PRIMARY RESEARCH PAPER

Effect of substrate particle size on burrowing of the juvenile freshwater pearl mussel *Margaritifera margaritifera*

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Abstract Juveniles of the endangered freshwater pearl mussel (FPM, *Margaritifera margaritifera*) live burrowed in stream substrate for the first years of their life. Fine sediments block water exchange within substrate and may cause juvenile mortality and recruitment failure. To better understand the connection between success of juvenile FPM and substrate particle size, it would be important to understand behavioural responses of FPM to varying substrate sizes at this critical life stage. We placed newly detached FPM juveniles in a 7-mm layer of sieved sand sorted into five sizes (< 120, 120–200, 200–250, 250–500 and 500–650 μm) each with 10 replicate dishes, 10 juveniles per dish, with burrowing status monitored for 96 h. Mean dish-specific proportion burrowed (PB) was significantly affected by substrate size, increasing from 52% in the finest sand to 98% in the coarsest sand. Furthermore, the significant substrate × time interaction was observed due to dropped PB (30-34%) in finest sand at 2–4 h time points. Thus,

results suggest a clear behavioural response by juvenile FPM to substrate size, with fine sediments triggering surfacing behaviour. Surfacing may indicate stress, can increase predation risk, and expose to drift and/or enable drift of juveniles.

Keywords Behaviour · Early juvenile stage · Endangered species · Sediment · Siltation · Unionida

Introduction

With their rapidly declining biodiversity, freshwater ecosystems include the most threatened types of habitats and biota worldwide (Ricciardi & Rasmussen, 1999; Dudgeon et al., 2006; Geist, 2011). One of the main reasons for this decline is habitat degradation, with the fine sediment load considered as a major pressure (Richter et al., 1997; Henley et al., 2000; Denic & Geist, 2015: Lopes-Lima et al., 2017). The freshwater mussels (Unionida) – globally the most endangered animal group (e.g. Bogan, 1993; Lydeard et al., 2004; Strayer et al., 2004; Régnier et al., 2009; Lopes-Lima et al., 2017, 2018) – are significantly dependent on the quality of stream/lake substrate because of their benthic ecology.

Freshwater mussels provide important ecosystem services contributing to nutrient cycling, water purification and bioturbation (Vaughn & Hakenkamp, 2001;

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Howard & Cuffey, 2006; Vaughn, 2018). Given their potentially high biomass and abundance (e.g. Kasprzak, 1985), the decline of freshwater mussel populations can have remarkable implications for ecosystem functioning (Howard & Cuffey, 2006; Strayer, 2014). Like many other freshwater bivalves, the freshwater pearl mussel, *Margaritifera margaritifera* (Linnaeus, 1758), has declined substantially and is highly endangered or threatened throughout most of its distribution range (e.g. Young et al., 2001; Moorkens, 2011: Lopes-Lima et al., 2017, 2018). Fulfilling the characteristics of an indicator, flagship, umbrella and keystone species, *M. margaritifera* is an ideal target species for conserving aquatic ecosystem functioning (Geist, 2010).

Margaritifera margaritifera has a larval stage, glochidium, which is parasitic on salmonid fish, *Salmo trutta* Linnaeus, 1758 and *S. salar* Linnaeus, 1758 (Salonen et al., 2016, 2017). After being released to the water by female mussels in autumn, glochidia attach to the body of host fish where they encyst. The glochidia remain attached to the host fish for several months during which they grow and develop into small mussels, excyst, drop off and start their benthic life as a juveniles (Young & Williams, 1984; Salonen et al., 2017).

The post-parasitic, juvenile stage of *M. margaritifera* burrows within the stream substrate for a period of several years, a phase which is considered to be critical stage in the life cycle of *M. margaritifera* (Bauer, 1988; Buddensiek et al., 1993) and all Unionida mussels. In many declining *M. margaririfera* populations, mussels are able to produce glochidia, but juvenile recruitment has not been reported for decades (Lopes-Lima et al., 2017; Oulasvirta et al., 2017). In addition, adult *M. margaritifera* are found over a wider range of physical conditions than juveniles (Buddensiek et al., 1993; Hastie et al., 2000), suggesting that juvenile *M. margaririfera* are more susceptible to adverse environmental conditions than the adults. Studies on the oxygen needs of juvenile freshwater mussels indicate that their behaviour and survival are affected at much higher oxygen concentrations than those affecting adult mussels (Dimock & Wright, 1993; Sparks & Strayer, 1998). Above all, dissolved oxygen concentrations are much lower within the substrate than in the free waterbody (Buddensiek et al., 1993; Strayer et al., 1997), and during summer period, temperatures within

the substrate are cooler than at the surface of the sediment (Gough et al., 2012). Thus, the interstitial habitat of juvenile mussels differs vastly from the surface environment inhabited by adult mussels and calls for examination on its own.

Several studies indicate that stream bed quality is the most important factor limiting the recruitment of *M. margaritifera* (e.g. Geist & Auerswald, 2007; Österling et al., 2010; Denic & Geist, 2015). Even slight changes in the stream hydrological processes influence the microhabitats within the stream bed and may result in critical habitat degradation. In their postparasitic phase, juvenile *M. margaritifera* depend on a continuously aerated interstitial space within the stream substrate (Buddensiek et al., 1993; Geist & Auerswald, 2007). Fine sediment depositions alter the substrate composition and change the suitability of the substrate for juvenile mussels (e.g. Wood & Armitage, 1997; Henley et al., 2000). Clogging the top layer of substrate, fine sediments impede exchange between the free water body and interstitial water (Munn & Meyer, 1988; Ryan, 1991; Geist & Auerswald, 2007), reducing the well-aerated habitat spaces required by mussel juveniles (Wood & Armitage, 1997; Geist & Auerswald, 2007).

Stream sites with successful *M. margaritifera* juvenile recruitment are characterized by coarser and better sorted substrates with low quantities of fines (e.g. Buddensiek et al., 1993; Hastie et al., 2000; Geist & Auerswald, 2007; Österling & Högberg, 2014). Thus, the negative dependence between the recruitment of juvenile *M. margaritifera* and fine sediments has been shown clearly, but juvenile behaviour when exposed to substrates of varying particle size has not been studied. Knowing the behavioural responses of *M. margaritifera* juveniles to different particle sizes of substrate would increase our understanding of the mechanisms behind the negative effects of fine sediments on juvenile freshwater mussels. This knowledge would also contribute to the research priorities for freshwater mussels listed by Ferreira-Rodríguez et al. (2019), one of them being species response to the effects of land-use change, such as sedimentation.

Burrowing behaviour of adult Unionida mussels has been studied from many angles (e.g. Lewis & Riebel, 1984; Di Maio & Corkum, 1997; Watters et al., 2001; Saarinen & Taskinen, 2003; Taskinen & Saarinen, 2006; Allen & Vaughn, 2009). However,

only a few studies have focused solely on the burrowing behaviour of juvenile Unionida (Yeager et al., 1994; Sparks & Strayer, 1998; Archambault et al., 2013, 2014; French & Ackerman, 2014; Kemble et al., 2020), and even fewer have examined the burrowing behaviour of margaritiferids (Bílý et al., 2020). Experimental studies on a total of six Unionidae species by Yeager et al. (1994) and Kemble et al. (2020), showed that almost all juveniles burrowed into test sediment within 20 min. Low oxygen, high temperature and drought have been found to cause surfacing and other stress behaviours in Unionida juveniles (Sparks & Strayer 1998; Archambault et al., 2013, 2014). To our knowledge, only one study prior to ours has examined the burrowing behaviour of juvenile *M. margaritifera*; Bílý et al. (2020) found recently that 1-year-old *M. margaritifera* tended to burrow to depth of 2–3 cm in 12 cm substrate tubes, installed in natural river bed, with sand grain size of 1.0–2.0 mm. Low oxygen conditions within the test substrate were related to surfacing and low survival rates of juveniles. However, burrowing behaviour of recently detached (0 years old) *M. margaritifera* juveniles has not been previously studied. The aim of the present study was to fill this gap, as it is important to know the behavioural responses of juvenile *M. margaritifera* to substrate quality immediately in the beginning of their benthic life.

In this study, we investigated experimentally the effect of substrate particle size on burrowing behaviour of freshly metamorphosed juvenile *M. margaritifera*. We aimed to determine how fast *M. margaritifera* juveniles burrow into the substrate and do they stay burrowed or re-surface. Our hypotheses were as follows: (1) Based on the results of comparative field studies on the relationship between *M. margaritifera* recruitment and sediment quality (e.g. Buddensiek et al., 1993; Hastie et al., 2000; Geist & Auerswald, 2007; Österling et al., 2010), and on the results of unionid juveniles indicating that surfacing behaviour of juveniles is a sign of stress (Sparks & Strayer, 1998; Archambault et al., 2013, 2014), we hypothesized that – in the current range of particle sizes – proportion of burrowed juveniles would be negatively related to fine substrates, and thus, the percentage of burrowed juveniles was predicted to be higher in coarse substrates. (2) Based on the previous results on burrowing of Unionidae juveniles (Yeager et al., 1994; Kemble et al., 2020), we hypothesized that

a large majority of *M. margaritifera* juveniles would burrow within 30 min of time.

Materials and methods

To receive juvenile *M. margaritifera*, 0 + year old brook trout, *Salmo trutta*, fry ($N = 500$, River Ohtajoki, tributary of River Iijoki stock, Finland) were transported to River Haapuanoja (tributary of River Iijoki), a known *M. margaritifera* river (Salonen et al. 2017), on September 9, 2006, and put into five fish cages. *M. margaritifera* is known to release glochidia during the last half of September in this area (Salonen et al. 2017). Fish cages were submerged in the river, to expose the fish to naturally shed glochidia, and covered for shading. On October 17, 2006, the fish were transported to a laboratory for inspection. Infection success was confirmed by sampling two fish, which were infected with 300–500 glochidia when the gills were inspected microscopically. The remaining fish were kept over winter in groundwater (collected from a well) in a 1000 l tank at 8°C and fed with commercial fish food pellets. The tank was siphoned every 2–3 days once the juveniles began dropping from the host fish in June, 2007.

On June 13, 2007, newly transformed juveniles ($N = 550$, mean length = 363 ± 11 μm) were transported in 1-l containers in 8°C groundwater to the laboratory of Department of Biology and Environmental Science, University of Jyväskylä, where the experiment was performed. Juveniles were acclimated to the new water conditions by slowly adding water from the River Ähtävänjoki (Finland) to containers. Water was added to the juvenile containers 1–3 times an hour for 24 h until all the groundwater had been replaced with river water.

Bottom substrate collected from a sandy patch of the littoral zone of Lake Jyväsjärvi (Finland) was washed, dried, and sorted with sieves into five size classes: (1) < 120 μm; (2) 120–200 μm; (3) 200–250 μm; (4) 250–500 μm; and (5) 500–650 μm. These size classes correspond substrate sizes from fine sand to coarse sand (grain size definitions: ISO 14688-1:2018, 2018). Each treatment was applied to ten replicates and ten juveniles were placed in each replicate, a 60 mm (diameter) × 15 mm (height) plastic dish. Each dish was filled with substrate and River Ähtävänjoki water so that the total height of

substrate and water was 12 mm, of which 7 mm was substrate. Experiment was performed at 17–18°C in 12 h dark:12 h light conditions without changing the water. After placing the juveniles in random order on substrates, using a pipette, juveniles were monitored by observing them under a dissection microscope with an additional light source. The visible, not-burrowed juveniles were counted at 0.5, 1, 2, 4, 72, and 96 h time points. After the experiment, surviving juveniles were counted; only one juvenile died during the experiment.

For statistical analyses, burrowing percentage, i.e. the proportion of burrowed individuals, was used as the response variable, and single dish (containing 10 juveniles) was used as the statistical unit. The effect of substrate size on the proportion of burrowed individuals was tested using repeated measures analysis of variance (RM-ANOVA) with time (0.5, 1, 2, 4, 72 and 96 h) as within-subject factor and substrate type (five substrate size classes) as a between-subject factor. To meet the assumptions of ANOVA, an arc-sin transformation was performed on proportion data before statistical analysis. If the sphericity assumption of RM-ANOVA was not met (Mauchly's $W = 0.569$, $P < 0.05$), the Greenhouse–Geisser correction for the F-statistics was performed. The assumptions of normality and equality of error variances were not completely fulfilled because in the coarsest substrate class 500–650 μm, all juveniles were completely burrowed at the last three time points. ANOVA is robust for normality assumption violation, but if homogeneity-of-variance assumption is violated in ANOVA, the significance level will be underestimated, increasing the risk of falsely rejecting the null hypothesis. However, e.g. when analysing the effect of time on the proportion of burrowed juveniles in the coarsest substrate, the null hypothesis was not rejected (see "Results"), suggesting that the homogeneity-of-variance assumption violation did not bias the results. Paired (Post Hoc) tests were performed to analyse differences between substrate size classes over the 96-h experiment and between time points within each substrate size treatment. P values of these paired tests were corrected using LSD (least significant difference) adjustment because of multiple comparisons. Statistical analyses were performed using IBM SPSS Statistics for Windows 24.0 (SPSS inc., Chicago, IL, USA). Means are given with ± standard error (S.E.) of the mean.

Results

Juveniles burrowed quickly in the beginning of the experiment. After 30 min, the mean ± S.E. proportion of burrowed individuals over all replicates was 80.6 ± 2.4%, ranging from 65 ± 5.4% in the finest sand to 95 ± 5.4% in the coarsest sand (Fig. 1).

When assessed over the whole experiment, the effect of substrate size on juvenile burrowing was statistically significant (RM-ANOVA, $F_{4, 45} = 127.77$, $P < 0.001$). During the 96-h experiment, the lowest mean (± S.E.) proportion of burrowed individuals (52.0 ± 2.8%) was in the finest sand substrate, size < 120 μm; pairwise comparisons with LSD adjustment revealed that the mean percent of burrowed juveniles in this substrate size class was statistically significantly lower than in any other (coarser) sand size ($P < 0.001$) (Fig. 1). Next lowest mean proportions of burrowed individuals, 72.3 ± 2.0 and 78.3 ± 1.6%, were observed in the substrate size classes of 200–250 and 120–200 μm, respectively, which did not differ from each other but differed significantly from other substrate classes in pairwise comparisons (LSD adjusted $P < 0.001$ in all cases).

The highest mean (± S.E.) proportions of burrowed individuals over the 96-h experiment, 94.2 ± 1.2% and 98.0 ± 0.7%, were observed in the coarsest substrate size classes, 250–500 μm and 500–650 μm, respectively (Fig. 1). Paired comparisons revealed that these two substrates differed from each other statistically only marginally significantly by their mean percent burrowed over the whole experiment (LSD adjusted $P = 0.057$), but they both differed significantly from all other substrate sizes (LSD adjusted $P < 0.001$ in all cases) (Fig. 1).

There was a statistically significant interaction between 'time' and 'substrate size' (RM-ANOVA, $F_{20, 225} = 2.599$, $P < 0.001$), indicating that the time-dependent changes in the burrowing percentages were not equal in all substrate size classes. In the finest sand substrate, the lowest proportion of burrowed juveniles (of the whole experiment), 30 ± 4.0%, was observed at the time point 2 h and the maximum percent burrowed in this substrate was 67 ± 4.7% at 96 h, which was observed at the end of the experiment (Fig. 1), repeated measures ANOVA performed for the finest sand substrate, < 120 μm, revealed a significant effect of time on the percent of burrowed juveniles ($F_{5, 45} = 8.154$, $P < 0.001$). Pairwise
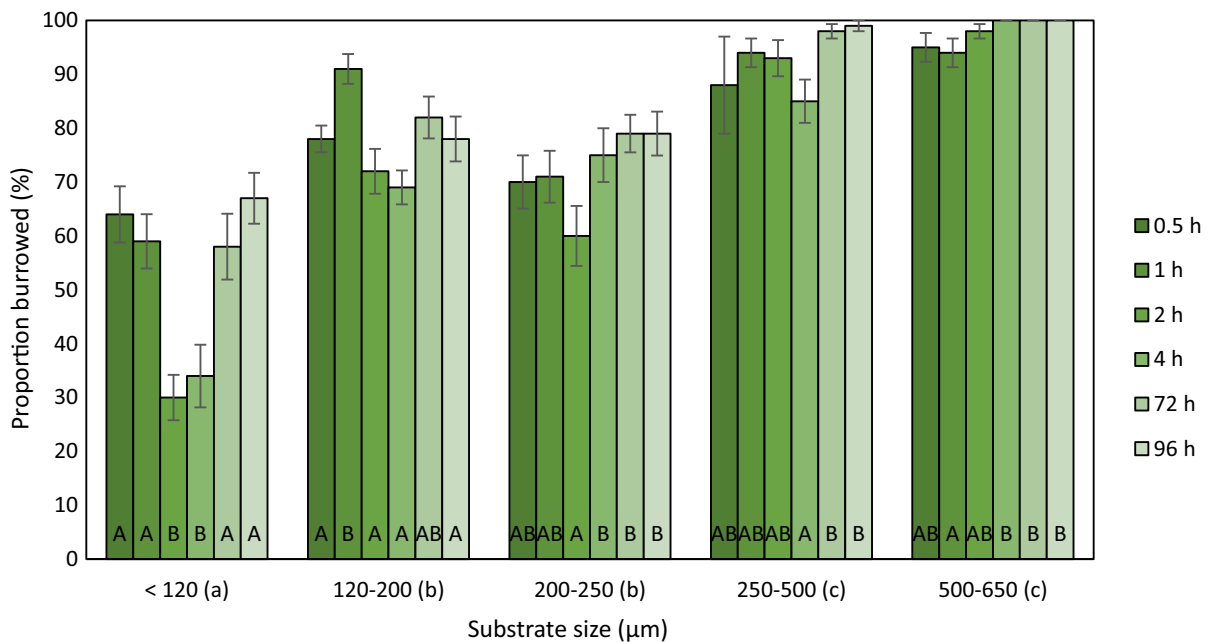
Fig. 1 Proportion (%) of burrowed juvenile *M. margaritifera* in five substrate sizes at six time points. Each bar represents mean (± SE) of 10 replicate dishes; each dish included 10 juvenile mussels. Time points (i.e. bars) marked with the same capital letter did not differ statistically significantly from each other within a substrate size class. Similarly, substrate sizes marked with the same small case letter (in parentheses) did not differ from each other by their mean proportion burrowed over all time points. Proportion of burrowed juveniles increased with substrate particle size

comparisons indicated that the time points 2 and 4 h did not differ from each other (LSD adjusted $P = 0.492$) while they both differ from all other time points (LSD adjusted P values ranging from $P < 0.001$ to $P = 0.020$). Also in the second finest sand substrate, 120–200 μm, there was a significant effect of time on juvenile burrowing (RM-ANOVA, $F_{5, 45} = 5.256$, $P = 0.001$) but the temporal pattern was totally different as compared to the finest sand substrate. In the second finest sand substrate, percent burrowed was the highest at the time point 1 h, differing significantly from all other time points (LSD adjusted P values ranging from $P = 0.002$ to $P = 0.019$) except for time 72 h (Fig. 1).

By contrast, in the coarser sand substrates, the statistically significant temporal variation in the percentages of burrowed juveniles was not found. There were no significant temporal differences in the percent of burrowed juveniles in the substrate size class 200–250 μm (RM-ANOVA, $F_{5, 45} = 2.018$, $P = 0.094$), in the substrate size class 250–500 μm (RM-ANOVA with a Greenhouse–Geisser correction, $F_{5, 45} = 2.109$, $P = 0.125$) or in the substrate size class

500-650 μm (RM-ANOVA with a Greenhouse–Geisser correction, $F_{5, 45} = 2.858$, $P = 0.072$). In the coarsest sand, the proportion of burrowed juveniles varied from 94 ± 2.7% at 1 h to 100.0% at 4, 72, and 96 h (Fig. 1). Thus, in the coarsest sand, all *M. margaritifera* juveniles were completely burrowed by 4 h from the start of the experiment and stayed completely burrowed after that.

These contrasting temporal patterns, especially the marked decrease in the percent burrowed at 2–4 h after the start of the experiment in the finest sediment as compared to the constant, high percent burrowed in the coarsest sand substrates probably explains the significant 'time' × 'substrate size' interaction observed in the data.

Discussion

We found that the proportion of burrowed juvenile *M. margaritifera* was dependent on the size of the available substrate. The hypotheses were supported as (1) most juveniles had burrowed in 30 min in all

substrate sizes and (2) more juveniles stayed burrowed in coarse sand than in fine sand. Thus, the burrowing speed was in the same range as in the previous studies with Unionidae juveniles (Yeager et al., 1994; Kemble et al., 2020). In addition, if surfacing behaviour of juveniles is considered as a sign of stress, as suggested by studies of Sparks & Strayer (1998) and Archambault et al. (2013, 2014), our results indicate that *M. margaritifera* juveniles were less stressed in the coarser sand substrates of the present experiment.

Despite able to burrow into the finest sand, juveniles resurfaced after a couple of hours, suggesting that juveniles have an ability to discriminate a poor habitat from a good one. Resurfacing from an unsuitable site not only exposes juveniles to the current with the risk of becoming dislodged and being washed away, but it also gives juveniles a chance to drift to a more suitable site, as also suggested by Bílý et al. (2020). Conversely, at suitable sites, it is beneficial for an individual mussel to avoid exposure to the stream current by staying burrowed. Laboratory experiments show that exposed juveniles are vulnerable to predation (Lin, 1991; Klocker & Strayer, 2004). Thus, if a suitable interstitial habitat is not available for burrowing, juveniles may be susceptible to an increased predation rate.

The results of this study are consistent with field studies in which fine sediments are associated with poor recruitment of juvenile *M. margaritifera* (Young & Williams, 1983; Hastie et al., 2000; Geist & Auerswald, 2007; Österling & Högberg, 2014). Deposition of suspended fine particles is known to deteriorate the microhabitats of interstitial layer by, e.g. lowering concentrations of interstitial dissolved oxygen (e.g. Munn & Meyer, 1988; Ryan, 1991; Geist & Auerswald, 2007). Juvenile freshwater mussels have been shown to be sensitive to low concentrations of dissolved oxygen (Dimock & Wright, 1993; Sparks & Strayer, 1998). The results of the present study as well as earlier work (Sparks & Strayer, 1998; Bílý et al., 2020) suggest that juveniles move to the surface as a response for avoiding an oxygen-depleted or otherwise unsuitable habitat. This hypothesis is in accordance with studies showing adverse physiological effects of fine sediments on freshwater mussels. Increased turbidity derived from fine sediments can interfere with filter-feeding (Aldridge et al., 1987), reduce growth (Österling et al., 2010) and lower

attachment and metamorphosis success (Gascho Landis et al., 2013) of freshawater mussels.

Recently, Bílý et al. (2020) showed experimentally that 1-year old *M. margaritifera* juveniles avoided deeper, low-oxygenated zones of the 12-cm substrate tube. Therefore, it is possible that the surfacing behaviour of *M. margaritifera* juveniles in fine sand in the present study was associated with decreased oxygen in fine sediment treatments. However, a steep oxygen gradient is not expected to take place in a relatively shallow (7 mm) sediment layer of our study. In addition, juveniles re-burrowed in the finest sediments during the course of time, which would indicate that substrate quality, rather that oxygen concentration, will explain the results. More importantly, our results verify that the burrowing tendency of young *M. margaritifera* is developed and effective as soon as they have detached from the fish host.

The results of this study indicate that newly metamorphosed juvenile *M. margaritifera* are, to certain extent, able to avoid poor habitats by sensing their surroundings within the substrate and surfacing at unsuitable sites. Further studies are needed to determine whether juvenile *M. margaritifera* are able to move horizontally within different types of substrates and whether their movement shows active habitat selection. Zajac & Zajac (2011) suggested that movements of adult *Unio crassus* Philipsson, 1788 were prompted by the avoidance of unsuitable sites, but the extent to which newly metamorphosed Unionida juveniles are able to influence their location is largely unstudied.

Our results show that substrate particle size is an essential measure of *M. margaritifera* habitat quality. If burrowing is assumed as a central feature of juvenile *M. margaritifera* behaviour, it was clear that—within the range of tested particle sizes—the coarse sand (500–650 μm) can be ranked as the best substrate for newly metamorphosed juveniles. In the coarse sand, all *M. margaritifera* juveniles were completely burrowed by 4 h from the start of the experiment and stayed completely burrowed after that until the end of the 96-h experiment.

Even though the present study did not examine juvenile survival related to substrate particle size, it is noteworthy that only one of the 500 newly excysted individuals died during the 96 h experiment. This suggests that the juveniles and the experimental

conditions were of equal, high quality in all treatments throughout the experiment.

Restoration of stream substrate is costly and requires long-term focus on catchment land use, but it is probably the most important conservation action for restoring threatened populations of *M. margaritifera.* The decline of Unionida mussels has prompted a number of studies on captive breeding of Unionida juveniles (e.g. Jones & Neves, 2002; Lima et al., 2012; Kunz et al., 2020; Hyvärinen et al., 2020). Culturing mussel juveniles can be the only way to conserve the most threatened populations and to retain the evolutionary and genetic potential of the species (Geist & Kuehn, 2005; Geist & Kuehn, 2005), but it cannot replace habitat restoration as a conservation measure. As suggested by Moorkens & Killeen (2014), introducing captive-bred juveniles to suitable stream sites could maintain an endangered population until long-term stream restoration has sufficiently improved the mussel habitat. Identifying stream sites with suitable substrate conditions is essential for the success of captive breeding initiatives aiming to restore the endangered populations of *M. margaritifera*. In providing new information about the behavioural responses of juveniles to varying substrate particle sizes, present study is a step towards understanding the essential habitat requirements of juvenile *M. margaritifera* – a prerequisite for successful conservation of this endangered species.

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