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

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ABSTRACT

Captive breeding is an effective conservation strategy, but it has risks, especially when a life history stage of an organism is bypassed. Freshwater mussels (Unionida) are critically imperiled, and their larvae are parasites on fishes. Traditional mussel captive breeding involves artificially infesting fishes with larvae (in vivo), but increasingly used in vitro methods allow larval metamorphosis in culture media, bypassing the parasitic stage. We provide the first comparisons of mussel performance between in vitro and in vivo methods in the wild and throughout the mussel life cycle using two mussel species. In six streams, survival and growth did not differ between in vitro- and in vivo-produced *Lampsilis cardium*. Metamorphosis of *Sinanodonta woodiana* differed sharply between two in vitro protocols (methods 1 and 2), but metamorphosis for method 2 was twice as high as in vivo. Survival and growth after eight days was lower for in vitro method 1 than method 2 and in vivo, showing that suboptimal in vitro protocols can have lingering effects on juvenile performance. However, survival and growth did not differ among methods by the end of the first and second growing seasons. Most importantly, in vitro-produced mussels survived, grew to maturity, and produced F_2 juveniles naturally on fishes, all at rates that did not differ from in vivo-produced mussels. We detected no strong side effects of bypassing the mussel host-fish stage, but this study illustrates the importance of assessing consequences of captive breeding methods for any organism in a variety of environmental and life history contexts.

1. Introduction

Captive breeding techniques are an integral part of many conservation programs (Ebenhard, 1995; Seddon et al., 2007), but they may produce individuals poorly adapted to the wild, and releasing those individuals can compromise the fitness of wild populations (Araki et al., 2007; Davis et al., 2020; Snyder et al., 1996). Furthermore, multiple captive breeding methods exist for some organisms, but the fitness of individuals produced by these methods may differ (Davis et al., 2020). Captive breeding and its effects are well-studied for some groups, including mammals (Pinder and Barkham, 1978), fishes (Attard et al., 2016; Fraser, 2008), and amphibians (Griffiths and Pavajeau, 2008). Consequences of captive breeding are less well-studied for invertebrates (Witzenberger and Hochkirch, 2011), but some evidence suggests that these groups also may experience negative outcomes related to this technique (Davis et al., 2020; Lewis and Thomas, 2001; Pearce-Kelly

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et al., 1998).

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

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

Despite the advantages of in vitro methods, they represent a major modification of the natural process of metamorphosis. Furthermore, in vitro methods require antibiotic treatment to control bacterial growth in the media and a CO_2-enriched atmosphere to regulate pH (Roberts and Barnhart, 1999). Consequently, in vitro methods have the potential to produce mussels that differ substantially from those produced in the wild or in vivo. In 96-hour laboratory toxicity tests, in vitro juvenile mussels were slightly more sensitive to several toxicants than in vivo juveniles, but the magnitude of the differences was within normal toxicity test variation, suggesting that juveniles produced by both methods responded similarly (Popp et al., 2018). However, no studies have evaluated differences between in vitro and in vivo mussel performance in the wild or throughout the mussel life cycle. In particular, the ability of in vitro-metamorphosed mussels to produce glochidia that can successfully attach, encapsulate, and metamorphose on fishes is not known. In vitro methods are increasingly widely used, and at least 60 species have been produced using these methods (Lima et al., 2012; Patterson et al., 2018; Kovitvadhi and Kovitvadhi, 2012). A better understanding of differences in performance between in vitro- and in vivo-produced mussels is necessary to evaluate whether in vitro production is appropriate for widespread conservation use.

We evaluated differences between in vitro- and in vivo–produced mussels concerning traits that directly affect the success of conservation efforts. First, we compared growth of in vitro and in vivo juvenile *Lampsilis cardium* after rearing to five months of age in a hatchery and subsequent survival and growth in the wild during three-month exposures in six streams. Second, we compared metamorphosis success, survival, and early juvenile growth among *Sinanodonta woodiana* produced by two different in vitro methods and in vivo on two host fish species. Third, we raised *S. woodiana* produced in vitro and in vivo to sexual maturity (18 months) in mesocosms and compared their survival, growth, and reproductive success, including natural recruitment of F_2 juveniles from fishes. Our results provide a comprehensive comparison of in vitro and in vivo mussel performance in a variety of contexts and throughout the mussel life cycle, which will allow a more informed assessment of conservation approaches for these imperiled animals.

2. Materials and methods

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

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

We produced juvenile *L. cardium* in vitro in 15 × 100 mm Petri dishes with 13.0 mL of a culture medium containing 3:1 parts by volume M199 cell culture medium (Sigma Aldrich M4530) and rabbit serum (Sigma Aldrich R4505), respectively. The medium was supplemented with 100 μg/mL each of rifampicin (Sigma Aldrich R7282), carbenicillin (Sigma Aldrich C3416), and gentamicin (Sigma Aldrich G1264); 1 μg/mL antimycotic amphotericin B (Sigma Aldrich A2411); and 10 μL/mL menhaden oil (Sigma Aldrich F8020). After flushing glochidia from marsupia, we cleaned them by rinsing with Eagles' minimum essential medium (Sigma Aldrich M5650), placed them in dishes, and incubated dishes at 1% CO_2 and 24 °C. On day 23 after start of the culture, we added sterile water at 1:4 by volume for 20 min then fully diluted dishes until they were clear.

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

We deployed mussels in streams from May 22 to June 10, 2015. We chose six study streams in Kentucky, USA, representing different ecological and conservation contexts. Elkhorn and Russell creeks are warm, well-buffered, productive streams in the Interior Low Plateaus physiographic province (drainage areas at the study sites = 1270 km^2 and 677 km^2, respectively; mean summer water temperature = 24.4 °C for both streams). The Green (4471 km^2, 23.2 °C) and Nolin (961 km^2, 21.3 °C) rivers are cooler, well-buffered, and moderately productive Interior Low Plateaus streams that are heavily influenced by karst. Horse Lick Creek (159 km^2, 20.1 °C) and the Rockcastle River (1564 km^2, 22.9 °C) are cool, moderately well-buffered, and less productive streams in the Appalachian Plateaus physiographic province. The conservation status of the mussel fauna varies widely among the streams from relatively healthy with ≤15% species loss (Elkhorn Creek, Green River) to essentially defaunated and >85% species loss (Horse Lick Creek, Nolin River; Haag et al., 2019).

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

We calculated proportional survival as the number of live individuals

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

2.2. Metamorphosis success and early juvenile performance

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

2.2.1. In vitro methods

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

We produced juvenile mussels using two different in vitro methods that differed in the volume of the culture medium, the initial number of glochidia, and maternal individuals used. In method 1, dishes received 17.5 mL culture medium and 872 ± 189 glochidia (mean ± SD). In method 2, dishes received 10.5 mL culture medium and 338 ± 147 glochidia (mean ± SD); these two methods represented similar numbers of glochidia/mL. We used glochidia from females A–C for method 1 and from females D–F for method 2, and we used 12 dishes for each female for a total of 36 dishes for each method. For both methods, we incubated all dishes at 5% CO_2 under UV light for 1 h prior to adding glochidia. We rinsed glochidia with sterile water to remove any remaining marsupia tissue fragments and placed them in dishes with sterile Pasteur pipettes.

We incubated all dishes at 5% CO_2 and 24 °C for 6 days after which metamorphosis was complete. On day six, we added sterile water to all dishes at 1:1 by volume. On day seven, we completely replaced culture media with sterile water and returned the CO_2 level to atmospheric. On day eight, we examined the contents of each dish under a stereomicroscope and quantified metamorphosis success as the number of active juveniles/the initial number of glochidia added to the dish.

2.2.2. In vivo methods

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

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

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

2.2.3. Early juvenile survival and growth

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

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

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

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

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

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

We examined differences in juvenile size among production methods at the end of the first growing season and winter with a single-factor ANOVA, with log-transformed mean length within each mesocosm pool as the response variable. At the end of the second growing season, we expressed growth as instantaneous growth as described previously and based on initial size at the end of the first growing season. We examined differences in growth among production methods during the second growing season with a single-factor ANOVA, with mean instantaneous growth within each mesocosm pool as the response variable. We examined differences in survival among production methods at the end of the second growing season with a single-factor ANOVA with arcsine-transformed survival in each pool as the response variable. We expressed recruitment as finite population growth rate (λ/yr) based on the final adult population size and the number of F_2 recruits produced in each pool. We examined differences in λ and recruit size among production methods with two separate single-factor ANOVAs with λ or log-transformed mean recruit size in each pool as the response variables. Statistical analyses were performed in R (v. 4.0.2, R Core Team, 2020).

3. Results

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

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

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

3.2. Metamorphosis success and early juvenile performance

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

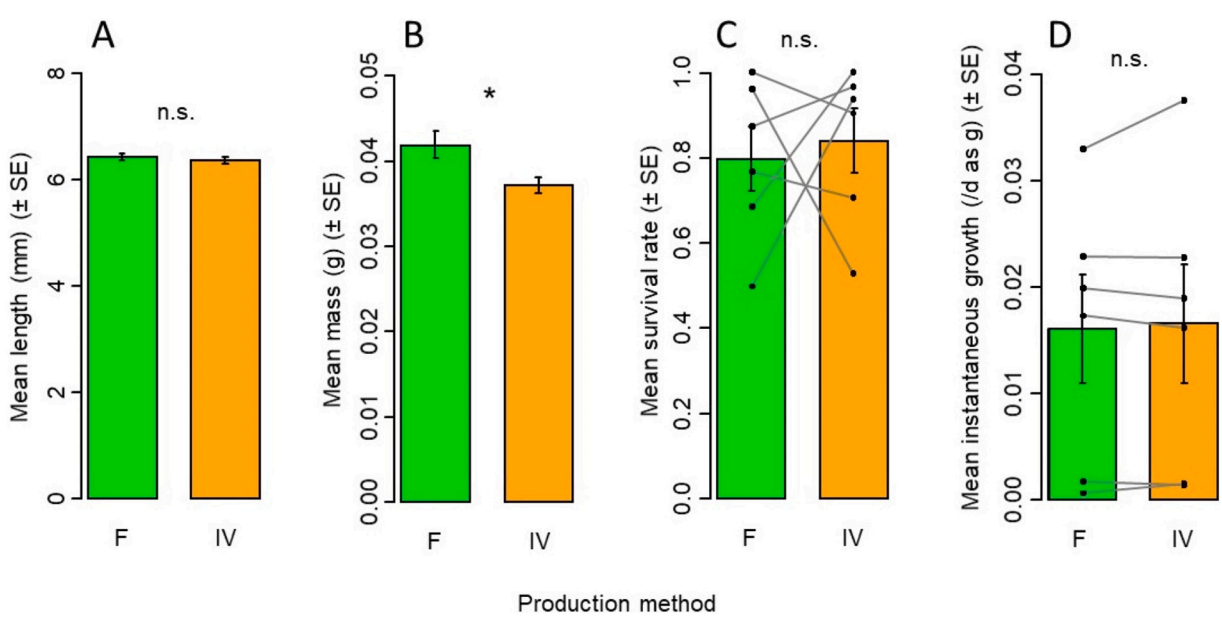

Fig. 1. Performance of in vivo-produced (host fish = *Micropterus salmoides* – F, green) and in vitro-produced (IV, orange) mussels (*Lampsilis cardium*). (A and B) Mean juvenile length and mass after five months rearing in hatchery conditions. (C and D) Mean survival and instantaneous growth during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

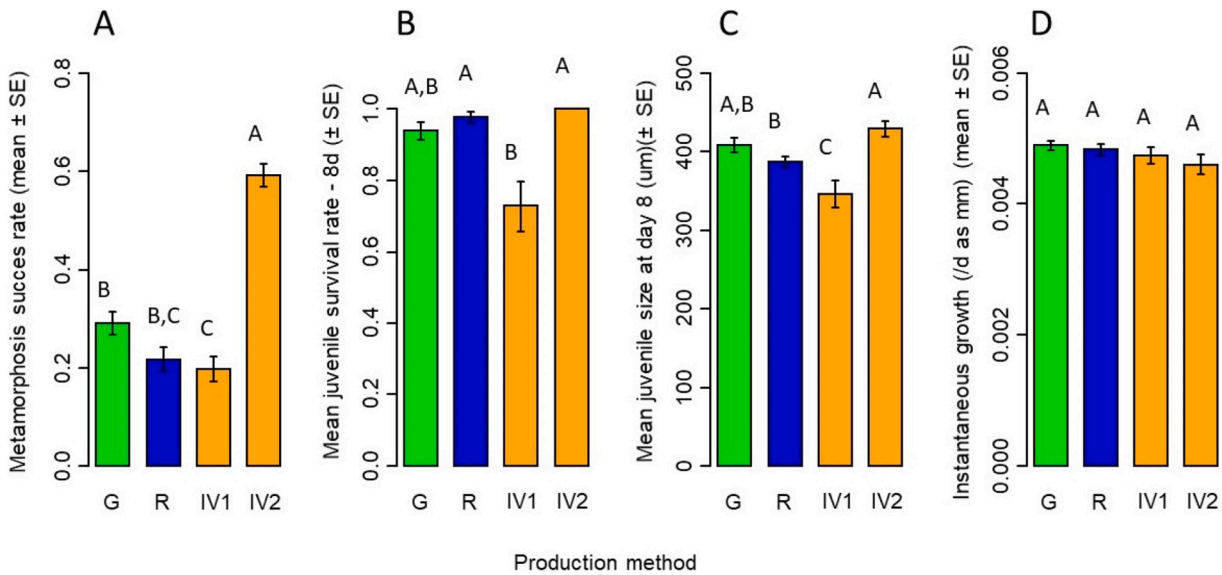

Fig. 2. Performance of in vivo-produced (host fishes = *Gobio gobio* – G, green; *Rhodeus amarus* – R, blue) and in vitro-produced (method 1 = IV1, method 2 = IV2 - orange) mussels (*Sinanodonta woodiana*). A) Proportion of metamorphosed larvae. B) Mean juvenile survival during 8 days post-metamorphosis.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

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

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

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

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

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

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

4. Discussion

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

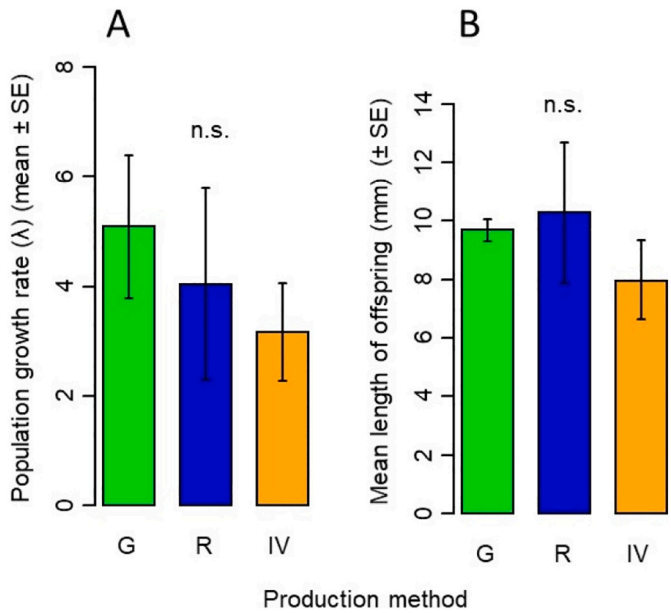

Fig. 3. Population growth rate (A) and mean lengths of F_2 juveniles (B) produced naturally on host fishes (*Scardinius erythrophthalmus*) by F_1 captively-bred mussels in mesocoms. F_1 mussels were produced by in vivo methods (*Gobio gobio*–G, green; *Rhodeus amarus*–R, blue) and in vitro (IV, orange).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

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

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

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

When an appropriate in vitro protocol is used, we found no important differences in post-metamorphosis performance between in vitro- and in vivo-produced juveniles of *S. woodiana*. The only significant difference we found between these methods was the smaller juvenile size for *Rhodeus*-produced juveniles compared with in vitro- produced juveniles, but this size difference disappeared by the end of the first growing season and winter. Most importantly, we showed that in vitro-produced mussels are capable of surviving and growing to maturity and producing F_2 juveniles naturally on fishes, all at rates that did not differ from in vivo-produced mussels. Sexual maturation of in vitro-produced mussels has been reported previously (Owen, 2009), but ours is the first study to compare in vitro- and in vivo-produced mussels throughout their life cycle.

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

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

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

of production methods.

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

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

CRediT authorship contribution statement

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

Declaration of competing interest

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

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