected and monitored for 8 days with aim to monitor their growth and mortality. 2 – 20 juveniles per each rearing method from each maternal individual were placed into a tray (54 trays total). Each tray was filled with 250 mL of dechlorinated tap water and held at 24°C. 150 µL of commercial algae mixture (Plankto Marine P, Grotech; cell density ~ 25 × 106/mL) was added into each tray daily. After the eighth day, surviving juveniles were counted and a subsample from each tray was preserved in ethanol for subsequent measurement of the length (longest dimension parallel to the hinge) of the juvenile.

Two separate two-factor ANOVAs (based on Type III sums of squares) were used to test differences in survival and final size of juveniles (arcsine-transformed and log-transformed, respectively) among propagation methods and maternal mussels. There was subsequent Tukey's HSD performed to examine pairwise differences among production methods (α = 0.05). Mean survival or size among all groups per each propagation method/maternal mussels in each treatment method was used as a response variable in each analysis (N = 18 treatment combinations).

3.1.2.4 Sexual maturity of reared mussels, their growth and reproduction success

A subsample of reared juveniles from each presented method was selected randomly and placed into an outdoor facility near Lužnice river (49°18'25"N, 14°30'15"E). 1500 juveniles from *G. gobio* host and 1500 from *R. amarus* host were distributed into 12 aerated water tanks (approximately 250 juveniles per tank) with river sand at the bottom (grain size 0.5–2 mm, layer thick 3–5 mm). There were also 6 similar tanks with juveniles produced by in vitro method with also 250 juveniles per tank (3 tanks per each in vitro method). Feed was delivered into tanks twice a day, 100 µm of filtrated river water was used as feed. Water had ambient river temperatures and mussels were kept in these conditions for the rest of the first growing season and for their first winter.

8 individuals from each of the 18 tanks were randomly selected at the start of the second growing season (30 March 2019, 290–308 days post-metamorphosis). Their length was measured, and they were marked individually. Subsequently, they were placed into twelve 700L pools with river water and sediment. Juveniles from in vitro method 1 and 2 were put together because their length didn't differ. In each pond there were 12 individuals from only one of the rearing methods (in vitro or in vivo with use of *R. amarus* or with use of *G. gobio*). Each rearing

method had 4 replication pools. To sum it up, there were 12 pools, 12 mussels per pool and 4 pools per rearing method. Individuals from 2 different maternal mussels were placed into each pool to ensure mixed maternal origin of the groups to eliminate potential effects of maternal origin.

Pools were aerated, water was exchanged 12 times per day (75% of water exchanged daily). Water flowing into the pools went through a ~100 μm filter to prevent transport of glochidia from the wild into the system but allow feed transport for mussels. Sperm could pass through the filter – there are reports of *S. woodiana* population from Lužnice river (Beran 2019) but with low densities and there was no record of current *S. woodiana* in 5 km upstream from the facility.

Pools were emptied on the 237th day of the second growing season (November 2019, 527–545 days post-metamorphosis). Mortality was recorded and sediment was examined (2 mm sieve) in search of F2 juveniles. The shell length of the found F2 juveniles was recorded.

3.1.2.4.1 Statistical analysis

We used single-factor ANOVA to test differences in juvenile size at the end of the first growing season and winter between propagation methods. The response variable was log-transformed mean length within each mesocosm pool for this analysis. Growth at the end of the second growing season was expressed as instantaneous growth as described previously and based on the initial size at the end of the first growing season. The possible differences in growth of the juveniles among production methods in the second growing season were tested by single-factor ANOVA as well and the response variable was mean instantaneous growth within each mesocosm pool.

Single-factor ANOVA was also used to evaluate differences in the survival of the juvenile mussels among propagation methods at the end of the second growing season; the response variable for this analysis was arcsine-transformed survival in each pool.

Recruitment was expressed as a finite population growth rate (λ/yr) based on the final adult population size and the number of F2 recruits produced in each pool. Differences in λ and recruit size among propagation methods were tested by two separated single-factor ANOVA, response variables were λ or log-transformed mean recruit size in each pool.

R version 4.0.2 (R Core Team 2020) was used for conducting all statistical analysis.

3.2 In situ monitoring of host parasitic compatibility and development of low-cost monitoring system (STUDY 3)

Our study Douda et al. (2020) aimed to create a low-cost easy to use device that would allow researchers to monitor aquatic animals in-situ and monitor falling of particles individually for each host or monitored specimen. Firstly, we tested the accuracy of the device in capturing all detaching particles by using pre-counted microplastic particles in laboratory settings. Secondly, we tested the device in the field in host-parasite experiments where we collected juvenile mussels detaching from fish hosts. The study presents two simple experimental designs that can benefit from use of such a device to illustrate the possibilities that this device opens for field studies. Here I present a summary of the methodology used.

3.2.1 Construction of the floating system

The device (fig. 4) consisted of a polystyrene floating board (width 50 mm), the board was layered on the up and bottom side with polypropylene sheets to protect it from external influences (width 5 – 10 mm). Under this topper there were 5 individual tanks (cages) for keeping the tested animals. Each tank had its own falling of particles collecting system. The individual tanks were held in place by a structure from polypropylene sheets (5 mm thick sheets). The individual tanks were regular commercially available plastic boxes (volume: 20 L, length x width x height: 34 x 22 x 28 cm; T-Box S, Keter Italia S.p.A., Italy). There was a gap between the topper and the individual tanks to ensure the water circulation, but the width of this gap had to be adjusted according to the tested animal to ensure that the animal wouldn't escape through this gap.

The falling of particles collecting system was based on airlift pumping. There was a rising pipe (PVC, 20 mm diameter) in each individual tank. The falling of particles collecting system needed pressured air to operate, compressor (100 W, airflow: 110 L min^{-1}; air pressure: 0.035 MPa, 102 W; Hailea ACO-009, China – used for 3 devices) placed on shore was needed to ensure the pressure. The pressured air was deposited (by a silicone tube with 4 mm inner diameter) to the bottom part of the rising pipe. This ensured that the tube sucked water from the bottom of the tank and delivered particles to the top of the falling particles collecting system where 90° bend was directing water into a sample collecting filter positioned into a fitted box. Thanks to the fact that water flowed through the filter cups continuously and the filters were in a box the collected water organisms could survive there until recovered by researchers during routine check and sampling. The silicone hoses used for delivering the pressured air were

connected to one common hose (135 mm inner diameter) delivering the pressured air from the compressor. Each individual silicone hose had its own two-way regulating valve to manually calibrate the same water flow for each individual tank.

The sample collecting filter cups were made from PVC pipe with 115 mm diameter and 65 mm height and with nylon screen. The nylon screen had specific mesh according to monitored particles. (The loop size was 139 μm in our study). The optimal angle of the collecting filter was 45° against the rising pipe outlet.

There was one feeding and calibration port for each individual tank at the top board (ending 5 mm above the board) on the opposite side of the rising pipe. The inner diameter of the port was 10 mm. These ports were used for deponing feed and other needed particles (depending on the research).

At the time of construction (2019), the cost of material for 35 tanks divided into 7 floating units was around 1105 USD. The equipment used for construction was a plastic welding heat gun, electric saw, screwdriver and an electric drill.

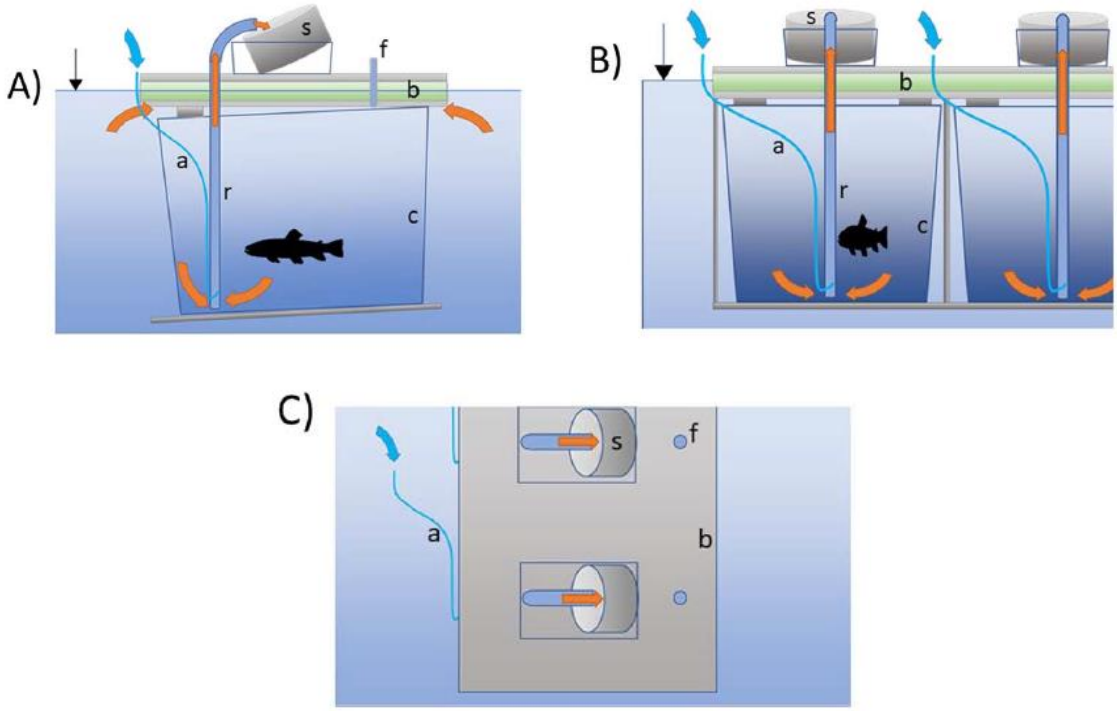

Fig. 4: Schema of the pontoon monitoring device. Side (A), front (B) and top (C) view. The device consists of these parts: a – air delivery hose, b – floating board, c – cage (individual tank), f – feeding port, r – rising pipe, s – filter cup. Arrows portray airflow (blue) and water flow (orange). Adapted from Douda et al. (2020).

3.2.2 Test of the system efficiency by microplastic particles

The test was performed in laboratory conditions. We used polyethylene microspheres – red Polyethylene Microspheres (1.12 g cc^{-1}, 500–600 μm, Cospheric – Santa Barbara, CA, USA). The fish species used for this experiment was crucian carp *Carassius carassius* (Linnaeus, 1758).

The fish originated from a laboratory breeding population from the Czech University of Life Sciences Prague. Before the experiment, the fish were placed into a 250L tank with a 15 °C water temperature and fed daily by pellets of commercial fish food (Pond Pellet, 5–6 mm; Tetra, Germany). The light-dark regime was 12:12 hours. The light regime and feed were the same during the experiment itself as well.

The system was placed into a laboratory tank (200 x 100 x 100 cm) with 1800 L of dechlorinated tap water at the same temperature as the tank where the fish were kept before placing into the system (e.g., 15° C). Five randomly selected animals were placed into the individual tanks of the system one day prior to the experiment to let the animals get accustomed to the new environment.

Food-grade surfactant (Tween 80 Biocompatible Surfactant, Cospheric, CA, USA) was used to ensure that the particles will not float or clump. We placed 106 – 114 particles into each individual tank. The counting of the recovered microparticles gathered at the sample collecting filters was performed 1, 6, 24, 48, 72, 96, 120, 144 and 168 hours after injecting the microparticles into the system. When the filters were taken out of the system for assessment, they were replaced with new ones.

3.2.3 Use of the system at host-parasite field experiment

The second test of the use of the floating system was in a field study. The animals used for this experiment were *S. trutta* and *M. margaritifera*. The particles that were collected by the system were juvenile *M. margaritifera* detaching from the fish hosts. The aim of the experiment was to evaluate the host-parasite compatibility of two different fish populations and two different mussel populations by monitoring the glochidia metamorphosis success rate.

The fish were caught by electrofishing (650 V, 4 A, pulsed D.C) in Živný potok stream (49°2' 39"N, 14°1'32"E, fish-A) and Častá stream (48°55'4"N, 13°40'27"E, fish-B). These localities belong to the Vltava basin and were selected because there is no *M. margaritifera* population nowadays which ensures no previous contact of the fish with glochidia. Fish were 6 – 13 days prior infestation individually marked and measured after anaesthesia (0.2 mL L−1; Merck

KGaA, Germany). After anaesthesia, each fish was equipped with one passive integrated transponder (PITs; Trovan ID100, 0.1 g in air, 12×2.1 mm; EID Aalten B.V., Aalten, the Netherlands) into the dorsal muscle. The fish were kept in a secured location in Šumava National Park hatchery – side-arm of Vltavský potok stream – until artificial glochidia infestation.

The *M. margaritifera* glochidia were collected in August 2018 from maternal individuals in the Vltava river basin. at localities at Blanice river (48°55'34"N, 13°5'12"E, Gloch-A) and Malše river (48°39'01.5"N, 14°28'00.3"E, Gloch-B). The maternal individuals were placed into 5L boxes with river water and were stimulated to release glochidia by low water level. The vitality of the glochidia was evaluated under a microscope. The glochidia were transferred to the place of infestation in a cooling container with 5L tanks with fresh river water. The mixture of glochidia from population Gloch-A was obtained from 35 females and from 5 females for population Gloch-B. Maternal individuals were placed back into the original locality after obtaining the needed glochidia. The infestation of fish hosts took place at Vltavský potok stream on the same day as the glochidia collection.

Common infestation baths with densities of 15 400 ± 3666 and 11 200 ± 3516 (mean ± SD) glochidia L^{-1} for populations Gloch-A and Gloch-B were used, respectively. The vitality of the glochidia was evaluated before the infestation by snapping reaction in NaCl solution (Roberts & Barnhart 1999). The average reactivity to the NaCl solution was 31% and 74% for populations Gloch-A and Gloch-B respectively.

Host fish were placed into the infestation bath for 15 minutes with a volume of 1L per fish. The control fish group (non-infested) went through the same handling process, but they were placed into water without glochidia. The fish hosts were released into semi-natural conditions after the infestation (side-arm of the Vltavský potok stream with a gravel and sand mixture and a nearby earth pond).

Monitoring of the detaching juveniles via our system started in June 2019 when the sum of temperatures reached the value that is usual for the start of *M. margaritifera* juveniles falling off the host (Hruška 1992).

Seven monitoring units with 5 individual tanks per each (35 individual tanks in total) were placed directly into a secured stream in the same locality as where the fish were held after the infestation until this monitoring of falling juvenile mussels. The fish were caught by electrofishing (details above) and placed into individual monitoring tanks where they were kept for 6 to 8 days.

The feeding ports were functional but not used due the fact that the stream had live aquatic invertebrates present and regularly found in the collecting filters. We assumed that the suitable feed is delivered into the tanks via natural water flow in the stream.

The samples collecting filter cups were examined every 1 – 2 days. The filter was exchanged for a new one and rinsed with river water. The content of the filter was then evaluated under a microscope (10 – 40x magnification) on the same day. The found juveniles were categorised as live or dead based on the presence of any movement. All live juveniles were sampled into 4% formaldehyde for future lipid quantification (see more at STUDY 4). The fish hosts were returned to the sampling site of their origin after the experiment.

Metamorphosis success during the monitoring period (percentage of live and dead juveniles collected) and rate of parasite detachment from the host (number of juvenile mussels per day^{-1} g^{-1} of fish body weight) were calculated.

The monitoring system efficiency of flushing live mussel juveniles was tested by placing 3 live uninfected fish into the system. 36 – 74 live mussel juveniles were delivered into the system via feeding port. The flushing of the juveniles was then monitored for 96 hours as described in the previous efficiency test (chapter 3.2.2).

Paired Wilcoxon rank-sum test was used for the determination of the presence of differences of juvenile detachment rate and metamorphosis success (arcsine-transformed proportion of viable juveniles) between combinations of host and mussel populations. Fish condition factor and temperature differences were tested by paired t-tests. R 3.5.2 (R Core Team 2019) was used for all statistical analyses.

3.3 Evaluation of health status and vitality

3.3.1 Evaluation of host quality based on quantification of lipid reserve in juvenile mussels (STUDY 4)

This chapter presents the methodology of research which publication is in preparation (Vodáková et al. in prep.). Therefore, here I present a detailed methodology of the study.

3.3.1.1 Collection of samples

S. trutta infested by *M. margaritifera* glochidia were placed into the system which allows collecting falling off juvenile mussels from each individua host separately. The samples were collected during sampling presented at STUDY 3 (June 2019) and placed into microtubes with 4% formaldehyde immediately after sampling and kept in 4 °C until analysis.

For the use in the publication that is in preparation, juveniles were described according to their original population as Malše (MAL) and Blanice (BL) and fish according to Blanice (BL) and Častá (CA) river where they were collected. These populations are homologous to populations used in STUDY 3 (mussels MAL = Gloch-B, BL = Gloch-A; fish BL = Fish-A, CA = Fish-B).

3.3.1.2 Laboratory analysis of the samples

The laboratory analysis of the sampled juveniles took place in December 2020 and January 2021. We selected 378 juveniles from collected samples for the fluorescence analysis. The sampled juveniles were randomly selected with the aim to equally distribute the number of analysed samples between combinations of host and mussel populations.

The first step was to clean the samples from formaldehyde that could interfere with the staining process. We placed the samples into a test tube with 35 mL of distilled water and put the test tubes on the shaker for 5 minutes. The shaking was repeated after the water exchange.

The used stain was Nile red. Firstly, the stock stain solution was prepared. The concentration of the stock stain solution was 100 µg/mL and it was kept at 5 °C for the maximum of one month. This stock solution was used for making daily staining solutions. The daily staining solution was prepared by diluting the stock staining solution with distilled water to reach a final concentration 5 µg/mL.

Analysed samples were placed into the daily staining solution for 30 minutes. Then samples were transferred into distilled water to remove redundant colouring. The photos using a fluorescence microscope (Nikon Eclipse Ni-E) had to be taken within 30 minutes after putting samples into the distilled water.

The hardware used for the microscopy was B-2A Longpass filter cube (Nikon). This filter cube had B excitation, 25 mm filter diameter, 470/40 nm excitation filter, 505 nm dichroic mirror, and 510 nm barrier filter. The software used for the microscopy and taking pictures of the juveniles was NIS-Elements software (Nikon). The exposure of the microscope's camera was set to 10 ms with 3.4x analogue gain. The data from taken photos was extracted by using features of the NIS-elements software. Data for each juvenile was extracted separately. The automatic selection of object (in our case selection of juvenile) was used for each photo and the selected area was subsequently manually corrected to align it with the juveniles' edges. Photos from different layers of the organism were taken for each juvenile. We manually selected the lowest and highest point of the juvenile. The software took a series of photos of the organism with 10 µm height between each picture. For the analysis we used the central layer of the

juvenile as the representative one (if the series of photos had an even number of layers, the mean of the values of two central layers was used for data analysis). The data extracted from the photos were the area of the juvenile, perimeter of the area, mean intensity of the emitted light and sum of the intensity of the emitted light.

3.3.1.3 Data analysis

Firstly, the length of the juveniles was analysed. A fitted linear model was used, the predictors fish population and mussel population with the fish ID were used as a random intercept for this model. The fluorescence data was analysed by fitting a similar model. Lipid content measured as the mean intensity from the fluorescence test acted as response variable for this analysis and predictors were fish population and mussel population. The time of processing of the samples each day was used as a random intercept alongside the fish ID because the Nile Red staining solution is affected by time from preparation and light exposition.
Models with intercepts were exchanged for simple additive models because of worse fitting (as per AIC – Akaike information criterion) and the interactions were not significant. To avoid overfitting the random effects were tested using a likelihood ratio test as indicated by Molenberghs & Verbeke (2000). Likelihood ratio test was used to test the significance of the fixed effects.
R version 4.0.1 (R Core Team 2020) was used for all performed statistical analyses. Packages lme4 and lmerTest (Bates et al. 2015; Kuznetsova et al. 2017) were used in tests for mixed effects models.

3.3.2 Health assessment of adult mussels based on quantification of glycogen reserves in soft tissue (STUDY 5)

This chapter presents modernisation of methodology by Naimo et al. (1998) and assessment of the spatial distribution and variance of glycogen among different types of soft tissues in mussels. Detailed methodology and step-by-step protocol can be found in Vodáková and Douda (2019).

3.3.2.1 Sampling

The method was tested by using samples from duck mussel *A. anatina*. This species is widespread in Europe (Lopes-Lima et al. 2017) and there is a possibility of its use in biomonitoring (Falfushynska et al. 2013). We used 6 adult animals from the Lužnice river,

Czech Republic (49°18'2"N, 14°30'14"E). Live captured individuals were transported to the lab in a 25L tank with river water and aeration. The tissue biopsy from the collected individual was performed the same day at the laboratory at the Czech University of life Science, Prague (Czech Republic) (November 6th, 2017). The tissue samples were collected from the posterior part of the foot, medial part of the mantel and medial part of the gills by using scissors. The tested animals were then sacrificed by cutting the adductor muscles to collect the anterior adductor muscle tissue as well. The aim was to collect samples in the range from 4 to 10 mg. This size of tissue samples (excluding the adductor muscles and gills) was determined as suitable for non-lethal biopsy by Naimo et al. (1998). We collected 68 samples with sizes between 2.17 and 12.68 mg (mean 7.36 mg). The samples were stored at –75°C for up to 40 days.

3.3.2.2 Standards and calibration samples

The samples were analysed by using a method based on Naimo et al. (1998) with a number of modifications – see supporting information section of Vodáková & Douda (2019). The bias of the modified methodology was tested by analysis of a sample set with known glycogen concentration e.g., *matrix standard*. Triplicate analyses of all matrix standards were used to determine the precision of the method – relative standard deviation (RSD). 11 *blank samples* from 3 analysed sets were used to estimate the method detection limit (MDL) according to the EPA (2016) guidelines. Each analysed set consisted of tissue samples, calibration standards and internal standards.

The calibration standards consisted of 40 mg of powdered oyster glycogen (type II, Sigma-Aldrich, St. Louis, Missouri) dissolved in 20 mL deionized water and then diluted to make solutions with concentrations 2 000, 1 000, 500, 250, and 125 mg/L of glycogen. These diluted solutions were prepared right before analyses.

The internal standards consisted of *in-house reference material* that was prepared according to Naimo et al. (1998). The homogenised foot tissue of six adult *A. anatine* individuals sampled on June 24th, 2017. The homogenization was ensured by adding 2 ml of 30% KOH (Penta, Prague, Czech Republic) per 1 gram of tissue into cryovial (Simport, Beloeil, Quebec) and then heated for 20 minutes in boiling water bath (RTC Basic, IKA, Wilmington, North Carolina). The boiled sample was vortexed for 10 seconds (MS2 Minishaker, IKA) and stored at –75°C until analysed. Before analyses the sample was thawed at room temperature and vortexed on each analytical date. The analyses were performed for 3 analytical days.

The reference material was used to make *spiked calibration standards*. Preparation of the spiked calibration standards was the same as preparation of the aqueous calibration standards but 10 μL of the in-house reference material was added to spike the solution instead of powdered oyster glycogen.

3.3.2.3 Analysis of tissue samples and glycogen quantification

The samples were placed into 3-mL cryovials. 100 μL of 30% KOH was added into the cryovials with tissue samples as well into 3-mL cryovials with blank solution and in-house reference material. 280 μL of 30% KOH was added into the cryovials with spiked sampled and aqueous calibration standards. The cryovials were boiled in a water bath for 20 minutes to ensure homogenisation of the samples. After boiling, 96% ethyl alcohol (Penta) was added to the cryovials. The added volume of ethyl alcohol was 1.5 times bigger than added KOH. The samples were boiled in a water bath for 15 minutes. After the second boiling, deionized water was added into the cryovials to the same total volume of the solutions. After this step, 2600 μL of the solution was removed from each cryovial. Subsequently, 390 μL of deionised water was added into each sample.

After homogenisation and dilution of the samples, the diluted samples were prepared for spectrophotometry. The needed coloration for spectrophotometric analysis was ensured by the addition of 40 μL of 80% phenol (Carl ROTH, Karlsruhe, Baden-Württemberg, Germany) and 2,180 μL of 96% sulfuric acid (Penta) into each cryovial. 250 μL of solution from each cryovial was pipetted into 96-well microplate (Anicrin S.R.L., Scorze, Venice, Italy). The plate was placed into a spectrophotometer to measure the absorbance of each solution. Absorbance values of triplicate samples of aqueous calibration standards were used to calculate the calibration slope for the calculation of glycogen concentration in the target samples. Each analytic day had its own calibration slope calculated with linear regression models.

We calculated the mean recovery of glycogen in spiked samples, the mean CV (coefficient of variance, 100·SD/mean) (SD) for each concentration of aqueous calibration standards, and the mean percentage difference between slopes. The calculation was performed from 3 replicated samples for each from 4 analysis dates according to Naimo et al. (1998).

3.4 Ecological impact of water borne pharmaceuticals on host-parasite relationship between mussels and their host (STUDY 6)

This chapter describes methodology of study published at Douda et al. (2019). See the paper for a detailed methodology.

3.4.1 Tested chemicals

The tested chemicals were tramadol hydrochloride (Sigma-Aldrich, USA) and methamphetamine (Lipomed, USA). Stock solutions had a concentration of 10 mg L^{-1} in ultrapure water of resistivity 18.2MΩcm, prepared by Young Lin AquaMax Basic 360 Series and Ultra 370 Series instruments that were purchased from Labicom (CZ)

Stock solutions were used for the preparation of the exposure solutions by diluting the stock solution with dechlorinated tap water. Exposure solutions had a concentration 1 μg L^{-1} (equal to 6.7 nmol L^{-1} of methamphetamine and 3.8 nmol L^{-1} of tramadol). The concentration of the studied chemicals in exposure solutions was determined by liquid chromatography with tandem mass spectrometry (LC–MS/MS; Thermo Fisher Scientific, USA).

The water samples were filtered (cellulose with 0.20 μm pores, Labicom, CZ) and kept at -20 °C until the laboratory analysis. Frozen samples were allowed to thaw at room temperature before the analysis. Internal standard was added to the samples and 20 μL of the sample were injected on a Hypersil Gold aQ column (50 x 2.1 mm; 5mm particles). It was coupled with an Accela 1250 LC pump and a TSQ Quantum Ultra Mass Spectrometer (Thermo Fisher Scientific).

The mobile phases for the LC–MS/MS analysis were acidified by formic acid (Labicom, CZ) acetonitrile (LC–MS grade purity, Sigma-Aldrich, USA) and ultrapure water. The calculation was based on the isotope dilution method (D3-tramadol, Lipomed, USA; D5-methamphetamine, Chiron). The limits of quantification (LOQs) were 0.008–0.013 μg L^{-1} for aqueous methamphetamine and 0.039–0.046 μg L^{-1} for tramadol.

3.4.2 Experimental animals

A. anatina glochidia and European chub *Squalius cephalus* (Linnaeus, 1758) were chosen as a fish host for this experiment. *A. anatina* was chosen because of its wide abundance in central Europe (Lopes-Lima et al. 2017) and *S. cephalus* is one of the suitable host species of this mussel (Douda et al. 2013) with big abundance in the region of central Europe (Kottelat & Freyhof 2007)

Maternal mussels were collected at Vltava River (48°56'55.7"N, 14°27'49"E for experiment I and 49°18'40.3"N, 14°30'8.6"E for experiment II). The sampling sites were selected with the aim to minimise the possibility of previous exposure to the tested pollutants – the localities were upstream from wastewater effluents. The mussels were transported into the laboratory at the Czech University of life Science, Prague (Czech Republic) where they were kept in aerated tanks. The collection of glochidia was performed 2 to 6 weeks after the capture of maternal individuals and performed right before the inoculation. The glochidia collection was done by rinsing the marsupia of female mussels with water using a syringe (Douda et al. 2013). Five maternal mussels were used to obtain glochidia per each experiment. Glochidia collected from each maternal individual were tested to estimate their viability by reaction to concentrated NaCl solution. The rate of viable glochidia was more than 95%. The glochidia from different maternal mussels were mixed into one glochidia mixture and rinsed with dechlorinated water to disperse clusters and then cleaned from the remaining marsupia tissue.

The fish individuals used as hosts were obtained from hatchery via local fish trade (Vodňany, Czech Republic) to ensure that the host had no previous contact with glochidia. All the fish were over one year old.

The source population (320 fish) was randomly divided into four groups with 80 fish per group and held in four 200 L tanks two weeks before the experiment. 2 tanks were used for exposure of the fish to the target chemicals and two were used as control groups with clean water. The risk of cross-contamination of control groups was minimized by placing these tanks in different laboratories. Fish used for the experiment were chosen randomly from each tank. 28 fish were used for tramadol and 32 for methamphetamine experiments.

3.4.3 Exposure of animals to the chemicals

Methamphetamine and tramadol were used for the study in nominal concentrations of 6.7 nmol L^{-1} and 3.8 nmol L^{-1} of pure compound respectively. The exposure period was 42 days and 24 hours for fish and glochidia respectively. The length of the exposure period and the concentration of chemicals were established based on the estimation of realistic natural conditions. The glochidia are able to successfully infect the host only for a few days after releasement by the maternal individual and sediment quickly. Ecologically relevant are only toxicity tests performed within 24 hours after releasing of the glochidia (Farris & Hassel, 2006). The measuring of actual concentrations of the target pollutants in water was performed at the start and the end of the exposure period in tanks with glochidia (4 exposure chambers) and it was performed six times during the exposure period of the fish. The water samples at the fish

tanks were collected before and after the water exchange. Water samples were stored at -20 °C until the laboratory analysis. Samples were thawed at room temperature prior the laboratory analysis. The samples were tested by LC/MSeMS after the addition of internal standard.

The exposure of glochidia was performed according to the standard design used in in toxicology glochidia tests (Farris & Hassel 2006). The exposure period was 24 hours and glochidia were placed into 300 mL glass beakers with 150 mL of solution covered with aluminium foil. The exposure period started within 3 hours after obtaining the larvae. There were approximately 1000 glochidia per beaker in seven to eight replicas per treatment and controls.

Tramadol and methamphetamine solutions were added into each fish tank with exposed fish after every water exchange. Fish host individuals were randomly selected from the tanks – 16 for the methamphetamine experiment and 14 for tramadol – for each exposed and control groups.

3.4.4 Data collection

Glochidia were tested for reactivity to NaCl stimulus, and their snapping activity was recorded as a marker of their viability. The time needed for the snapping action to occur was also recorded for each tested glochidium.

Samples of 30 – 50 glochidia were collected (16 per methamphetamine experiment – 8 exposed and 8 control, 28 per tramadol experiment – 14 exposed, 14 controls). The sample was placed under a microscope (10x magnification) into a Petri dish with 30 mL of dechlorinated tap water. The sample was video recorded and NaCl solution (200 µL with concentration 130 g L^{-1}) was added after 2 minutes from the start of recording. The recording was stopped 2 minutes after this addition.

The remaining tested parameters were based on the controlled infestation of the host by the glochidia. Full factorial design was used to create the combinations of exposed and control groups of the partners. The glochidia concentration in the infestation batch was an environmentally relevant density value. The infestation was performed in individual 3L tanks (AHAB, Pentair Aquatic Habitats, FL, USA) and infested fish hosts were monitored for 24 hours in the same type of tank. Fish were placed out of the exposure tanks two hours before the infestation and placed into 1–2 L of aged tap water in the individual tanks. 1090 ± 271 (mean ± SD) glochidia were added into each tank for the infestation. The infestation period was 15 minutes. Subsequently, fish were transferred into glochidia-free individual tanks. The glochidia from the infestation tank were collected to count the real concentration of each individual infestation bath. After 24 hours of monitoring, fish hosts were overdosed with anaesthetics and

dissected for quantification of attached glochidia. The detached glochidia from each individual tank were collected as well.

The spatial distribution of attached glochidia on the host was calculated individually and represented as gill ratio. The next calculated parameter was the attachment rate of the glochidia. The number of initially attached glochidia was divided by the number of glochidia added into the initial infestation bath to calculate the attachment rate. The last parameter was infection success. This parameter was defined as the proportion of initially attached glochidia that remained attached during the entire 24 h monitoring period. The total number of used fish was 32 in the methamphetamine experiment (8 per treatment) and 28 fish in the tramadol experiment (7 per treatment).

3.4.5 Statistical analysis

The assumption of homogeneity of variance and normality of the data set was tested by Levene's test and subsequently a Kolmogorov-Smirnov test before the statistical analysis itself. Welch's two sample t-test was used to compare water quality and reactivity of the exposed and unexposed glochidia. Generalized linear models (GLM, logit link function and a quasi-binomial error structure) were performed to compare the infection parameters of the individual hosts among treatments. Attachment rate, gill ratio, and infection success were presented as dependent variables and treated as binary.

Tukey's post hoc test using the glht function in the R package multcomp (Hothorn et al. 2008) was used for multiple comparisons – pairwise differences among host species. R 3.5.0 software package (R Core Team 2018) was used for all the performed analyses.

3.5 State of the art of the *Sinanodonta* spp. research (STUDY 7)

The overview of the research paper published on *Sinanodonta* spp. (Modell 1945) research was performed during work on a review paper (Douda et al. under review) focused on *Sinanodonta* spp. with emphasis on their invasiveness. A systematic overview of the literature started by searching a list of papers on SCOPUS database by the string "Sinanodonta or woodiana" in August 2022. This string was also used to search papers using the old name of species S. *woodiana* which used to be *Anodonta woodiana*.

All available results were manually classified into categories of theme and country (country of main author's affiliation and place of research in case of field studies outside of main author's country of affiliation). Duplicates, secondary literature, and studies outside the scope of the

review article were eliminated from the data set (for example studies focusing on different species). The final data set consisted of 266 publications.

Publications were sorted into these eleven primary categories: genetics, biogeography, ecology, ecotoxicology, anatomy, commercial use and aquaculture, ecosystem services, conservation, physiology, reproductive biology, systematics. Secondly, papers centred around the *Sinanodonta* spp. invasion in any way was divided into separated secondary categories that were: prevention, distribution, impact, control, genetics, ecology in the invasive range.

It is common for studies to cover more than one presented category. Each study was placed into only one category that represents the main topic of the paper. The categorisation was based on the consensual evaluation of at least two study coauthors.

3.6 Ethics statement, acknowledgment and fundings

All the sampling and experimental work were in compliance with the current laws of the Czech Republic Act No. 246/1992 coll. on the Protection of Animals against Cruelty which derived from the Directive 2010/63/EU and required qualification according to Law no. 246/1992, § 17, art. 1.

The number of host fish and all methods used to comply with the reduction, replacement, and refinement principle in animal experimentation. In addition, I would like to thank local nature conservation authorities for providing access to the research area and facilities and for providing needed permits.

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4 Results and Discussion

Performed experiments are closely related (fig. 5) by topic, tested animals and central European region. Some of the experiments were performed in tandem design, for example juveniles used for STUDY 4 were a subsample of juveniles collected during STUDY 3. This collective approach enables us to obtain a robust data set that can be viewed from various angles and gives us connections and insights that could not be possible within simple designs.

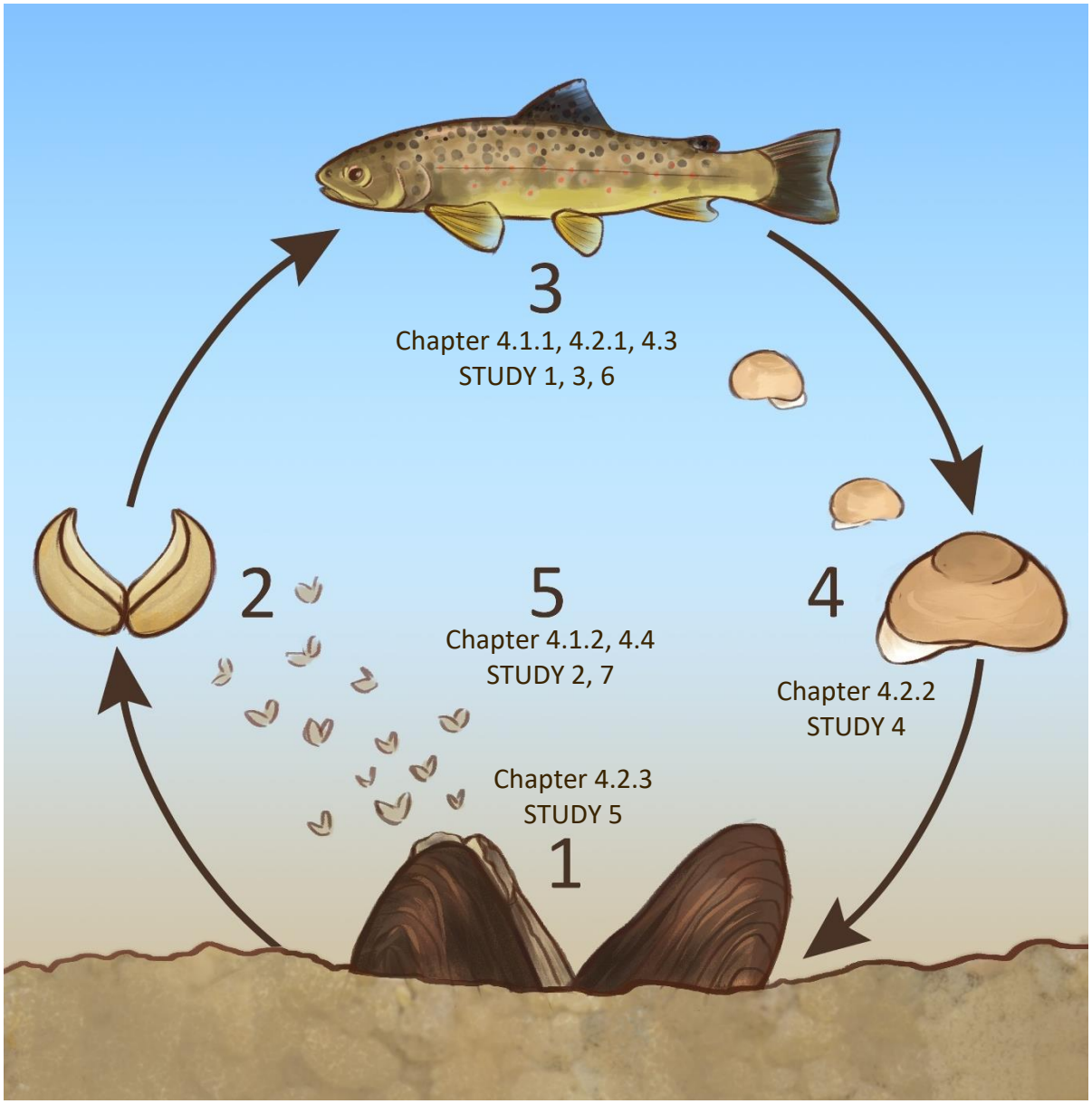

Fig. 5: Presented studies are shown in context of host-parasite life cycle. 1 – Adult mussels (buried in sediment, releasing glochidia): Health assessment of adult mussels based on quantification of glycogen reserves in soft tissue (Vodáková & Douda 2019); 2 – glochidium; 3 – host fish (that glochidium attaches on): a) Effect of medium composition in an early stage of *M. margaritifera* in vitro cultivation ((Escobar-Calderón et al. 2020); b) In situ monitoring of host and parasite compatibility and development of low-cost monitoring system (Douda et al. 2020) c) Ecological impact of water borne pharmaceuticals on host-parasite relationship between mussels and their host

(Douda et al. 2019); 4 – detaching juvenile mussel: Evaluation of host quality based on quantification of lipid reserve in juvenile mussels (Vodáková et al. in prep.); 5 – complex studies describing whole life cycle: State of the art of the *S. woodiana* research (Douda et al. under review), b) Assessment of the effect of in vitro rearing on subsequent vitality of the mussels population (Douda et al. 2021). Original illustration by the author of this thesis (Vodáková 2023).

4.1 Rearing of mussel juveniles under laboratory conditions

4.1.1 Effect of medium composition in an early stage of *M. margaritifera* in vitro cultivation (STUDY 1)

The study focusing on in vitro rearing of *M. margaritifera* evaluated several aspects of the medium and their effect on the rearing success in the first 11 days of cultivation. Here I present the most important results of the study, for detailed results please see Escobar-Calderón et al. (2020). You can see the overview of the results shown by box plots in fig. 6.

Tested aspects were the type of serum (calf, horse), lipid source (ELM, fish liver oil) and taurine content (mediums with added taurine or without). The experiment was conducted in two trials (Trial 1 and Trial 2) and there were 3 quantifications each time (second, fifth and eleventh day of the cultivation). The medium effect was evaluated by the average rate of developing glochidia in the dishes and final length of the glochidia.

Both trials had almost the same average proportion of developing glochidia (Trial 1 = 32.38 ± 17.39%, Trial 2 = 33.59 ± 27.67%). The type of serum had a significant effect on both trials, but the effect was not consistent. A significantly higher proportion of developing glochidia was recorded for each serum among the quantification dates and one of the used serums cannot be pointed out as clearly more suitable.

The second tested media compound was the lipid source. The only quantification where fish oil had a higher rate of developing glochidia was the third quantification of Trial 2 (ELM = 18.68 ± 17.26%, fish oil = 21.21 ± 22.33, GLMM: $z = 0.292$, $P = 0.77$) but the difference was not significant. Contradictory, ELM performed significantly better in 3 quantifications.

Last but not least, the effect of taurine addition was also evaluated during the experiment. Taurine had no significant effect on the average rate of developing glochidia in Trial 1. The only significant difference for taurine was found during the first quantification of Trial 2.

The final length of the glochidia wasn't significantly related to any of the monitored medium compounds. Moreover, the average final length was smaller than the average initial length of the glochidia.

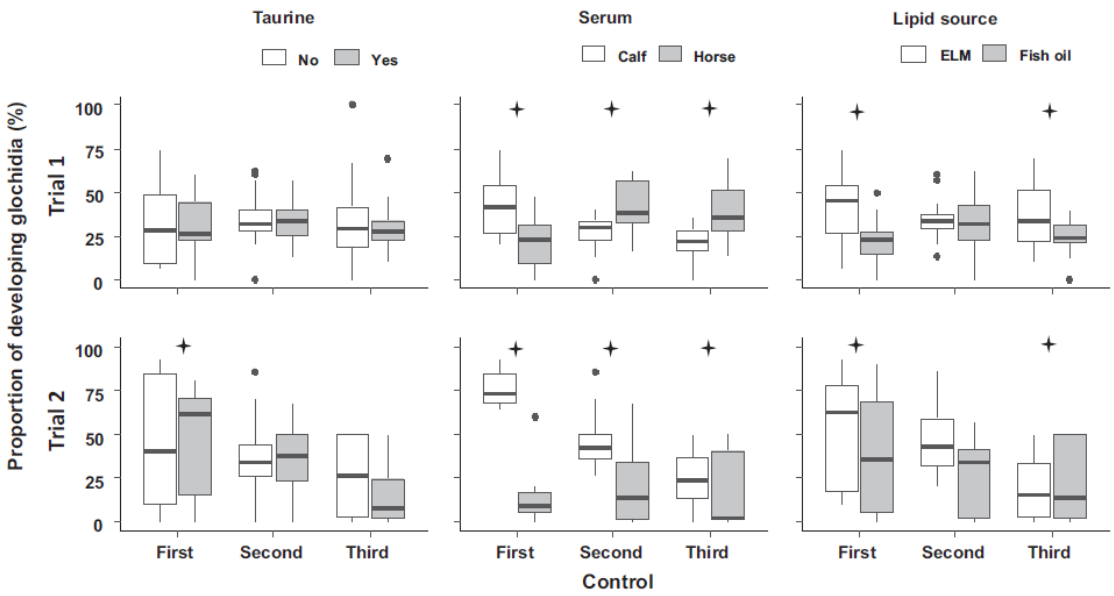

Fig. 6: Effect of medium components on the rate of developing glochidia (*Margaritifera margaritifera*) during three subsequent quantifications. Effect of taurine addition, type of serum (calf, horse) and type of lipid source (ELM – emulsified lipid mixture, Fish oil). Stars are marking statistically significant differences ($p < 0.01$). Published at Escobar-Calderón et al. (2020).

To sum it up, the effect of taurine was found to be statistically significant at only one from 6 performed quantifications. The type of serum had a significant effect during the whole process, but the results were not consistent and the type of serum which performed better varied during the experiment. The aspect that had a significant effect on improvement of the rate of developing glochidia for the majority of the study (except two quantifications) was the use of ELM as a source of lipids.

Glochidia usually don't grow during the parasitic stage and metamorphosis but there are exceptions. The final size of the detached juvenile can be even seven sizes of the initial glochidia length in some species (Barnhart et al. 2008) and *M. margaritifera* grows significantly during the parasitation stage as well. It can grow in length even by 670% during the larval development (Chowdhury et al. 2018; Castrillo et al. 2022). The growth during metamorphosis is recorded mostly for species with small glochidia such as *M. margaritifera* (Barnhart et al. 2008). The in vitro rearing of such glochidia is not possible nowadays. The only method with partial success was performed by (Taskinen et al. 2011) with the use of a combination of in vitro and in vivo rearing. The reason for the problematic rearing of species with growing glochidia during the larval stage is probably their higher nutritional demand during the parasitic stage (Kern 2017; Wen et al. 2018).

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, the importance of taurine for the development of freshwater larvae is supported by for example a study by Wen et al. (2018). Ther results show taurine as a promising additive for culture mediums. They reared glochidia of pink heelsplitter *Potamilus alatus* (Say 1817) which is a host-specialist freshwater mussel with glochidia growing during metamorphosis. They suggested that taurine could be an important factor for the success of their method based on the fact that they had better results (e.g., higher growth rate) by using fish plasma with higher taurine level. Our data didn't support this theory; however, it can play an important role in later stages of development or can vary based on osmotic conditions of the environment that the glochidia is exposed to during the parasitical stage.

Freshwater environment is mostly hypoosmotic and adult freshwater molluscs have relatively low levels of taurine concentration in soft tissues in comparison with marine molluscs (Simpson et al. 1959; Huxtable 1992). On the other hand, there is a possibility that glochidia are exposed to hyperosmotic conditions during the parasitic development due to the contact with plasma and other fluids of the host body. More research is needed to fully understand the interaction and chemical influences between host body and encysted glochidia.

The effect of different lipid sources on the rate of developing glochidia was significant in four out of 6 quantifications. Finding the best lipid source could be one of the key factors in a successful in vitro rearing of glochidia since lipids are important in the development of marine bivalve larvae (Bayne 1976) and lipid reserves gained during glochidia development is connected to the growth rate of the juvenile mussels (Douda 2015). There were some studies using culture mediums without lipid compounds (Isom & Hudson 1982; Dimock JR & Wright 1993; Fisher & Dimock 2006) and negatively affecting lipid reserves in reared juveniles (Tankersley 2000; Fisher & Dimock 2006). Nowadays it is common to add 50 µL of fish oil per dish in the medium (Lima et al. 2012). Fish oil is insoluble in the culture medium, and it can cause complications that are even more probable in long term cultivation – *M. margaritifera* has several months of parasitical stage (Castrillo et al. 2022). The fish oil tends to create droplet congregations which results in uneven oil distribution in the dish and possibly causes malnutrition of a portion of cultured glochidia. The undissolved oil could even create a layer on top of the medium, negatively affecting gas exchange between medium and the CO_2 rich atmosphere in the incubator and therefore affect the O_2 availability and pH in the dish.

There are ways to solve these problems. Owen (2009) reported the use of mixture from serum, menhaden oil and rifampicin with positive outcomes (Lima et al. 2012; Kern 2017). Solution

presented in our study (Escobar-Calderón et al. 2020) is the use of ELM – emulsified lipid mixture. The ELM can be mixed with the culture medium evenly and our results suggest that it can be successfully used as a lipid source in in vitro culture. We have to stress that our experiment focused only on a short part of the early period of the in vitro rearing of the *M. margaritifera* glochidia and their reaction to different types of mediums. More research with longer exposure to the medium or with the use of different species is needed to support the theory of ELM as a suitable lipid source for such use and validate it as a viable compound of culture medium for routine use in in vitro rearing protocols of glochidia.

Last but not least, the inconsistent effect of the type of serum on the rate of developing glochidia has to be addressed. The effect of the type of serum was statistically significant in each quantification. In Trial 1, the statistically significant increase of the portion of developing glochidia was shown for horse serum in two from three quantifications. This result is in contrast with data from Trial 2 where the calf serum performed significantly better based on the rate of developing glochidia in each quantification. Taskinen et al. (2011) used newborn calf serum in his cultivation medium but suggested use of different serums such as fish plasma. The fish plasma used in other studies was shown as suitable even in cases when the used fish plasma wasn't obtained from host species (Uthaiwan et al. 2002; Lima et al. 2012) the other hand, some more specialised species could require plasma from specific species they parasite on for development (Lima et al. 2012). It is highly possible that adding fish plasma of *S. trutta* or *S. salar* into the culture medium for rearing *M. margaritifera* (according to population) would be beneficial, but it brings significant methodological challenges. The rearing of *M. margaritifera* glochidia is very long and it is needed to keep the dishes uninfected and obtaining the supply of needed volume of such serum can be complicated.

An important result of this study is also the fact that the culture medium can be tested without the successful completion of the whole metamorphosis process of the larvae. The evaluation of the medium compounds was based on reactions of the glochidia to the medium during a relatively short initial stage of the parasitical phase and statistically significant effects of different medium mixtures were found despite the fact that the glochidia didn't achieve the juvenile stage. One big problem with in vitro culture of glochidia is the fungal infestation of the dish (Ryan et al. 2022). This problem would be even more prominent in future successful in vitro methodology for rearing *M. margaritifera* because it would be a months-long period of cultivation. A commonly used fungicide is Amphotericin B. It was used in our culture medium mixture as well. According to Ryan et al. (2022) it could be toxic to glochidia if used in large concentrations. This finding shows the importance of also testing the possible effects of the

antibiotic and antifungal compounds of the medium on the glochidia. In addition, the possible effect of chronic exposure to these compounds should also be tested in case of species with long cultivation period in the future.

4.1.2 Assessment of the effect of in vitro rearing on subsequent vitality of the mussel population (STUDY 2)

Study focused on in vitro cultivation aimed to clarify if the in vitro rearing of glochidia has effect on their subsequential survival, growth, and reproduction. The model species (*S. woodiana*) has a short metamorphosis phase and reaches sexual maturity rapidly. The overview of the results by boxplots is shown in fig. 7. The detailed results can be found at Douda et al. (2021).

Every used rearing treatment successfully produced live juveniles but there were significant differences in metamorphosis success among propagation methods ($F_{3,132} = 21.95$, $P < 0.0001$) and maternal individuals. ($F_{5,132} = 7.13$, $P < 0.0001$). Meanwhile the effect of interaction between production method and females was not statistically significant ($F_{9,132} = 1.10$, $P = 0.365$). Larger variability was found among production methods than among maternal individuals.

Metamorphosis success of the mussels was the highest when using in vitro method 2 (back-transformed least square mean = 0.508) among the used rearing methods (in vitro method 1 = 0.251, *Gobio* = 0.282, *Rhodeus* = 0.197) after accounting for differences among maternal mussels. Moreover, in vitro method 2 significantly differed in the metamorphosis rate from the rest of the used methods.

Next analysed aspect was survival rate of the juvenile after the parasitic stage. There were significant differences in juvenile survival rate among the production methods ($F_{3,9} = 4.77$, $P = 0.030$;) but no significant differences were found among used maternal mussels ($F_{5,9} = 0.31$, $P = 0.894$). The juvenile survival rate for method 1 was significantly lower (back-transformed least square mean = 0.781) than for using *Rhodeus* live host (0.996) or in vitro method 2 (0.999). Size of produced juveniles was also evaluated. This parameter has shown significant differences among propagation methods ($F_{3,9} = 21.28$, $P = 0.0002$). The propagation method with significantly lower juvenile size than another method (back-transformed least square mean = 332.6 μm ± 29.8 SD) was in vitro method 1. On the other hand, the size at the end of growing season and instantaneous growth during the second growing season did not differ among production methods.

The F2 juveniles were recorded in all observational ponds with variation of parameters among the propagation methods. These parameters were population growth rate (λ) and number of recruits. However, the differences were not statistically significant.

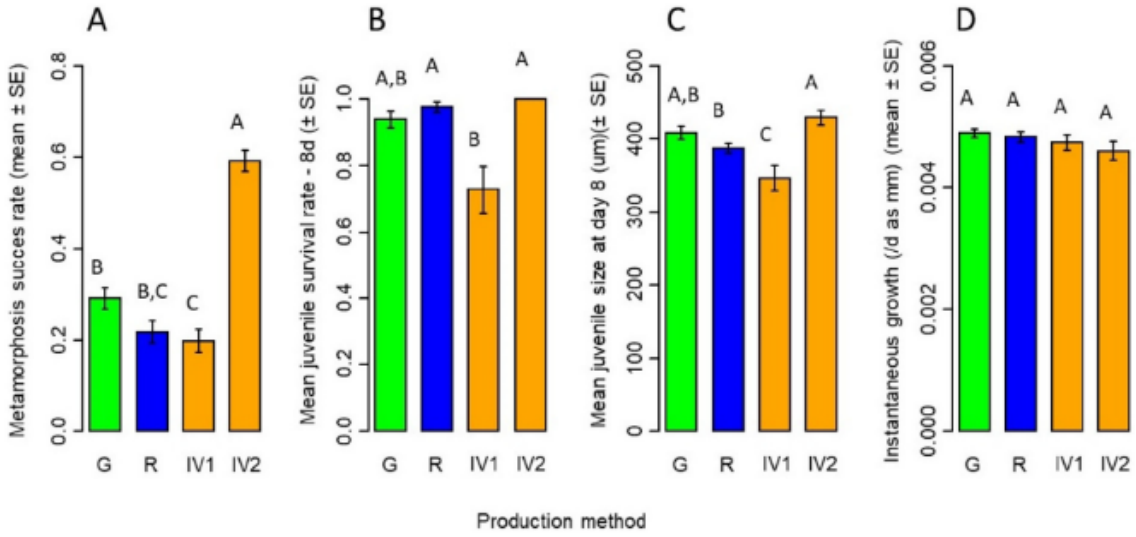

Fig. 7: Performance of *S.woodiana* juveniles reared by in vivo methods (using hosts: G – *G. gobio,* R – *R. amarus*) and in vitro methods (IV1 – method 1, IV2 – method 2). A) Proportion of metamorphosed glochidia; B) Mean survival rate of juvenile mussels during an 8 days period after metamorphosis; C) Mean mussels juvenile size after 8 days post-metamorphosis period; D) Instantaneous growth during second growing season. Letters among the box plots mark significant differences among propagation methods (ANOVA, Tukey's HSD test, α = 0.05). Published at Douda et al. (2021).

To sum it up, there were differences between in vitro and in vivo reared juveniles and the distinction was caused by differences in in vitro cultivation mediums. In vitro method 1 had lower metamorphosis success rate and early survival and growth of the mussels than in vitro method 2, we can assume that these differences were caused by different level of medium in the culture dishes (method 1: depth ~ 3.4 mm, method 2: depth ~ 2.0 mm). The larger depth of the medium could inhibit gas exchange in the dishes. We also cannot rule out a possible effect of unknown physiological or genetic aspects. Other reason for these differences in the results can be the density of glochidia in the dishes - method 1 had more glochidia per dish than in vitro method 2, on the other hand the ratio of glochidia to volume of the medium was similar among the methods. An important finding is that simple alteration of the culturing method can have a big effect on the success of the cultivation of the glochidia. On the other hand, the

differences in growth and survival between juveniles reared by in vitro method 1 and 2 evened out at the end of the first growing season.

One of the biggest advantages of in vitro cultivation of the glochidia is that it can produce a higher number of juvenile mussels than in vivo production methods (Lima et al. 2012). Metamorphosis success of in vitro method 2 in our experiment was approximately twice as high as that from in vivo method. With the use of appropriate in vitro propagation method this vast difference in yield can be beneficial particularly in conservation programs. The appropriate method also ensures no difference in post-metamorphosis performance of the reared individuals between in vivo and in vitro propagation according to our data. Significant difference was found in the size of juveniles produced using *Rhodeus* as live fish host – these juveniles were smaller than the in vitro reared juveniles. This difference evened out by the end of the first growing season.

Next important result is that juveniles produced by in vitro method can survive in the long term and even sexually mature and successfully produce F2 generation by using live fish hosts naturally, furthermore the rates did not differ from rates of in vivo reared mussels. Owen (2009) reported sexual maturation of mussels produced by in vitro methods before, but our study reports the comparison of in vitro and in vivo reared mussels in a long-term perspective of the life cycle. In a study performed on *A. anatina* by Escobar-Calderón & Douda (2019) the growth of animals produced by in vitro and in vivo methods did not differ after 8 days and in a similar study by (Kern 2017) using giant floater *Pyganodon grandis* (Say 1829) the same result was obtained even after five months of the observation. These studies are in accordance with our findings. There are even results showing that juveniles produced by these two methods didn't different significantly in toxicological experiments (March et al. 2007; Popp et al. 2018). There are contrasting results of some studies. Studies on paper pondshell *Utterbackia imbecillis* (Say 1829) and *L. fasciola* show significantly lower survival and growth for mussels produced by in vitro method than for mussels produced by in vivo methods after fourteen and eighty days, respectively (Fisher & Dimock 2006; Fox 2014). The reason for these contrasting results can be explained by the in vitro method itself. The in vitro methods in these studies had lower survival rate and growth similarly to in vitro method 1 used in our study (that was less efficient than in vitro method 2 in our study). However, we were not able to evaluate the possible effect of the methods used in the studies of *U. imbecillis* and *L. fasciola* on the results. It is necessary to find an optimal in vitro method and its standardisation for comparative studies on in vitro and in vivo propagation of mussels for further understanding of the different effects that these two approaches could have on the animals and their future generations.

In addition, we were not able to compare performances of animals produced by in vivo and in vitro methods in captivity with wild populations that are produced in fully natural conditions. We can compare our results with reports from the wild. Growth of *S. woodiana* produced in our study was similar to the reported growth of wild China population (Zheng & Wei 1999). Our results differ from in vivo produced *S. woodiana* in China (Chen et al. 2015) in some aspects. The comparison of the size of the juveniles from our study with (Chen et al. 2015) data differs. The size in eight days of propagation was similar (mean length = 0.425 mm in our study versus 0.468), our recorded size was smaller at 308 days (24.5 mm in our study vs. 57.2) and larger at the end of the comparison period e.g., after 434 days (76.9 mm in our study vs. 58.2). There is limited availability of estimation of population growth of mussels and to obtain such an information for wild populations is extremely difficult, however there are some estimations that we can compare our data with. The mean population growth of in-vitro reared mussels in our study was $\lambda = 3.17$/yr which is similar and even a little bit higher than λ reported for wild mussel populations produced in hatchery ponds with similar host abundance as in our experiment (mean $\lambda = 2.0$/yr at 1 fish/mussel). High variability of recruitments among ponds was the next similarity to our study (Haag & Stoeckel 2015). Despite challenges that occur in obtaining data of populations and population growth in the wild, more research is needed that will compare wild populations and the effect on in-captivity rearing of mussels on their vitality, growth and other long-term aspects.

An important factor that should be considered is that the metamorphosis success can vary even in case when a suitable live host is used (Douda et al. 2017a). There were no significant differences found at adulthood between mussels produced on *G. gobio* and *R. amarus*. *S. woodiana* is host generalist therefore this result was expected. There were differences found between the performance of mussels produced from glochidia from different females and these differences were not connected to the production method. The maternal mussels were obtained in one population and there were no explanations found for the differences in performance of their F1 generation. These results show us a new important place for research and aspect that has to be considered in mussel rearing programs.

The performance and its evaluation in captive bred animals after release to the wild, and such assessments in F1 generation as well, are highly important in conservation programs but not so common practice. Our study shows in vitro as a usable tool for mussel conservation and also stresses out the importance of performance evaluation of F1 generation of captive bred animals in the frame of their environment and life cycle.

4.2 Monitoring of parasitic stage success and vitality evaluation

4.2.1 In situ monitoring of host parasitic compatibility and development of low-cost monitoring system (STUDY 3)

This experiment focused on the development of a low-cost in-situ monitoring device for monitoring and collecting of detaching particles from individual animals (in our case glochidia from each individual fish host). The device was first tested in laboratory conditions with *C. carassius* and the deposition of microplastic particles. Here I present the highlights of the results, for detailed results please see Douda et al. (2020).

First important result of the laboratory test on the efficiency of the pontoon system was no mortality of *C. carassius*. There weren't even any observed injuries of the skin or fins. Efficiency of flushing out of the microsphere was 95.9 ± 4.5% (± SD) in the first 24 hours after inserting the particles into the system. The last particles were recovered 120 – 144 hours after the start of the test. Particles recovered within 72 – 144 hours after insertion to the system were mechanically damaged. That suggests that these particles went through the digestive tract of the fish.

Secondly, the system was used in a field experiment to collect juvenile *M. margaritifera* juveniles detaching from fish hosts *S. trutta*. There was no recorded mortality and injuries of the host fish placed into the system in the field either. Even the condition factor of the monitored fish did not change ($P > 0.05$) during the monitored period. The flushing efficiency was tested by adding live mussel juveniles into the system and the recapture rate was 88,1% – 100% and 90.4% – 98.6% within first 24 hours after addition. Furthermore, the temperature of the water was same inside and outside of the system ($P > 0.05$; mean difference ± SD: 0.05 ± 0.08 °C) which allowed us to get as close as possible to the natural conditions of the captured fish host.

In total, 2377 *M. margaritifera* juveniles were collected. There were significant differences found in the detachment rate between fish hosts infested by glochidia from different mussel populations. Hosts infested by glochidia from Gloch-A population had significantly ($P < 0.001$) lower detachment rate of juvenile mussels (0.01 ± 0.02 juveniles day^{-1} g^{-1}) than hosts infested by glochidia from population Gloch-B (0.68 ± 0.79 juveniles day^{-1} g^{-1}). Meanwhile, there was no significant difference in juvenile detachment rate between populations of the hosts ($P > 0.05$).

The metamorphosis success results corresponded with the results of juvenile detachment rate, but the difference was not statistically significant ($P > 0.05$).

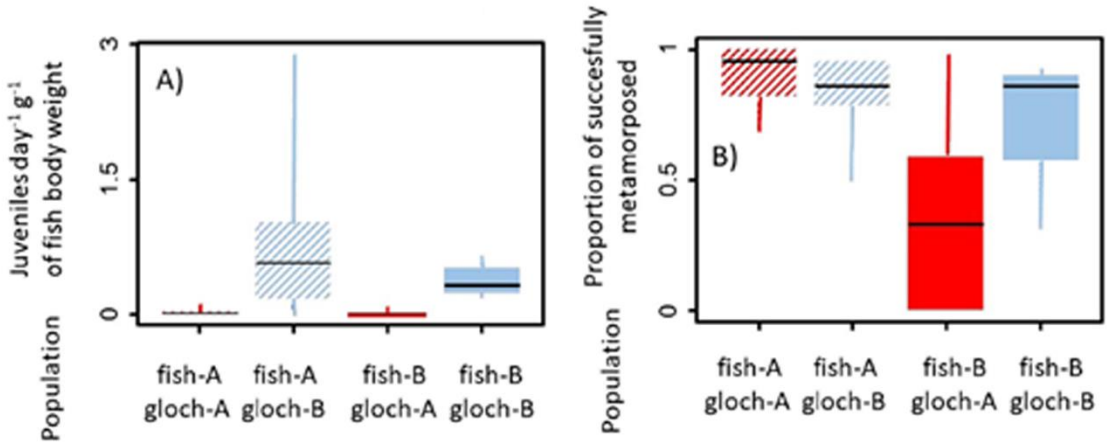

Fig. 8: *M. margaritifera* juveniles detachment rate per gram of hosts body weight (A). Proportion of successfully metamorphosed glochidia collected by the pontoon monitoring device during 14 days of monitoring period (B). The median, interquartile range and min/max for different combinations of source populations of mussels (blue, red) and fish (hatched, full flat colour) are displayed. Adapted from Douda et al. (2020).

The glochidia from population Gloch-B performed better in our experiment. That can be caused by immunological mechanisms (Rogers-Lowery et al. 2007) or simply by higher quality of the glochidia from population Gloch-B than from population Gloch-A. The possible difference in the quality of the glochidia is also supported by the initial glochidia vitality test. The low glochidia quality is a common problem in the propagation of freshwater mussels (Patterson et al. 2018). In conservation programs, it is key to find the most efficient source populations for breeding activities.

The results didn't show differences in the ability of fish from two different strains to successfully host *M. margaritifera* glochidia. Visual representation of key results is shown by boxplot at fig. 8. It is important to stress that this topic needs more complex study design to also consider the effect of glochidia viability. Even with lower overall success of Gloch-A population, both host populations can be considered as physiologically compatible with the mussel populations.

Our results demonstrate that the used monitoring system is suitable for host-parasite experiments evaluating differences in physiological compatibility of combinations of populations of mussels and fish hosts and that it is possible to detect these differences by using the system presented in the study Douda et al. (2020). The system was proven to be usable for

live fish without causing harm or mortality to the animals in short term studies with no significant effect on fish condition index. Moreover, it allows us to study this interaction in semi-natural conditions very close to natural ones (e.g., same length of photo period, water quality and temperature, even access to natural feed for the host to some extent – there were larger invertebrates found flushed into the filter cups). The floating device is a low-cost novel approach that can be used in a variety of fields like a wide range of parasitology studies, studies of microplastic digestion or nutrition research with need of faeces analysis (Nelms et al. 2019) and not only by using fish but also other aquatic animals (amphibians or macroinvertebrates). The presented device is especially useful in cases where long distance transport of animals would be needed or in cases where acclimatisation to laboratory conditions is problematic (Calisi & Bentley 2009) or the laboratory isn't able to provide suitable conditions for the target species.

There are studies showing that long term exposure to laboratory conditions can alter biological factors in adult mussel – their growth can be lower, metabolism can change, and mortality can be higher (Roznere et al. 2014; Patterson et al. 2018). This enhances the possible benefits of using methods that allow us to study mussel biology in the field in natural or at least semi-natural conditions. Possible applications of the presented monitoring device can be expanded by specific alterations.

The main use of the floating device presented in this study is application in conservation of freshwater mussels. It can be used in a number of activities targeting towards bivalve conservation. Evaluation of glochidia metamorphosis success in connection with a variety of aspects is crucial research for the management of successful conservation programs – e.g., for the management of host resources and the assessment of conservational units (Modesto et al. 2018).

Other possible use of the floating monitoring device in mussels research is the study of their parasites. Parasites from class Trematoda (Rudolphi, 1808) use molluscs as their intermediate host in which they produce their free-living larvae known as cercariae. In field conditions, it is problematic to estimate a population of these free-living larvae. Cercaria production is estimated by placing mollusc into a container with water and the produced larvae are counted over time (Taskinen 1998). Modified systems like the one presented in our research could put these experiments in the field.

The device can also be used for educational purposes. In our case, the device was placed in a secured fish hatchery of Šumava National Park. Public visits are allowed in the hatchery and the monitoring floating device was used to highlight the importance of host-parasite

relationship and its research in conservation of mussels to the visitors. Due to the small cost of the device and easy construction it can also be used for demonstration of research methods to bring the public closer to the topic.

It is important to stress that the device provides seminatural conditions. It doesn't allow the animal to perform some natural behaviours, such as interaction with substrate or migration to foraging areas. It has to be considered if these limitations can have an effect on the results of a specific study. We would also like to emphasise the importance of disinfection of the whole device between experiments and especially between locations to prevent animal and disease transmission.

In conclusion, the presented device for in-situ monitoring of animals and detaching particles was shown as a useful tool for collecting data and samples for evaluation of host-parasite compatibility of mussels and fish hosts without causing harm to the captive animals. The device presents a novel approach to parasitology in the field and could largely contribute to the increase of volume and type of data that can be obtained in environmental parasitology and feeding ecology. New emerging field of research of microplastic pathways in nature could also benefit from this approach. The system could be enhanced by using modern monitoring technology (for behavioural data for example). There is a need for more research and tests to optimise the device for specific requirements of different types of studies.

The biggest advantage of this device is its low-cost design with good portability and easily made construction that allows researchers to build the device on their own and make changes that suit their goal best. It can also be used in the education of the public by placing the device in a publicly accessible area in combination with info signs or in a place with guided tours – like for example the hatchery of a National Park. We believe that this device will be an easy way to obtain new types of field data and will be beneficial for a wide range of researchers.

4.2.2 Evaluation of host quality based on quantification of lipid reserve in juvenile mussels (STUDY 4)

Subsample of 378 collected juveniles from STUDY 3 was later analysed by using fluorescence microscopy to quantify the lipid content in each individual juvenile mussel (fig. 9). Juveniles were also measured, and their metamorphosis success was evaluated to represent common approaches to the evaluation of compatibility between host and the glochidia. Study presenting these results is in preparation (Vodáková et al. in prep.).

Firstly, the metamorphosis success rate was evaluated. The estimated average *M. margaritifera* juvenile detachment rate was 0.58 ± 0.76 juveniles day^{-1} g^{-1} of fish body weight. The proportion

of successfully metamorphosed glochidia was 79.43 ± 17.75% during the peak period of juvenile collection. There was no significant effect of either fish or mussel population in terms of the proportion of successfully metamorphosed glochidia (ANOVA, all $P > 0.05$).

Juveniles originating from the Malše population were larger (Malše 361.34 ± 29.76 μm, Blanice 358.73 ± 37.48 μm), however there were no statistically significant effects found for mussels of fish population with mixed effects model. The population combinations also didn't show any significant results. However, the Malše juveniles were larger if they parasited on Blanice fish (363.96 ± 27.07 μm) than if the host was from the Častá population (359.40 ± 31.56 μm). Contradictory, Blanice juveniles collected from Častá fish hosts were larger (72.43 ± 24.652) than from sympatric Blanice fish (352.40 ± 40.68 μm).

On the other hand, the lipid data have shown different results. Significant effect of host fish population on the lipid reserves of juvenile mussels was found by using a mixed effects model. Meanwhile, there was no effect of the mussel population detected. Juveniles attached to Blanice fish had higher average lipid reserves (Mean fluorescent Intensity: 101.50 ± 15.24) than the ones attached to Častá fish (MFI: 97.01 ± 15.72) regardless of mussel population. Differences in lipid reserves among mussel populations were low (MFI: 98.43 ± 14.47 for Blanice and 99.816 ± 16.22 for Malše). The results are shown by box plots in fig. 10.

The fluorescence lipid quantification was shown to detect significant differences that were not shown by analysis of the length data. The results indicate that the fluorescence lipid quantification method could be more sensitive in distinguishing differences between fish populations than commonly used methods.

Our results suggest that lipids quantification is a promising tool for easy enhancement of experiment design in host-parasite studies of fish and mussels relationship. Lipids serve as energetic reserve (Palais et al. 2011; Douda 2015) in mussels and it is possible to use them as a marker for the evaluation of vitality and health status of mussel juveniles (Tankersley 2000; Douda 2015; Gibbs et al. 2021). Lipid analysis in juvenile mussels brings qualitative approach into field where quantitative methods are mostly used nowadays (e.g., survival and metamorphosis rate or quantification of attached glochidia on the live fish host) (Dodd et al. 2005; Douda et al. 2014; Huber & Geist 2017; Marwaha et al. 2021). We also cannot leave out the use of genetic methods which are more on the qualitative side of the methods spectrum and are increasingly used in research over the past decades (Marwaha et al. 2021). Other widely used method is measuring the length and growth of the juveniles (Österling & Larsen 2013).

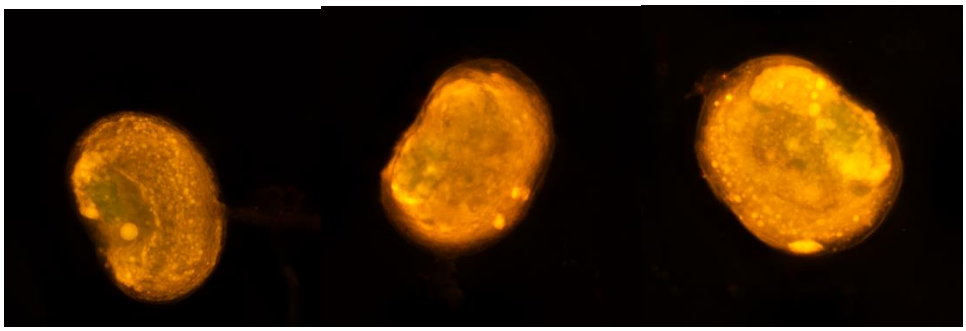

Fig. 9: Example of distribution of lipids in *M. margaritifera* juveniles. Juveniles that detached from *S. trutta* were stained by Nile Red solution and placed under fluorescence microscopy. The stained lipids emit light during fluorescence microscopy such as shown in the pictures. Size of the juveniles was 361.34 ± 29.76 µm and 358.73 ± 37.48 µm according to the original maternal population (Malše, Blanice, respectively).

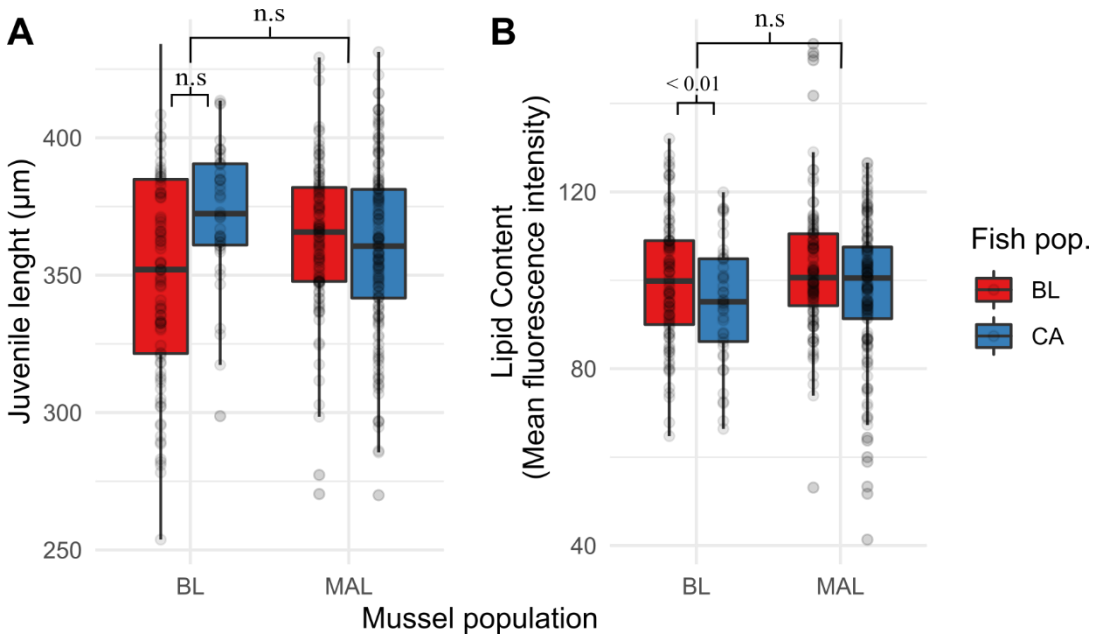

Fig. 10: Boxplot of the final juvenile length (A) and juvenile lipid content (B) per host fish and mussel population. Juvenile *M. margaritifera* detached from fish host *S. trutta* and stained by Nile Red solution that stains lipids in soft tissues and emits light when placed under fluorescence microscopy. The lipid content was estimated based on mean fluorescence intensity (e.g., intensity of the emitted light by the stained lipids) and length was measured from photos obtained during the fluorescence microscopy. Mussels originated in the population from Blanice (BL) and Malše (MAL) rivers, they were used in combination with two populations of hosts – populations from Blanice (BL, red) and Častá (CA, blue) rivers.

The qualitative approach to evaluation of health and vitality of juvenile mussels with combination of more traditionally used methods could give us new point of view into the enigmatic decline of freshwater mussel populations (Haag 2019) and to low recruitment (at

some localities even not occurring recruitment) of *M. margaritifera* (Geist & Kuehn 2008; Österling et al. 2008; Taskinen et al. 2011; Simon et al. 2015; Boon et al. 2019).

There are other qualitative approaches to the evaluation of physiological compatibility of mussels and fish hosts, but they provide results in a longer time period than the presented lipid assessment. These methods are for example evaluation of reproduction success of F1 generation and monitoring of vitality of F2 generation (Douda et al. 2021). The lipid quantification provides us with rapid results in comparison with these methods. Moreover, the age of sexual maturity is strongly dependent on the used mussel species so the length of such research can vary from a few months to a few years. For example, *S. woodiana* reaches sexual maturity within half a year (Douda et al. 2021), however species with long life span, such as *M. margaritifera* presented in our experiment, reach sexual maturity after approximately 20 years (Bauer 1987). Due to this fact, the qualitative approach based on evaluation of performance of F1 or even F2 generation is extremely unsuitable for research of this and similar species.

Moreover, the presented method is relatively easy to perform with a small team and can be used in combination with other easy methods of host-parasite compatibility testing. A combination of several simple analyses and methods in one experiment design provides robust data sets. The combination of the presented field monitoring system (STUDY 3) that allowed us to collect individual data for each fish host for quantitative analysis of glochidia metamorphosis success and juvenile length, and subsequent qualitative analysis based on lipid content of collected juveniles mussels gave us a wide set of connected results.

M. margaritifera is critically endangered in the target region (Šumava, Czech Republic). The few populations can be found on only small fragments of the original area and the populations are mostly separated. *M. margaritifera* is flag species for the conservation of oligotrophic basins (Simon et al. 2015). The life cycle of the species presents obstacles in the conservation research. This species has a life span between 80 to even 100 years with possible reproduction in even up to age 75 years. On the other hand, juveniles bury themselves in the sediment until they reach adulthood (Bauer 1987; Simon et al. 2015) which makes monitoring of the juvenile population practically impossible. Moreover, the spawning period is limited to a very short time (Hastie & Young 2003) and glochidia release happens simultaneously in two days in the whole population with specific timing according to the environmental conditions (Hastie & Young 2003).

Despite the conservation efforts in the target location of our study (Šumava National Park) there is still practically no natural recruitment observed in the area. Sporadic successful reproduction

(e.g., juvenile production) was observed at only two localities (Malše and Blanice) (Simon et al. 2015). These populations were used as source populations for glochidia in our experiments. The lipid reserves statistically differ among these two populations according to our results. The populations are geographically separated, and our data suggests that the Malše population has more vital glochidia than the Blanice river populations. Mixing of the population could have unknown effects on the vitality of the juveniles, reproduction success in general, and the quality of the next generations.

The use of an unsuitable host could cause a bottleneck effect and alter the genetic structure of the parasitic species population (Taeubert & Geist 2017). We can assume that the naturally cooccurring host population should be the most suitable host (Taeubert et al. 2010; Taskinen & Salonen 2022). The latest results published by Taskinen & Salonen (2022) comparing host suitability of salmonids from allopatric and sympatric *M. margaritifera* populations didn't show that the allopatric fish host would be better hosts to the glochidia. This result also supports the theory of local adaptation between mussels and their cooccurring host fish. But our data don't support these suggestions.

Our results did not find any significant effect of population combinations. The lipid data showed the Blanice fish population as a better host. It could be due to untested physiological or condition factors. The next reason could be simply the small number of fish hosts used in the study (378 juveniles were analysed but only from 12 fish hosts). The fact that there was no difference shown by other approaches (length and metamorphosis success of the mussels) could be caused by the fact that the fish aren't genetically significantly different (Douda unpublished data) even though they are from different populations geographically. That means they belong to one group from a biological point of view. This is probably the result of the long-term fish management in the area.

One possible outcome of our results is the importance of focusing on the vitality of each individual fish host in the selection process for mussels breeding programs. In a study performed by Österling & Larsen (2013) a positive relationship was found between the length of the *M. margaritifera* shell and the condition index of their host fish. We can assume that the condition of the individual fish is connected to its energetic reserves and moreover it could have a direct effect on the amount of nutrition that the host could provide to the attached glochidia. Contradictory, a more vital host could have a stronger immune response to the glochidia infestation at the start of the parasitic process (Österling & Larsen 2013).

An aspect that should be taken in account in host selection is also age of the individuals (Marwaha et al. 2019). Older fish from the wild population have a higher probability of previous

glochidia infestation than younger fish from the same populations. This could lead to a stronger immune reaction. There is a number of studies showing that adaptive immunity response developes in fish after encounter with glochidia (Dodd et al. 2005; Douda et al. 2014; Donrovich et al. 2017; Modesto et al. 2018) and it is highly possible that this is also happening to salmonids after interaction with *M. margaritifera* (Hastie & Young 2001). Age has an effect even for naïve hosts – e.g., that never encounter glochidia – according to a study by Marwaha et al. (2019). They compared glochidia attachment on naïve *S. trutta* individuals of age 0+ and 1+. Condition of 0+ hosts negatively correlated with load of attached glochidia on the individual host. In contrast, hosts from 1+ group had opposite results (there was a positive correlation between the condition of the host and the load of attached glochidia). Another aspect of glochidia's success on the host is host size. Studies evaluating this factor have mixed results (Marwaha et al. 2019). There are suggestions that the role of age and size of the host (positive effect of bigger and older fish) is getting less important over the parasitation phase with insignificant effect at the end (Hastie & Young 2001). The larger size of the host could be better due to the larger area of gills where glochidia can attach, on the other hand, bigger fish could have a stronger immune response and a large amount of attached glochidia could detach because of it.

The problematics of the host-parasite relationship between fish and unionids is a widely studied topic. There is a need to focus more on qualitative approaches and globally appliable methods and findings. We should look for more physiological markers that can be used as biomarkers in studies like this one to get more robust data sets for conducting detailed outcomes and seeing the problematics from more points of view to fully understand the topic. Deeper knowledge of host-parasite compatibility problematics and the effects of multiple aspects on the problematics is crucial for the optimisation of conservation programs.

4.2.3 Health assessment of adult mussels based on quantification of glycogen reserves in soft tissue (STUDY 5)

The glycogen (primary energetic reserves in mussels) quantification in several types of soft tissues of mussels was performed. The analysed tissue types were foot, mantel, gills, and adductor muscle. Modified spectrophotometric methodology based on Naimo et al. (1998) was used and the precession of the simplified methodology was tested as well. Detailed results are available at Vodáková & Douda (2019). Visual representation of key results is provided in fig. 11.

To sum up the glycogen quantification in mussel soft tissues, the glycogen concentration in samples of *A. anatina* soft tissue were 5.6 mg – 61.1 mg/g wet tissue (mean 20.7 mg/g, SD 10.7). The mean glycogen content in the biopsied samples was 0.15 mg (SD, 0.07). The calculated method detection limit (MDL) was 0.049 mg of glycogen in the whole analysed sample which corresponded with 0.0075 mg of glycogen in the final solution analysed in final spectrophotometry.

Methodology used was tested by the recovery of samples with known concentration. The mean recovery of glycogen in spiked samples was over 80% for each of the used concentrations of aqueous calibration standards. The specific numbers were 85%, SD 13; 82% SD 27, 109%, SD 20 and 83%, SD 25 for concentrations 125, 500, 1000 and 2000 mg/L respectively with CVs 7.7% (SD, 2.7), 10.5% (SD, 3.4), 7.4% (SD, 4.5) and 7.9% (SD, 1.6), respectively.

Significant glycogen concentration variance among tissue types was found (two-way ANOVA: $F_{3, 44} = 6.9$, $P < 0.001$). Higher glycogen concentration was in gills (26.1 mg/g, SD = 9.0), followed by adductor muscle (20.2 mg/g, SD = 8.6) and mantle (20.1 mg/g, SD, 16.1). The lowest glycogen concentration was found in foot tissue (18.7 mg/g, SD = 4.9) (fig. 11).

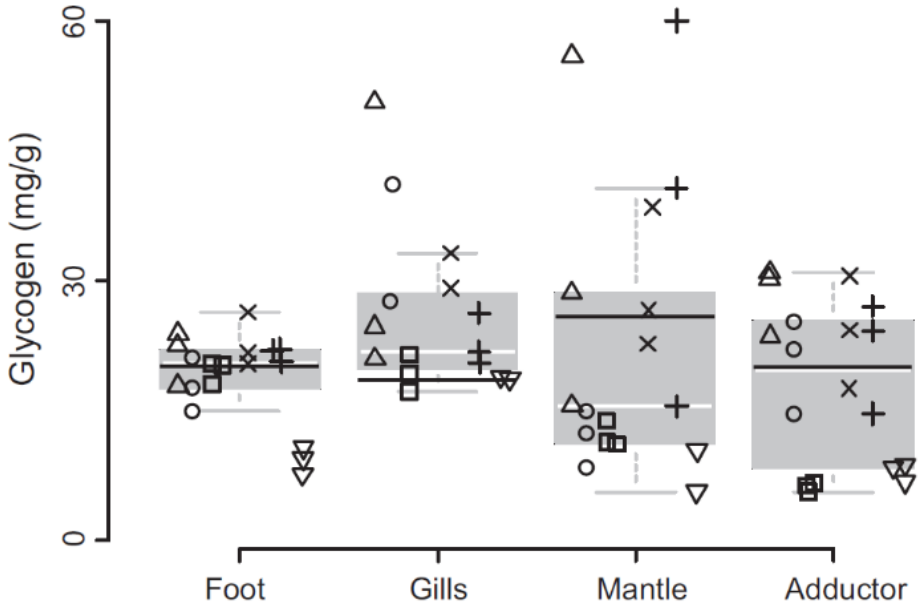

Fig. 11: Glycogen content in different types of tissues of duck mussel. Box plots show the mean (black line), median (white line), interquartile range and minimum and maximum values (without values > 1.5x interquartile range). Different symbols show values measured for different individual mussels. Published at Vodáková & Douda (2019).

Significant effect of individual mussels was also found (two-way ANOVA: $F_{5, 44} = 22.0$, $P < 0.001$). Furthermore, the variation in body distribution of glycogen was suggested by significant interaction term between tissues and biopsied individuals (two-way ANOVA: $F_{15, 44} = 3.3$, $P < 0.01$).

To sum it up, the simplified modified methodology of glycogen quantification in soft tissue based on the method by Naimo et al. (1998) was proven to be reliable and precise for the evaluation of differences in glycogen distribution between different types of soft tissues in *A. anatina* and differences between glycogen body distribution between individual mussels.

The results are in line with previous studies (de Zwaan & Zandee 1972; Naimo & Monroe 1999) showing significant differences in glycogen concentration among different types of soft tissues of Unionida. The most variable glycogen level was found in the mantle meanwhile the glycogen concentration is most stable in foot tissue (fig. 11).

Even though there is relatively high variability of glycogen concentration within the same tissue type, the results show significant differences in glycogen body distribution between individuals. New valuable insight could be obtained by sampling more than one type of tissue per individual in condition assessment studies in future and the evaluation should be tissue specific. It also gives us a new critical point of view on studies where only one type of tissue was sampled.

Sampling was lethal in this study, but it was proven that biopsy of foot or mantle tissue can be performed non-destructively and have no effect on subsequent mortality in the long term (Berg et al. 1995; Naimo et al. 1998). The effect of biopsy of gill and adductor muscle or the effect of sampling multiple types of tissues at once on mussel wellbeing should be tested.

The modification to the used method made it more economical by reducing the material consumption. The needed volume of sulfuric acid was reduced by 56.4% and only one 3-mL cryovial is needed for the process before placing the solution into the multi-well microplate instead using 3 different types of test tubes for each sample. Moreover, the method can be performed by one person and one analytic set can hold up to 60 samples (including calibration solutions). The reduction of hazardous waste is an important result of these simplifications.

Glycogen can be linked to several factors and the connection between its level, body distribution and other factors needs clarification. The glycogen level and body distribution changes annually (de Zwaan & Zandee 1972), the food availability changes in annual cycle (Albentosa et al. 2007; Cordeiro et al. 2016) which we can assume has a big influence on mussel glycogen reserves. These reserves and their utilisation are also related to the reproductive cycle (Lemaire et al. 2006). The glycogen could be broken down to create glucose that is used for development of gametes during mating season (Martínez-Pita et al. 2012; Ke & Li 2013).

Catabolizing glycogen reserve is also needed when glucose level declines in haemolymph or due to stress (Fritts et al. 2015a). Due to the least factor glycogen level in tissues can be used to evaluate stress under various conditions (Anacleto et al. 2013; Cordeiro et al. 2016, 2017). Glycogen level reacts to changes much quicker than changes in the growth of the individual or survival rate of the populations (Sim-Smith & Jeffs 2011), because of that it can be used as a biomarker in ecotoxicological studies (Hazelton et al. 2014). Our methodology simplification allows a routine application of this methodology. In addition to that, the results highlight the need for tissues specific evaluation in the case of research focused on mussel energetic metabolism.

It needs to be stressed out that this study has a pilot character and further research is needed to clarify the most suitable organ, gender, and stage of life cycle for nonlethal analysis. Furthermore, it is necessary to deepen our understanding of the connection between glycogen and other factors listed above (such as various annual cycles and types of stress) and the problematics of glycogen body distribution and its variation among individuals.

4.3 Ecological impact of water borne pharmaceuticals on host-parasite relationship between mussels and their host (STUDY 6)

The effect of asymmetrical exposure to water borne pharmaceuticals (tramadol and methamphetamine) on aquatic animals was tested by using the interaction between glochidia and their host as an endpoint. Hosts and parasites were exposed to these chemicals in environmentally relevant concentrations for species specific time period. Subsequently, a multifactorial experiment with asymmetrical exposure was performed to test the hypothesis. Detailed results of this study are presented in Douda et al. (2019) and a visual representation of key results is presented by fig. 12.

The concentrations of tramadol and methamphetamine were consistent in analysed water samples with one exception – tramadol concentration slightly decreased in samples from the glochidia exposure experiment at the end of the 24 hours exposure. The concentration of studied pharmaceuticals in water samples from control groups were below LOQs.

The parameter that significantly differed was the gill ratio of the glochidia attachment. There was found an effect of the host exposure to the methamphetamine in this parameter. Gill ratio was 72.29 ± 19.27% for exposed host and 62.54 ± 16.45% for unexposed (GLM: $F_{1,30}=4.84$, $P < 0.05$). Results in the tramadol experiment didn't show a clear effect of the exposure to the gill ratio but the effect of the interaction was significant (GLM: $F_{1,24} = 6.63$, $P < 0.05$). This

result means the glochidia attachment was affected in case of exposure or non-exposure of both glochidia and host.

The results of infection success were in line with the gill ratio results. It was increased on hosts exposed to the methamphetamine (78.66 ± 11.28%, unexposed 59.60 ± 15.77%) with significant differences found (GLM: $F_{1,30} = 21.57$, $P < 0.001$). Moreover, glochidia exposure also was found to have a significant effect on infection success (72.57 ± 18.05% exposed, 65.69 ± 14.43% unexposed) (GLM: $F_{1,29} = 5.02$, $P < 0.05$). In the tramadol experiment, the differences in infection success were caused by increased mortality of glochidia in the initial 24 hours period in the exposed group in comparison with the group that was not exposed to the chemical (survival rate 58.65 ± 10.91%, 71.89 ± 12.97%, respectively) (GLM: $F_{1,25} = 7.83$, $P < 0.01$). Meanwhile, exposure of the host of interaction had no significant effect.

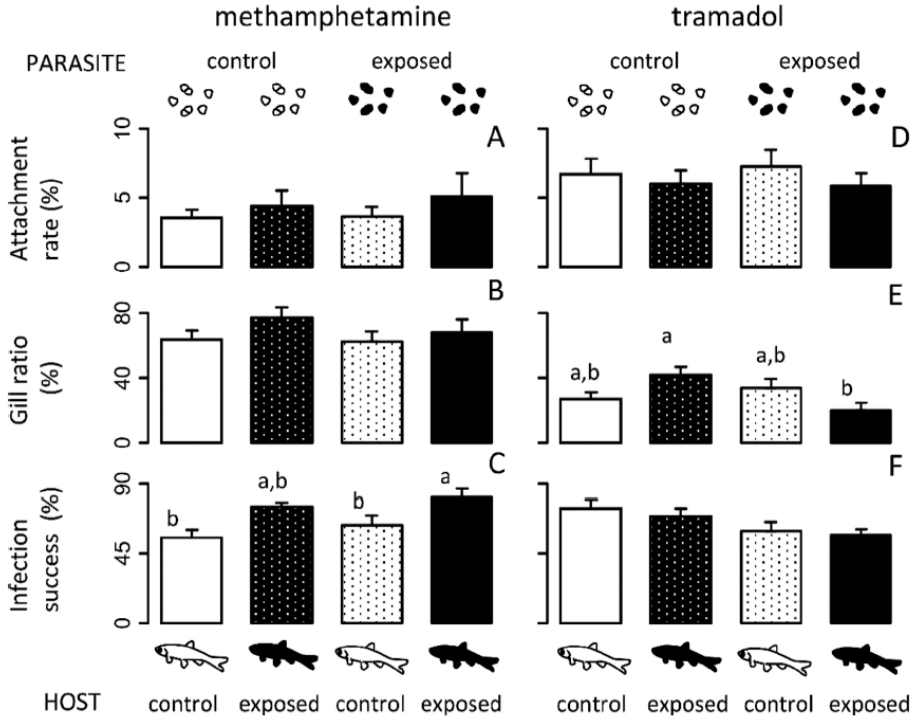

Fig. 12: Host-parasite interaction between *A. anatina* and *S. cephalus* after exposure to methamphetamine and tramadol for full factorial design of exposed and control group partners. Toxicity endpoints were sublethal interaction parameters: A, D – proportion of attached glochidia to the host; B, E – proportion of glochidia attached to the host gills; C, F – proportion of encapsulated glochidia. Different letters mark statistically significant results of multiple comparisons. Published at Douda et al. (2019).

To sum it up, the animals were exposed to the toxicants in joint and unilateral exposure design to inspect the possible ecological effects of the presence of pharmaceutical pollutants on the host-parasite relationship. Our results show that preceding contact with pollutants has an effect

on the interaction between fish host and glochidia, specifically on the spatial distribution of the attached glochidia on the host as well on the infection success of the glochidia.

Growing number of studies support the assumption of ecological impact of pharmaceuticals in freshwater ecosystem (Fong & Ford 2014; Brodin et al. 2014; Sancho Santos et al. 2020; Santos et al. 2021).

The host-parasite relationship dynamic affects both host and parasite and the approach used in this study can be applied to different studies working with other species and their host-parasite interactions. The asymmetrical exposure enables us to distinguish individual effects on the hosts and on the parasites. Specific pollutant concentrations that have no effect on the host could affect the parasite and vice versa and the result of interaction between the host and parasite can shift (Morley 2009).

Assessment of the effect of the psychoactive chemicals in water is important for the management and conservation of unionids. Glochidia viability evaluated by the snapping reaction is commonly used as a reliable toxicity indicator (Farris & Van Hassel 2006) and is used as a standard marker. Our study showed the importance of testing the effect of the specific studied toxins on the host and their interaction as well because our results showed no effect of the pollutants on the glochidia viability but there was an effect on other monitored factors (such as infection success and gill ratio). We can assume that the glochidia viability may not be sensitive to some pollutants. Moore & Bringolf (2018) found that the viability can be unaffected while the infectivity is reduced.

The alteration in the spatial distribution of the attached glochidia can have a subsequent effect on the host. A higher proportion of the glochidia was attached on the gills of the host in the exposed group compared to the unexposed. The attachment of glochidia to the gills of the host can affect the host's respiration and interact with the immune system (Alvarez-Pellitero 2008; Crane et al. 2011; Slavík et al. 2017). On the other hand, glochidia may be unable to finish metamorphosis on the same body parts of the host (Akiyama et al. 2018). Alteration of this factor of the host-parasite interaction can subsequently alter the population levels by changes in the survival rate of the glochidia. This is also supported by our results in which the infection success was higher for the exposed animals in the methamphetamine experiment. The significance of these alterations in nature cannot be predicted based on our results and more research is needed to estimate the magnitude of this change.

More research is also needed to pinpoint the pathway of the alterations. Data presented in this thesis cannot estimate if the changes are based on the effect of the pollutants on the immune system, physiology, or behaviour. The spatial distribution of attached glochidia among the host

body is linked to several known factors such as stress and concentration of stress hormone – cortisol (Dubansky et al. 2011; Douda et al. 2018; Nelson & Bringolf 2018). On the other hand, tramadol and methamphetamine are known for having an effect on fish behaviour, locomotion, physiology and hatching (Liao et al. 2015; Sehonova et al. 2016; Sancho Santos et al. 2020; Santos et al. 2021). One of the main defence against parasites in a host is their behaviour (Daly & Johnson 2011). I cannot comment on the possible link between potential behaviour alterations and changes in spatial distribution of attached glochidia since no behavioural test was performed during the study.

There is limited knowledge of the effect of water borne pharmaceuticals on molluscs and their larvae. It was found that physiological biomarkers connected to oxidative stress and protein damage defence in *D. polymorpha* can be influenced by pharmaceuticals (Contardo-Jara et al. 2011) and pharmaceuticals can also have an effect on the locomotion of marine gastropods (Fong & Molnar 2013).

An important aspect of the study is also the use of asymmetrical exposure. In nature, the level of concentration of pharmaceuticals in water fluctuates specially and in time according to input. This fluctuation means unevenly exposed populations and individuals with different concentrations and length of exposure. According to Burns et al. (2018) concentrations of pharmaceutics in River Foss (United Kingdom) can vary up to 20-fold in the range 21 – 456 ng L^{-1} in the time frame of a single year at one sampling locality. In the same study, the annual median of the concentrations of studied chemicals was in range 31 – 177 ng L^{-1} with monitoring of 5 sampling sites within a 20 km section of the river. The difference is the life cycle and locomotion behaviour of the different species in affiliated relationships can possibly enhance the exposure asymmetry.

In the study, we presented results for such asymmetrical exposure to sublethal concentrations of pollutants that can be assumed to happen in natural conditions. The effect of various conditions, different pollutants and species should be the focus of subsequent studies. This study also aims to highlight the importance of considering the effects on affiliated species in research on the ecological impact of different chemicals on biota and the importance of an evaluation of these effects on multi-species level.

4.4 State of the art of the *Sinanodonta* spp. research (STUDY 7)

This thesis presents studies focused on specific topics from theme of the host-parasite relationship between Unionida and their hosts. This inter-species interaction can be also

influenced by invasive species. Such species is for example *S. woodiana*. Here I present a review paper we worked on as well. This paper is currently at the minor revision stage of the publication process in Hydrobiologia. The paper is focused on invasion of *Sinanodonta* spp. – specifically *S. woodiana* has the potential to affect not only the host-parasite interaction of native fish and mussel species by competition for the host (Donrovich et al. 2017) but also the glycogen reserves of adult unionids by food competition (Douda & Čadková 2018). The in-situ monitoring pontoon system presented in STUDY 3 could also be used for monitoring this species. The analysis of glycogen reserves in adults presented in STUDY 5 could be used for example for comparison of the nutrition state of native species at localities with cooccurring *S. woodiana* and without this invasive species. *S. woodiana* is used as model species in STUDY 2. In addition, this species can serve as model species for study of biological invasions and therefore there is space dedicated to the synthesis of research focused on the model species. Here I present an overview of the state of the art research focused on *Sinanodonta* spp.

The systematic literature overview consisted of 266 articles divided into 11 primary categories. Most of the papers were in the following categories: ecology, ecotoxicology, biogeography. 73 articles (27.4 %) were centred around invasive biology. Majority of these articles were in category distribution; the second strongest category was impact and ecology in the invasive range. There were only two studies covering the topic of prevention and no article about control of the invasive bivalves. The distribution of articles among categories is shown in fig. 13.

The evaluation of article distribution among topics showed us gaps in research of the *Sinanodonta* invasion. Some of the topics aren't covered for logical reasons. For example, the lack of articles about prevention and control of the invasion can be caused by the nature of the invasion process of the species. *S. woodiana* is present in almost whole Europe with suitable climate (Paunovic et al. 2006; Popa et al. 2007; Munjiu & Shubernetski 2008; Pou-Rovira et al. 2009; Lajtner & Crnčan 2011; Sîrbu et al. 2016; Donrovich et al. 2017; Bespalaya et al. 2018; Kondakov et al. 2018, 2020b, 2020a; Beran 2019), many parts of Asia and was reported even at central America (Watters 1997; Bogan & Schilthuizen 2004; Bolotov et al. 2016; Vikhrev et al. 2017; Bogan et al. 2021). Articles covering the topic of prevention would have to be focused on region without large *S. woodiana* invasive occurrence such as North America where it was found only in one locality (Bogan et al. 2011) or Africa where its spread started only recently (Mabrouki & Taybi 2022; Bensaad-Bendjedid et al. 2023).

Mussels are benthic animals and due to that fact, it is almost impossible to remove the invasive mussels from the water bodies with reasonable means and without harming other parts and species of the habitat. One of the methods would be hand-picking the animals but with the high

abundance of the species in nature (Kraszewski & Zdanowski 2007; Dobler et al. 2022) it is not possible to perform this on an effective scale. The only place where eradication of the species was performed with probable success was USA, New Jersey in the year 2019 (Benson 2023). This locality has unique features, due to these features the approach is not repeatable in most other localities with *S. woodiana* occurrence. The eradication was performed in one single secured pond and there could be surviving populating downstream from the pond (Benson 2023).

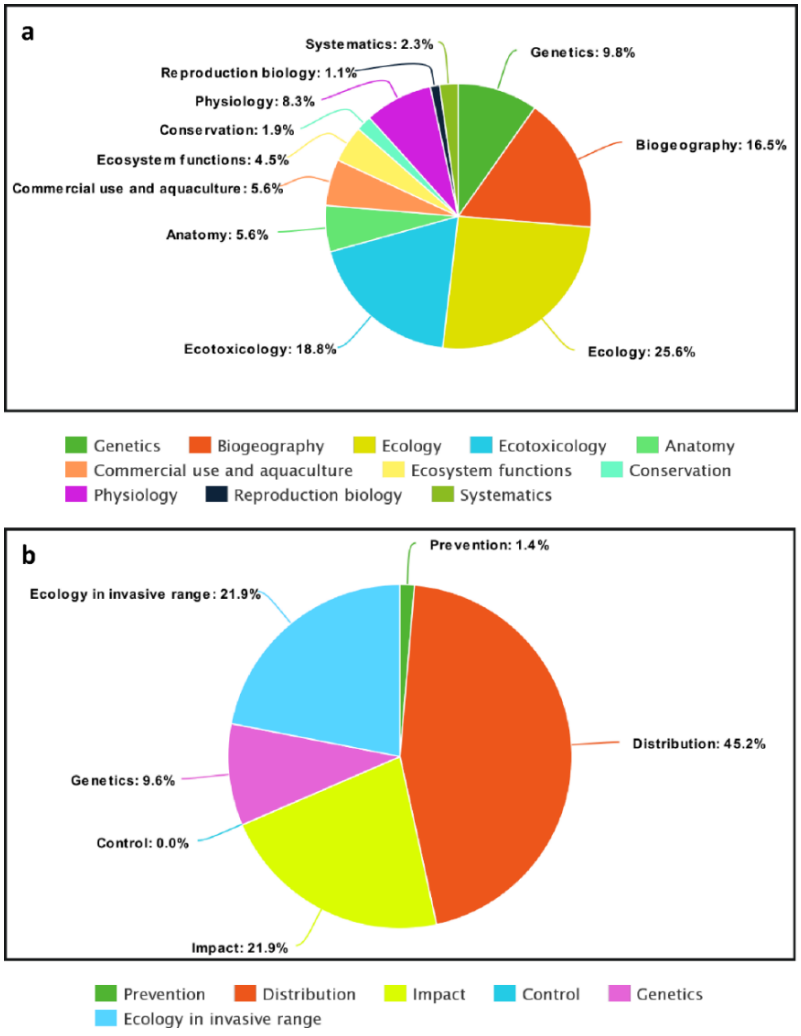

Fig. 13: Distribution of research papers among categories. Categorisation of papers from SCOPUS database searched by string "Sinanodonta or woodiana" in August 2022 (with the subsequent exclusion of papers not focusing on *Sinanodonta* spp.). a) categorisation of all papers into general category; b) categorisation of papers focusing on biological invasion of *Sinanodonta* spp. into categories of focus in the framework of *Sinanodonta* spp. invasion. (Douda et al. under review).

The aspects of the management of invasion topic should be further studied and despite the facts upper there are still management options to explore. There could be potential benefits of *S.*

woodiana such as use as fish feed (Konieczny et al. 2021) or in human diet (Stangierski et al. 2021). The next possible use is as easily accessible model species for host-parasite studies or in ecotoxicological studies and for biomonitoring as other mussel species are used nowadays (Douda et al. 2019; Vereycken & Aldridge 2023). We also cannot overlook *S. woodiana* contribution to the ecosystem by providing ecosystem services as mussels in general in areas where native mussel species cannot be found due to worsen conditions (Gutiérrez et al. 2003; Sousa et al. 2009; Bielen et al. 2016; Vaughn 2018; Zieritz et al. 2022).

45.2% of articles from studies focused on invasiveness of *Sinanodonta* spp. are covering distribution of the species. Distribution reports are basic knowledge needed for monitoring the invasive spread of species and for estimation of future pathways and expansion of the species and for understanding possible future pathways of similar organisms.

4.5 Authors contribution to the publications presented in the thesis

Chapters	Details	Contribution	Status
3.3.2 4.2.3 STUDY 5	**Vodáková B**, Douda K. 2019. Variation in Glycogen Distribution among Freshwater Bivalve Tissues: Simplified Protocol and Implications. Journal of Aquatic Animal Health **31**:107–111.	**First author** Organisation of samples collection and analysis; experiment design; writing (lead)	Published (2019)
3.3.1 4.2.2 STUDY 4	**Vodákova, B.** et al., Determining priority conservation linkages in an endangered freshwater mussel-fish relationship using lipid gains of parasitic larvae.	**First author** Field samples and data collection; laboratory analysis organisation, realisation and design; writing (lead)	In preparation
3.2 4.2.1 STUDY 3	Douda K, Escobar-calderón F, **Vodáková B**, Horký P. 2020. In situ and low-cost monitoring of particles falling from freshwater animals: from microplastics to parasites. Conservation Physiology **8**:1–10.	Calibration of the system; daily sample collection and data recording in field during field test of the system; construction of the system; laboratory work during laboratory test of system efficiency	Published (2020)
3.1.1 4.1.1 STUDY 1	Escobar-Calderón F, **Vodáková B**, Douda K. 2020. Early responses of in vitro cultured fish-growing glochidia: The effects of taurine, lipids and sera on Margaritifera margaritifera. Aquaculture Research 51:1069–1076.	Laboratory work during in vitro rearing of the mussels (medium exchange, glochidia quantification and determination of developing glochidia)	Published (2020)

3.1.2 4.1.2 STUDY 2	Douda K, Haag WR, Escobar-Calderón F, **Vodáková B**, Reichard M, Chen X, McGregor M, Yang J, Lopes-Lima M. 2021. Effects of in vitro metamorphosis on survival, growth, and reproductive success of freshwater mussels. Biological Conservation 254 (108964) DOI: 10.1016/j.biocon.2021.108964.	Laboratory work during in vitro rearing of the mussels (medium exchange, glochidia quantification and determination of developing glochidia)	Published (2021)
3.4 4.3 STUDY 6	Douda K, Zhao S, **Vodáková B**, Horký P, Grabicová K, Božková K, Grabic R, Slavík O, Randák T. 2019. Host-parasite interaction as a toxicity test endpoint using asymmetrical exposures. Aquatic Toxicology 211:173–180.	Assistance during experiments (glochidia exposure and controlled host infestation)	Published (2019)
3.5 4.4 STUDY 7	Douda K, Zieritz A, **Vodáková B**, Urbańska M, Bolotov, IN, Marková J, Froufe E, Bogan AE, Lopes-Lima M, Review of the globally invasive freshwater mussels in the genus *Sinanodonta* Modell, 1945. Hydrobiologia, in revision.	Initial work on paper (structure and manuscript base for further detailed work of coauthors), data extraction and categorisation of all papers published on the topic for overview of *Sinanodonta* spp. research	Under review

5 Conclusions and Recommendation for Scientific and Technical Development

This thesis presents the outcomes of various studies connected by the host-parasite relationship of fish and unionids in central Europe.

The aim to modernise glycogen level analyses in bivalve soft tissue originally by Naimo et al. (1998) resulted in a decrease of material and workload consumption by the method. These reductions also mean more cost-efficient methodology. This study serves as a pilot study for subsequent research of the connections between glycogen level in the soft tissue and life, sexual and annual cycles of the animals.

Second simple biomarker described in this thesis is lipid content in juveniles analysed by fluorescence method. The used method was shown as more sensitive in distinguishing between host population than measuring juvenile length and estimation of metamorphosis success. This study was conducted in collaboration with National Park Šumava and the results will have a direct impact on the ongoing conservation program and its future research efforts. There is space for improvement of the used method. Different possible stains should be tested and even the possibility of staining in-vivo to eliminate the need of sacrificing juveniles of endangered species. Moreover, the link between host condition and condition of *M. margaritifera* juveniles remains to be tested in deeper details.

The developed low-cost monitoring and sample collection system was proven to be well designed for the target purpose with possible use in other fields after specific alterations. The system provides opportunity for a wide range of research topic in aquatic field and the possible alterations for different use or use on different species is an open window for future research.

This thesis presents two studies focusing on in vitro rearing of Unionida. The study focusing on the start of the *M. margaritifera* cultivation has shown the effect of different compounds of the used medium. The results of this study provide information that will help to build on in subsequent research aiming to successfully rear *M. margaritifera* juveniles via in vitro approach. Different species can benefit from the results as well, since the already developed in vitro protocols can be optimized and improved by applying such tests of used medium compounds as shown in this thesis. The aim to compare in vivo and in vitro reared juveniles and their performance and performance of their subsequent generation was completed. Our results have shown that the in vitro rearing could be used as a promising conservational tool since the performance wasn’t worsened by the artificial rearing. However, the differences in performance found between juveniles originating from different maternal individuals show us

space for follow-up research to clarify mechanisms behind this result to ensure the best possible outcome from artificial rearing programs that could be part of conservation efforts.

The objective to test the effects of tramadol and methamphetamine on the host-parasite relationship of fish and mussels via asymmetric exposure was successfully fulfilled. Our results can be used as argument in debate about the risks of pharmaceuticals in treated sewage water that should be brought to public knowledge. The effects of these chemicals on juveniles reared on exposed hosts and originating from exposed mothers could be tested in future research, as well as the effect of chronic exposure on mussels and their host and juvenile mussels reared on chronically exposed hosts or originating from chronically exposed maternal mussels.

Last but not least, the overview of research focused on *Sinanodonta* spp. invasion is presented in this study. Our results show that there is a lack of literature focusing on the prevention of its spread. There are still regions with no occurrence of this invasive mussel, and it would be beneficial to focus on prevention in these regions.

6 References

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