Hydrobiologia
https://doi.org/10.1007/s10750-022-04865-1

PRIMARY RESEARCH PAPER

Assessing methods for mitigating fungal contamination in freshwater mussel in vitro propagation

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Received: 1 June 2021 / Revised: 15 March 2022 / Accepted: 16 March 2022

Abstract Many freshwater mussel species are critically imperiled, and propagation is essential for species 'recovery.' Fungal contamination can negatively affect in vitro propagation of freshwater mussels; thus, we investigated methods of mitigating fungal contamination. Specifically, we tested the effect of medium replacement frequency and antifungal (Amphotericin B) concentrations on risk of fungal contamination and transformation success of two species, *Alasmidonta heterodon* and congener, *Alasmidonta undulata*. We observed a trend of increased contamination risk in treatment groups that received medium replacements every 1 or 2 days (vs. every 3 days), but this trend was not statistically significant. We found that contamination risk differed significantly across Amphotericin B concentrations. Although contamination severity reduced transformation success, we found no significant difference in transformation success based on medium replacement frequency. Amphotericin B concentration was negatively correlated with transformation success, suggesting toxicity at higher concentrations. Additionally, we identified the fungal pathogen (*Candida parapsilosis*), the first knowledge of this fungus during mussel culture. Our findings suggest that low contamination results in high probability for

Handling Editor: Manuel Lopes Lima

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Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10750-022-04865-1.

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Published online: 07 April 2022

transformation success; however, there is a tradeoff in higher concentrations of Amphotericin B. This study offers new ideas for improving mussel propagation techniques that may better control fungal contamination and increase transformation success.

Keywords In vitro propagation · Unionidae · Dwarf wedgemussel · Triangle floater · *Pathogen* · Culture contamination

Introduction

Since the early 1900s, propagation and population augmentation or reintroduction have been used to restore freshwater mussel (Bivalvia, Unionida) populations (Lima et al., 2012). Historically, propagation mimicked natural reproduction, using host fish to transform glochidia to juveniles, a process called in vivo propagation (Patterson et al., 2018). While host fishes are still used for propagation, in the last 40 years, methods have been developed to propagate freshwater mussels with growth medium in lieu of host fishes to transform glochidia to the juveniles. Effective medium for culture varies by species and may consist of different amounts and types of serums, lipids, amino acids, antifungals, antibiotics, and vitamins (Owen, 2009). In vitro propagation methods often use glochidia more efficiently than in vivo methods, obtaining higher transformation rates than in vivo propagation (Lima et al., 2012). When metamorphosis success was compared in one study of *Sinanodonta woodiana* (I. Lea, 1834), in vitro methods produced higher transformation compared to in vivo methods (Douda et al., 2021). These higher juvenile outputs are especially important for species with low fecundity where maximizing production yield is critical for restoration efforts. Although there is some concern regarding the overall fitness of in vitro-produced juveniles (Popp et al., 2018), research found no significant difference in length or mass of in vivo versus in vitro-propagated *S. woodiana* juveniles after one growing season, no difference in length of *Lampsilis cardium* Rafinesque, 1820, and only a 12% difference in mass of *L. cardium* after 5 months (Douda et al., 2021). Furthermore, when in vivo and in vitro animals were used to produce 2nd-generation juveniles on fish, there was no difference found in population growth rate or mean length (Douda et al., 2021).

Several species of freshwater mussels have been successfully propagated using in vitro methods (Owen, 2009; Lima et al., 2012); however, one key challenge to successful propagation is management of fungal contamination (Owen et al., 2010). Fungi are ubiquitous and live on our bodies, artificial materials, soil, water, and air; however, depending on the amount and species, fungi can be harmful to organisms in the environment (Trofa et al., 2008; Newbound et al., 2010). Fungal contamination causes poor growth in culture (Macleod & Langdon, 2010) and can kill glochidia before they transform, which creates logistical challenges when removing transformed juveniles from culture medium (Monte McGregor, Aquatic Scientist/Malacologist with the Kentucky Department of Fish and Wildlife Resources, personal communication, Frankfort, Kentucky, March 15, 2019). Building blocks for minimizing contamination include good aseptic techniques, an organized workspace, use of healthy cells (glochidia), and consistent monitoring of the contamination (Ryan, 1994). To mitigate contamination during propagation, procedures typically include frequent (as often as daily) changes of medium (Monte McGregor, personal communication, March 15, 2019) and a low dose of the antifungal, Amphotericin B (Owen et al., 2010). Amphotericin B is a polyene antibiotic that was the first commercially significant antifungal drug (Gallis et al., 1990; Lemke et al., 2005) and has been widely used in a variety of cell cultures and to treat fungal infections in human patients (Steimbach et al., 2017) in addition to propagation of freshwater mussels (Owen et al., 2010). Currently, Amphotericin B is administered as a comprehensive antifungal, as the species of fungi growing in culture is currently not known (Monte McGregor, personal communication, March 15, 2019). In medicine, Amphotericin B is given in low doses to patients combating fungal infections because the drug is accompanied by dose-limited toxicities and can cause harm to the patient if given in high enough amounts (Hamill, 2013). It is presumed that a similar harmful effect would cause low transformation in freshwater mussels and thus many formulas call for a low concentration of Amphotericin B (0.67–5 µg/ml) to control fungal contamination (Keller & Zam, 1991). However, a 50 µg/ml dose of Amphotericin B did not detrimentally affect transformation of the cockscomb

pearl mussel [*Cristaria plicata* (Leach, 1814)] (Ma et al., 2018). It is unknown how Amphotericin B concentration impacts transformation success in other species. Although transformation success does not necessarily equate to ultimate juvenile survival of propagated mussels, to date, no studies have quantified the effect of fungal contamination on mussel transformation or identified the species of fungi infecting mussels in vitro.

The goal of this research was to determine how fungal contamination and methods of mitigation influenced transformation success of two *Alasmidonta* species, *Alasmidonta heterodon* (I. Lea, 1830), and *Alasmidonta undulata* (Say, 1817). Our objectives were to (1) determine which methods of contamination mitigation—frequency in changes of medium and concentrations of the antifungal Amphotericin B—caused the least contamination risk, (2) identify the species of fungus in experimental dishes, and (3) assess how different methods mitigation affected mussel transformation success. This research will contribute to the improvement of in vitro propagation methods for freshwater mussels by developing ways to mitigate contamination and maximize transformation.

Methods

Study species

This study focused on *A. heterodon*, a small (<45 mm) mussel found in isolated distributions along the Atlantic slope of the UUSA from New Hampshire to North Carolina, where it is federally protected under the United States Endangered Species Act (USFWS, 2007). *A. heterodon* is considered a host generalist and has a maximum theoretical life expectancy of 12 years (Michaelson & Neves, 1995). To minimize experimentation on a federally listed species, two experiments used *Alasmidonta undulata* as a surrogate for *A. heterodon*. *A. undulata* is closely related to *A. heterodon* and has similar life history and ecological traits (e.g., habitat requirements), although *A. undulata* are slightly larger than *A. heterodon* and have higher fecundity. The species are often sympatric in the northern Atlantic Slope of the USA (Nedeau, 2008). *A. undulata* is protected in many states or considered vulnerable throughout much of its range (Cordeiro, 2011).

Broodstock collection, housing, and glochidia extraction

We conducted two experiments to address the study objectives where we examined how each experiment and their treatments impacted contamination severity and transformation success. Experiment 1 examined frequency in changes of medium for *A. heterodon* glochidia and Experiment 2 examined different Amphotericin B concentrations of *A. undulata*. For Experiment 1, two gravid *A. heterodon* were collected via view bucket and snorkeling from the Mill River in Whately, Massachusetts in March 2019 and brought to the U.S. Fish and Wildlife Service's Cronin Aquatic Resource Center (CARC) in Sunderland, MA. Mussels were housed in a recirculating system with weekly water changes and held at 2 °C until day 0 of the experiment on June 13, 2020.

For Experiment 2, six gravid *A. undulata* were collected from the Squannacook River in Townsend, Massachusetts in November 2019 using a combination of snorkeling and view buckets. Mussels were held in silos (adapted from Chris Barnhart; Missouri State University) in the river so that the glochidia could fully mature before their use in experiments. The mussels were moved to CARC on January 2, 2020, where they were housed in a recirculating system with daily water changes. The temperature was increased from 1.5 to 12 °C over 5 days for the start of the experiment on January 7, 2020. All mussels were measured, tagged, and cleaned before use.

Glochidia were extracted from each mussel by rupturing one (for *A. undulata*) or both (for *A. heterodon*) gills with a water-filled syringe with a 22G needle. Glochidia from the ruptured gills from each female were kept in separate beakers with 100 ml of sterile water. Glochidia density and viability were tested using the salt test following Neves et al. (1985). Five, 200 µl subsamples were taken from each beaker and 1 drop of salt solution (NaCl) was added to each subsample of glochidia. Glochidia that closed after the addition of salt indicated that the glochidia were viable and those that did not were considered not viable. Percent viability for each subsample is calculated using the following equation:

$$\left(\frac{\text{Open before salt addition - Open after salt addition}}{\text{Open before salt addition + Closed before salt addition}}\right) \times 100.$$

The mean of the 5 subsamples was calculated to determine viability for each mussel. The viability of *A. heterodon* used in Experiment 1 were 97.7% and 100.0%, resulting in an average viability of 98.9%. The viability of *A. undulata* used in Experiment 2 were 89.1%, 90.4%, 96.1%, 97.1%, 100.0%, and 100.0% for the 6 mussels, resulting in an average viability of 95.3%. Additionally, glochidia from the same 5 subsamples were counted and used to volumetrically estimate the number of viable glochidia from each gravid female.

After testing viability and volumetric estimates, glochidia from each mussel were combined into a single beaker filled with the filtered medium solution (Table S1). On a sterile, clean bench, the medium was continuously agitated to prevent glochidia from snapping shut on one another. Debris from the mussel and non-viable glochidia were removed by repeatedly decanting the medium off the top as the glochidia settled to the bottom. Additional debris and open glochidia were removed with a pipette.

For both experiments rabbit serum (33%) was added to the base solution (67%) (Table S1). Prior to use, the rabbit serum was heat treated by warming it in a 56 °C hot water bath for 30 min to help prevent fungal and bacterial contamination in the culture (Barile, 1973). Then the basal medium and serum were brought to a pH of 7.65 by adding small amounts (< 1 ml) of sodium hydroxide (NaOH) solution until the medium reached the desired pH. All medium and serum were stored at a temperature of − 30 °C and brought up to match the temperature of the glochidia before use.

Experiment 1 procedures

Experiment 1 examined how frequency of changes in the medium impacted contamination severity and transformation success of *A. heterodon* glochidia. There were 3 treatments based on the frequency in changes of medium: every 1 day (control, $n = 5$), every 2 days ($n = 5$), or every 3 days ($n = 3$) as well as two nonexperimental dishes. Base medium (5 ml, Table S1) and approximately 150 glochidia were added to each replicate petri dish (60 mm × 15 mm). During incubation, dishes were kept in a sterile incubator at 23 °C with 1.5% CO_2 and were only opened on days coinciding with changes of medium. All changes of medium took place on a laminar flow horizontal flow bench, while the researcher was wearing gloves sprayed with 70% isopropyl alcohol to minimize risk of contamination. Live glochidia were removed from the old medium using a sterile pipette and placed in a new dish with clean medium to rinse the glochidia. Then, the glochidia were moved to a new dish of medium and placed back in the incubator.

The level of microbial contamination severity was assessed daily prior to changes of the medium using a scoring system (Table S2). Dishes were removed from the incubator for a short time (< 10 min) and observed under the microscope to assess for contamination. The contamination score ranged from 0 to 5 where a score of '0' indicated the fungus was not detectable in the dish and where a score of '5' indicated the fungus was intertwined with the glochidia and could only be separated through invasive and time-consuming measures. On the day a dish was given the highest fungal contamination score of '5,' it was removed from the experiment, and the dish was given a score of '5' daily for the remainder of the experiment.

Starting on day 11 of the experiment, 10 glochidia were taken out of 2 nonexperimental dishes and diluted with filtered (0.1 µm), chlorine-free water that was warmed to 23 °C (to match the temperature of the incubator) to assess for transformation. One hour after full dilution, the dishes were given a fresh dose of water to promote movement and glochidia were counted under a microscope and assessed for transformation. A glochidium was considered transformed if it was closed and dark in color, with developed adductor muscles. Once at least 50% of the glochidia were considered transformed, all the experimental dishes were diluted. To dilute glochidia from the medium, 1.5 ml of filtered, chlorine-free water (at pH of 7.65 and 23 °C) was added to each dish. After 15 min, an additional 3 ml of water was added to each dish, then after another

15 min, 4 ml of water was added. At 45 min, using a wash bottle with the same water, the glochidia were gently spun to the center of the dish and slowly (~2 min) diluted from medium to sterile freshwater. Dishes were left on the clean bench overnight to let the glochidia acclimate and then moved to a recirculating system the next day. Transformation success was determined prior to dilution; they were not re-counted after dilution.

Experiment 2 procedures

Experiment 2 examined how different Amphotericin B concentrations impacted the level of contamination severity and transformation success. Treatments varied in Amphotericin B concentration: 0 µg/ml (control), 1 µg/ml, 3 µg/ml, 5 µg/ml, and 10 µg/ml. Prepared glochidia were transferred to 25 dishes and divided into the 5 treatments, with 5 replicates per treatment and approximately 100 individuals in each replicate. Fungal contamination was assessed at the same time each day in the same manner as Experiment 1, and media changes for this experiment took place every 3 days.

Starting on day 12 of the experiment, 10 glochidia were taken out of 2 nonexperimental dishes (with 1 µg/ml of Amphotericin B) and diluted to assess for transformation. On day 13, more than half of the individuals moved in sterile freshwater after 30 min (indicating transformation), so all individuals were removed from the medium. Replicate dishes were diluted at the same time as described in Experiment 1 placed back in the incubator (at 23 °C) overnight and the next day 5 ml of fresh, sterile water was added to each dish. Each dish incubated for approximately 11 h before its second observation. The mussels were counted and assessed for transformation success at the second observation and was considered 'transformed' if it displayed bright adductor mussels and a dark interior.

Identification of microbial contamination

To identify the species of fungal contamination growing in the dishes, discarded samples of contaminated medium from 3 dishes (two 10 µg/ml dishes and one 3 µg/ml dish from Experiment 2) were poured into separate, sterile bottles and observed under a microscope. Two replicates of each yeast sample were cultured on potato dextrose agar for 4 days in an incubator prior to DNA extraction.

Replicates of yeast and filamentous fungi DNA were extracted using both the Qiagen DNeasy Plant Mini Kit and the DNA Immunoprecipitation (DIP) method. Polymerase chain reaction (PCR) was performed using the Eppendorf Mastercycler Gradient (Hamberg, Germany), and the quality of the DNA was tested using standard gel electrophoresis. PCR was performed using Eukaryotic ITS and LSU primers 5′-CGT AAC AAG GTT TCC GTA GGT GAA C-3′ 5′-GTT GTT ACA CAC TCC TTA GCG GAT-3′ (Robideau et al., 2011). Samples (4 yeast, 2 filamentous, and 1 negative control) were sent to the Psomagen Inc. (formerly Macrogen) for DNA sequencing. The reverse complement of the sequences were taken using the tool Reverse Compliment tool (https://www.bioinformatics.org/sms/rev_comp.html) and then the forward and reverse nucleotides for the DNA sequences were aligned using the EMBOSS Water tool (Madeira et al., 2019). DNA sequences were then examined using SeaView (version 5.0.2, Gouy et al., 2010) which identified the longest strings of nucleotides. The Basic Local Alignment Search Tool (Altschul et al., 1990) compared the longest nucleotide sequences to those in the databases from the National Center of Biotechnology Information and calculated match likelihood to known samples in the database.

Data analysis

Fungal contamination risk

We assessed the risk of fungal contamination across treatments for each of the two experiments using Kaplan–Meier survival analysis followed with Log-rank post hoc comparison across treatments (Rich et al., 2010). Kaplan–Meier survival curves were fitted to the time of first observation (i.e., days) of a given level of contamination, and treatment variables were evaluated as time-independent covariates of contamination probability (i.e., replacement time in Experiment 1 and Amphotericin B concentration in Experiment 2). We used this approach to evaluate the effect of medium replacement (Experiment 1) and Amphotericin B concentration (Experiment 2) for risk of any fungal contamination (i.e., a contamination score ≥ 1). Treatment groups for both experiments were treated as categorical variables and each

dish served as a single replicate. Data were right censored such that no dish was followed in the analysis after the first positive observation. Dishes where no specified contamination was observed were given a 0 for contamination score with a follow-up time as t_x+1, where t_x is the day when the trial was terminated ($t_x=12$ in Experiment 1 and 13 in Experiment 2). Kaplan–Meier Survival curves and post hoc Log-rank tests were fitted using the 'survival' package in R (Therneau, 2021). We compared statistical differences across Kaplan–Meier curves using the Log-rank method, with a level of significance a = 0.05 (Rich et al., 2010). We further assessed differences between each treatment level and the control using pairwise Log-rank tests and incorporated a Bonferroni-corrected a to control for a Type I Error (Colak et al., 2017), where a = 0.017 for Experiment 1 (n = three total comparisons among 3 groups) and a = 0.005 for Experiment 2 (n = 10 total comparisons among 5 groups). Our incorporation of the Bonferroni method is a conservative adjustment to avoid Type 1 Error for two reasons: first, the Bonferroni method is among the more conservative multiple comparison post hoc analyses for the Log-rank test in survival analysis (Colak et al., 2017) and second, we adjusted a for all possible comparisons among treatment groups, not just comparisons between the control and each subsequent treatment group.

Transformation success

To assess how the level of contamination and various methods of mitigation influenced transformation success, we used a generalized linear mixed model (GLMM) with a logistic regression framework and a logit link transformation. Models were fit using the glmmTMB function from the 'glmmTMB' package in R (Brooks et al., 2017). All glochidia data were expressed as a binary response where 0 = non-transformed glochidia (i.e., failure) and 1 = transformed glochidia (i.e., success). This gave the dataset a hierarchical structure, which allowed for individual glochidia to be nested within a dish, which allowed each dish to function as a random effect in all models (following Hazelton et al., 2013).

To evaluate the effects of fungal contamination on mussel transformation success, we created 3 categories of covariates: time to contamination (i.e., onset), contamination frequency (i.e., prevalence), and the maximum contamination score for each dish, which resulted in 9 contamination covariates for evaluation (Table S3). Time to contamination consisted of four covariates, including days to any contamination present and days to contamination score of 1 or higher, 2 or higher, and 3 or higher. For replicates that did not achieve a specific contamination score during the experiment, we assumed the time to contamination was t_x+1, where t_x is the length of the trial in days (trial length = 12 days for Experiment 1, and 13 days for Experiment 2). Four covariates for contamination frequency were calculated based on the number of days that a replicate was scored at a given contamination level: any contamination present, contamination score above 1, contamination score above 2, and contamination score above 3. Maximum contamination was calculated as the highest contamination score during the experiment.

To reduce variables included in the final logistic regression models, we calculated Pearson correlation coefficients (r) for all contamination covariates and percent transformation for each experiment. Percent transformation was calculated as the proportion of juveniles successfully transformed divided by the number of total glochidia placed in each dish at the start of the experiment. We selected the contamination covariate from each category (i.e., time to event, contamination frequency, and maximum contamination) with the highest Pearson r with transformation success to be included in logistic regression models. We also incorporated treatments of contamination mitigation as a predictor variable for each experiment. In Experiment 1, we modeled contamination mitigation treatments as nominal variables with the following categories: Daily Medium Change (reference level), Alternate Medium Change, and Three-Day Medium Change. For Experiment 2, we modeled treatment as a continuous variable of Amphotericin B concentration (0, 1, 3, 5, and 10 µg/ml). Models were compared using Akaike Information Criteria with a correction for small sample size (AIC_c) using the 'aictab' function in the 'AICcmodavg' package in R (Marc Mazerolle, 2020). We chose to interpret models with the highest AIC_c weight of evidence (AIC_{wi}) up to a cumulative weight of evidence of 0.70 for

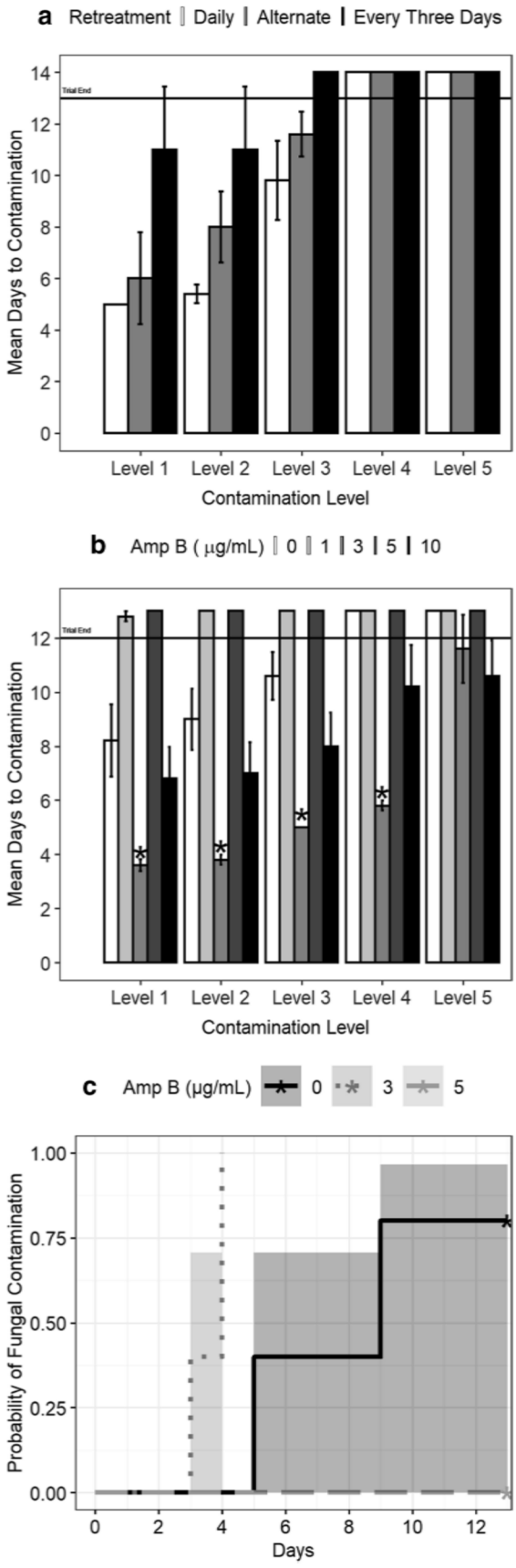

Fig. 1 Kaplan–Meier-restricted mean times to fungal contamination across all contamination levels: **a** Comparison of media change frequency (Experiment 1) and **b** comparison of Amphotericin B concentration (Experiment 2) measured through Kaplan–Meier survival analysis. Groups represented with "*" are statistically significant from control group at that level of contamination through Log-rank post hoc pairwise comparison, with a Bonferroni-adjusted $a = 0.005$. **c** Kaplan–Meier event curve comparison of effects of Amphotericin B concentration on probability of fungal contamination for all levels of contamination. Horizontal line on a & b demarcates trial termination

parameter estimation. Parameters from all top models were assessed using standard errors and level of significance ($P < 0.05$).

Results

Experiment 1

In the evaluation of fungal contamination risk across different frequencies of media changes, we observed 83 contamination events across 182 total observations (Table S4). Contamination onset (i.e., day of first contamination) occurred first in the treatment group with daily changes of medium (min = 3 d, mean ± SD = 5.2 ± 4.92 d, $n = 4$), followed by the treatment where the medium was changed every other day (min = 5 d, mean = 5.0 ± 0.0 d, $n = 4$). The treatment where the medium was changed every three days also had contamination occurring first in day 5; however, most replicates had contamination onset much later (min = 5 d, mean = 11.0 ± 5.19 d, $n = 3$). Mean prevalence of contamination (i.e., number of days experiencing contamination) was also greater with daily (7.8 ± 1.20 d) and alternate day changes of medium (8.60 ± 4.83 d) than in the treatment with medium replaced every three days (0.33 ± 0.57 d, Table S5). As determined by the Log-rank test, differences in Kaplan–Meier time to event curves were not significantly different between treatments at fungal contamination level 1 ($\chi^2 = 5.2$, df = 2, $P = 0.075$), level 2 ($\chi^2 = 5.9$, df = 2, $P = 0.053$), or level 3 ($\chi^2 = 3.2$, df = 2, $P = 0.2$). Test statistics for fungal contamination levels 4 and 5 were not calculable as there were no observations at these levels (Table S5). Restricted mean days to each contamination level demonstrate earlier onset of contamination in daily and every other day media changes when compared every three-day

changes (Fig. 1a), although these were not statistically different.

Experiment 2

For the experiment comparing concentrations of Amphotericin B, there were 81 contamination events out of 325 total observations. One treatment (5 µg/ml) did not exhibit fungal contamination, 1 treatment (1 µg/ml) exhibited minimal contamination (i.e., only 1 replicate received a contamination score of 1 on the final day, the rest were 0), and 3 treatments (0 µg/ml, 3 µg/ml, and 10 µg/ml) had variable fungal contamination (Table S4). The 3 µg/ml treatment had the earliest mean onset (3.6 ± 0.55 d) and the highest prevalence of contamination (9.4 ± 0.55 d), followed by 10 µg/ml (onset = 6.80 ± 2.95 d, prevalence = 4.40 ± 3.36 d) and 0 µg/ml (onset = 8.20 ± 3.35 d, prevalence = 2.20 ± 1.64 d, Table S6). There were 3 replicates where contamination was so severe that glochidia could not be parsed out from the contamination and had to be removed from calculating transformation success (1 dish from the 3 µg/ml treatment and 2 dishes from 10 µg/ml treatment), but these replicates were still used in calculating Kaplan–Meier time to event curves because they were right censored. Log-rank tests of Kaplan–Meier event curves comparing treatments were significant for fungal contamination levels 1–4 with a $P < 0.001$ and df = 4 (Table S6. Level 1: $\chi 2 = 39.3$; level 2: $\chi^2 = 38.6$, level 3: $\chi^2 = 31.7$; and level 4: $\chi^2 = 20.30$). However, the 3 µg/ml group was the only treatment significantly different from the control group (0 µg/ml) in pairwise Log-rank tests with a Bonferroni-adjusted $a = 0.005$ and df = 1 (Level 1: $\chi^2 = 8.7$, $P = 0.003$; Level 2: $\chi^2 = 8.5$, $P = 0.003$; Level 3: $\chi^2 = 9.0$, $P = 0.003$; Level 4: $\chi^2 = 8.5$, $P = 0.003$). Figure 1b illustrates Kaplan–Meier-restricted mean time to event for all contamination levels and demonstrates progression of time to event for each increase in contamination level. Amphotericin B concentration 5 µg/ml was the best control across all levels of fungal contamination and is compared to the 0 µg/ml control and 3 µg/ml treatment group in Kaplan–Meier curve for contamination level 1 (Fig. 1c). While the 5 µg/ml group was not significantly different than the control in pairwise Log-rank tests ($\chi^2 = 6$, df = 1, $P = 0.01$), differences between the 3 and 5 µg/ml were significant at the adjusted $a = 0.005$ level of significance ($\chi^2 = 8.7$, df = 1, $P = 0.003$).

Fungal identification

Standard gel electrophoresis was performed on the PCR products of 4 yeast, 2 filamentous, and 1 negative control sample; this revealed weak bands for the filamentous fungi with the Qiagen DNeasy Plant Mini Kit method and no bands with the DIP method, so the filamentous samples were not sent out for sequencing (Fig. S1). 3 replicates of the yeast had strong bands on the gel and were sequenced using Psomagen services, revealing a 100% identity and a 100% query match to the fungus *Candida parapsilosis* (Ashford) Langeron and Talice.

Transformation success in Experiment 1 ranged from 3.2 to 69.4% across all treatments ($n = 13$ dishes). Overall transformation was highest with medium changes every 3 days (57.7% ± 1.8), followed by changes every 1 day (53.5% ± 6.0%), and changes every 2 days (19.0% ± 28.3%) (Fig. 2a). The top model predicting transformation success included 1 random effect (dish) and a single fixed effect (days where contamination score was above 2), which held 71% of the AIC_c weight of evidence (Table 1). For every 1-day increase in contamination prevalence above 2, an associated 44% decrease (i.e., odds ratio of 0.56, Table 2) in transformation probability is expected (Fig. 2b).

In Experiment 2 model comparison indicated that the top model predicting transformation success included a random effect of dish and 2 fixed effects: Amphotericin B concentration and onset of contamination score higher than 3 (Table 1). For each 1 µg/ml increase in Amphotericin B, glochidia were 28% less likely to transform (log odds = −0.322 ± 0.09), and for each daily increase in the onset to contamination level 3, glochidia were 1.34 times as likely (or 34% more likely) to transform (log odds = 0.279 ± 0.101) (Table 2, Fig. 3a, b). Transformation ranged from 0 to 95.5% and varied across all treatments: 0 µg/ml (91.3% ± 4.7%), 1 µg/ml (84.0% ± 2.1%), 3 µg/ml (69.6% ± 39.2%), 5 µg/ml (73.5% ± 6.1%), and 10 µg/ml (37.3% ± 35.2%) (Fig. 3c).

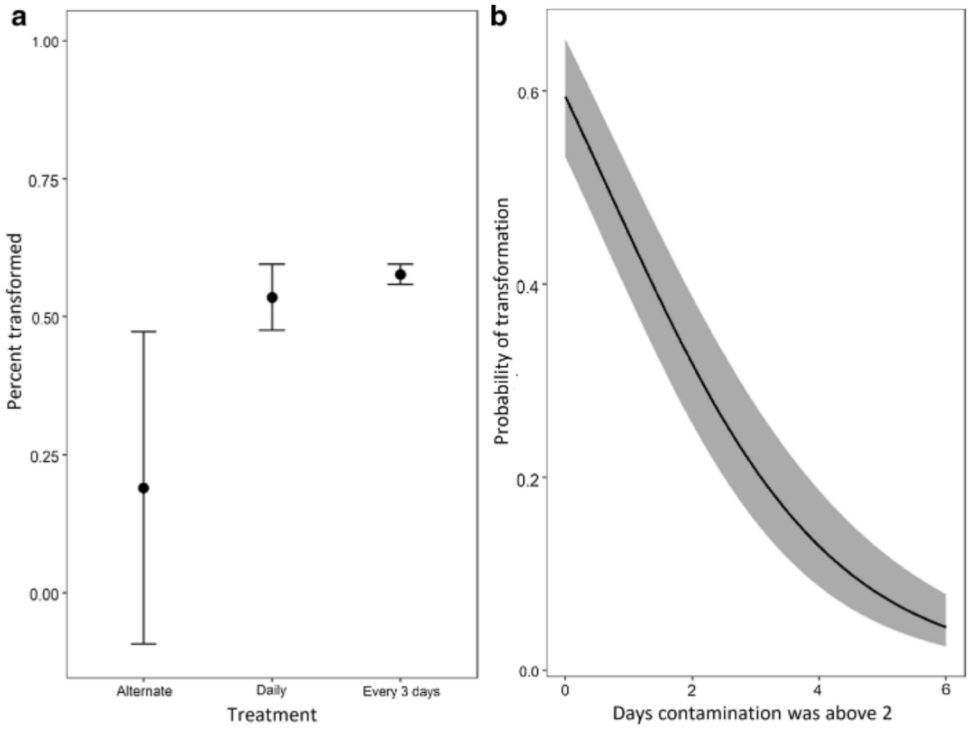

Fig. 2 Experiment 1: **a** percent transformation across different medium change treatments with standard deviations, and **b** top model representing influence of contamination severity on probability of transformation with 95% confidence intervals

Table 1 Comparison of generalized linear mixed models predicting transformation success across both experiments

Experiment/model covariates	*K*	AIC_c	ΔAIC_c	AIC_{Cwi}	*Cumulative* w_i	Log Likelihood	Pr (> chisq)	
Experiment 1								
Frequency, above 2	3	2151.35	0.00	0.71	0.71	− 1072.67	< 0.001	***
F(treatment) + frequency, above 2	5	2154.47	3.12	0.15	0.86	− 1072.22	< 0.001	***
F(treatment) + frequency, above 1	5	2154.83	3.49	0.12	0.98	− 1072.40	< 0.001	***
Frequency, above 1	3	2158.48	7.14	0.02	1.00	− 1076.24	< 0.001	***
F(treatment) + Maximum score	5	2167.29	15.95	0.00	1.00	− 1078.63	< 0.001	***
Onset, of 2	3	2172.76	21.41	0.00	1.00	− 1083.37	1	
F(treatment) + Onset, of 2	5	2173.47	22.12	0.00	1.00	− 1081.72	1	
Maximum score	3	2174.53	23.18	0.00	1.00	− 1084.26	1	
F(treatment)	4	2178.58	27.24	0.00	1.00	− 1085.28	1	
1	2	2185.76	34.41	0.00	1.00	− 1090.88		
Experiment 2								
Treat + onset, of 3	4	1949.7	0.00	0.70	0.70	− 970.82	< 0.001	***
Treat + maximum Score	4	1952.8	3.15	0.14	0.84	− 972.40	1	
Treat + frequency, contamination pres	4	1953.6	3.96	0.10	0.94	− 972.81	0.0037790	**
Treatment	3	1954.9	5.28	0.05	0.98	− 974.47	< 0.001	***
Onset, of 3	3	1958.2	8.57	0.01	0.99	− 976.11	< 0.001	***
Maximum score	3	1960.0	10.34	0.00	1.00	− 977.00	1	
Frequency, contamination present	3	1962.2	12.50	0.00	1.00	− 978.08	1	
1	2	1964.0	14.38	0.00	1.00	− 980.02		

All models included replicate as a random effect

K number of parameters, AIC_c Akaike information criterion with a correction for small sample sizes, ΔAIC_c change in Akaike information criterion with a correction for small sample sizes, AIC_{Cwi} Akaike information criterion with a correction for small sample sizes weight of evidence, *Cumulative* w_i cumulative Akaike information criterion weight, *Pr (> chisq)* associated p-value of Chi-Square test statistic

Table 2 Top generalized linear mixed models predicting transformation success across both experiments

Variable	Estimate	Std. error	Odds ratio	*z*-value	*p*-value	
Experiment 1: days above 2 + (1|Dish)						
Intercept	0.314	0.112		2.815	0.00488	**
Days where contamination score was above 2	−0.573	0.048	0.56	−11.844	< 0.001	***
Experiment 2: treatment + days to 3 + (1|Dish)						
Intercept	−0.964	1.259		−0.766	0.444	
Treatment	−0.322	0.090	0.72	−3.570	< 0.001	***
Days to a contamination score of 3	0.279	0.101	1.32	2.751	0.00595	**

All models included replicate as a random effect

Std. Error standard error, *z-value* number of standard deviations away from the mean, *p-value* level of significance

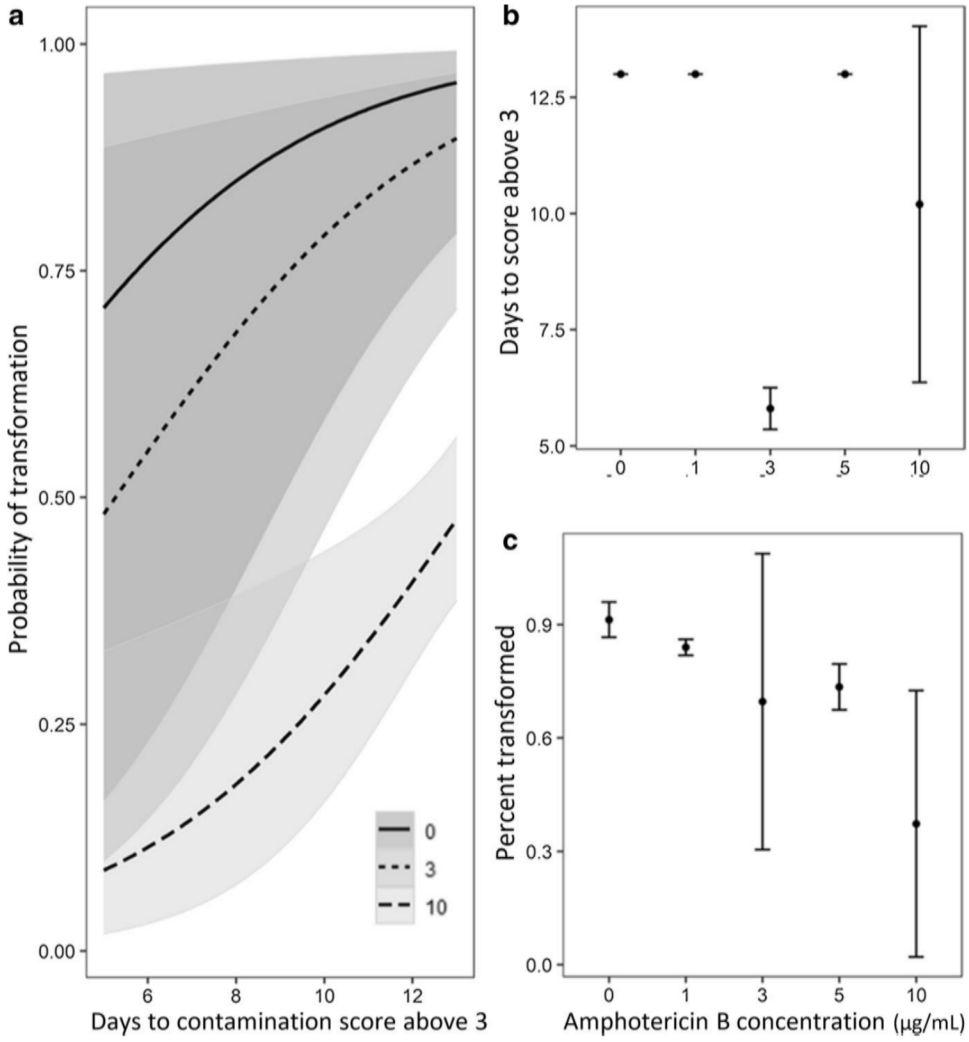

Fig. 3 Experiment 2: **a** top model for influence of contamination severity on probability of transformation based on Amphotericin B level (solid line = 0 µg/ml, short-dashed line = 3 µg/ml, long-dashed line = 10 µg/ml) with 95% confidence intervals, **b** days to a contamination score above 3 across different concentrations of Amphotericin B with standard deviations, and **c** percent transformation across different concentrations of Amphotericin B with standard deviations

Discussion

Mitigation of contamination

We were successful in mitigating contamination in culture using several methods. We found that dishes with medium changes every 3 days were consistently able to stay relatively sterile, although these comparisons were not statistically significant at $a = 0.05$. Increased contamination may be likely because the frequency that dishes were exposed to airborne contaminants was minimized (Ryan, 1994). This result is in contrast to dishes with changes of medium daily or every 2 days which experienced earlier and higher intensity of contamination. The high contamination may be due to the high frequency of potential exposure to contamination (opening the dish), while simultaneously being exposed to contamination for longer periods of time compared to dishes with daily changes of medium. These results suggest that growth medium should be changed infrequently when dishes are sterile and glochidia densities are low (43–57 glochidia per 3.5 ml of medium) (Ma et al., 2018) to minimize exposure to contamination. However, it should be noted that the antifungal, Amphotericin B, depletes after 3 days in medium when incubated at 37 °C (Thermo Fisher Scientific, personal communication, Waltham, Massachusetts, October, 15, 2019).

Time to all levels of contamination varied across different concentrations of Amphotericin B. Surprisingly, dishes with the highest doses of Amphotericin B did not have the lowest contamination. The minimum inhibitory concentration needed to impede 50% (MIC_{50}) of *Candida parapsilosis* rests somewhere between 0.13 and 1 µg/ml (Tóth et al., 2019), meaning a concentration of 10 µg/ml should have mitigated this fungal contamination better than lower concentrations of Amphotericin B. We found that higher concentrations of Amphotericin B did not necessarily prevent a higher level of contamination, but may have changed how the contamination manifests. In treatments with lower Amphotericin B that experienced contamination (0 and 3 µg/ml Amphotericin B), the fungus was yeasty and covered the whole dish ubiquitously. This expression of fungal contamination was also nearly impossible to eradicate once it appeared. In these dishes, the risk of contamination would be lower the next day after a change of medium, but inevitably just as pervasive 2 days after the change. On the other hand, in the dishes that had a higher concentration of Amphotericin B (10 µg/ml), the fungus expressed itself in a pseudofilamentous form. This difference in fungal expression may be due to an attempt by the fungus to thrive in a more stressful environment with a higher concentration of Amphotericin B (10 µg/ml) than dishes with a lower concentration of Amphotericin B (0 and 1 µg/ml) (Tóth et al., 2019). The filamentous form of fungi promotes more damage to cells than yeast (Németh et al., 2013), as filamentous fungi can invade cells, allowing the fungi to further proliferate within the cell (Tóth et al., 2019).

Source and mechanism of fungal impact

The identification of the presence and type of fungi is important because it allows for specific treatment of the fungus (Ryan, 1994) and broad-spectrum antifungals, like Amphotericin B, often fail to mitigate target fungi (Leifert & Cassells, 2001). *Candida parapsilosis*, the fungus identified in the dishes, is found in hospitals (Trofa et al., 2008), households (Zupančič et al., 2018), and in natural environments (Weems, 1992). This fungus manifests as a yeast and in a pseudohyphae (filamentous) form (Németh et al., 2013). It is possible that the fungus appeared in the dishes due to contamination exposure from human contact, as it is one of the most common fungi isolated under the fingernails of people (Trofa et al., 2008). However, changes of medium and assessments were made while wearing gloves and working under a properly sterilized clean bench each time the dishes were removed from the incubator, so it also seems probable that the fungus came from the gravid mussels and transferred to their glochidia. The natural ecology of *C. parapsilosis* remains poorly understood (Kurtzman et al., 2011) and its natural habitat has been undefined to date (Zupančič et al., 2018). The fungus has been documented in soil, marine (Trofa et al., 2008), brackish (Libkind et al., 2017), and freshwater (Maideros et al., 2012; Zupančič et al., 2018) environments. Infections by the fungi have also been documented in different animal hosts (de Aguiar Cordeiro et al., 2017). Although there have been no records of this fungus inhabiting unionids, fungi are an important component of a mussel's diet (Weber et al., 2017), and it is reasonable that fungus

may populate in the gills and glochidia of gravid mussels.

Candida parapsilosis can produce chemicals that exert cytotoxic effects on the cells of other organisms (Robledo-Leal et al., 2014). It is known for its ability to adhere to biotic and abiotic surfaces through colonization (Tóth et al., 2019), which could cause further harm to its host. This fungus also proliferates in medium with high levels of glucose or lipids (Pereira et al., 2015), which are both ingredients in the medium (Table S1). After the fungus adheres itself to a surface, the fungus will form a biofilm that provides protection to the fungus against antifungal substances and immune responses from the host (Nett, 2016; Silva et al., 2017). Under stressful conditions, such as high CO_2, low O_2, and in the presence of a serum, a filamentous expression of *C. parapsilosis* will form (Tóth et al., 2019). The filamentous form has greater virulence and can more quickly and readily damage the host than the yeast form (Németh et al., 2013). As *C. parapsilosis* causes damage to its host, the fungus may actively proliferate within the host cells, allowing the fungus to rapidly cause even more damage (Tóth et al., 2019).

Effects of contamination and mitigation methods on mussel transformation

We hypothesized that increased contamination would result in reduced mussel transformation success. In Experiment 1, treatments that had the highest contamination (those that received a change of medium every 2 days) had the lowest transformation success and dishes with the lowest contamination (with a change of medium every 3 days) had high transformation success, supporting the idea that frequent exposure to contamination is harmful. Additionally, changes of medium itself may be harmful to developing glochidia by disrupting their development (Kovitvadhi et al., 2002; Lima et al., 2006; Ma et al., 2018). In this study, daily changes of medium had moderate contamination and high transformation success, likely because the glochidia were getting fresh medium every day, which mitigated frequent exposure to contamination. Owen (2009) tested medium change frequency on the paper pondshell [*Utterbackia imbecillis* (Say, 1829)] and found dishes without changes of medium had lower transformation compared to mussels with changes of medium, a finding that contradicts our results. However, the treatments also had different concentrations of Amphotericin B (1 vs. 5 µg/ml), so it is possible that the differences in transformation success could be related to the concentration of Amphotericin B rather than the frequency of medium changes (Owen, 2009).

The concentration of Amphotericin B also influenced transformation success; dishes with high concentrations (10 µg/ml) had lower transformation success than dishes with low concentrations (0–1 µg/ml) of Amphotericin B. This may have been due to a dose-limited toxicity of Amphotericin B, as it is toxic to humans in high doses (Hamill, 2013) and is presumably toxic to glochidia in higher concentrations. However, transformation success was generally high overall regardless of Amphotericin B concentration. Ma et al. (2018) tested a much higher concentration of Amphotericin B (50 µg/ml) with the cockscomb pearl mussel (*Cristaria plicata*) and found no hindrance on transformation success when compared to much lower concentrations. It is important to note that none of the prior studies evaluated contamination risk and thus they could not assess the extent to which contamination affected transformation success across different antifungal concentrations.

Differences in the broodstock species (*A. heterodon* vs. *A. undulata*), timing of broodstock collection, and timing of the experiment may explain differences in transformation success between Experiment 1 (41.2% ± 24.8%) and Experiment 2 (71.2% ± 27.6%). In the first experiment, adult mussels were held in the lab from March 2019 until June 2019 before they were used for propagation. Naturally, gravid *A. heterodon* release their glochidia as early as April (McLain & Ross, 2005), so it is possible that the mussels produced less fit glochidia because they were held for too long. This decrease in fitness may have translated to lower transformation of their glochidia. On the other hand, the gravid *A. undulata* used for Experiment 2 were collected in the fall and held in mussel silos in the river before propagation. Glochidia for Experiment 2 were used for propagation in January, before they would have released naturally in the wild, which may have promoted fitness and overall transformation in this experiment. Future research is needed to determine if timing of propagation and holding time of gravid mussels impacts the transformation rates of glochidia over time.

Approaches for mitigating *C. parapsilosis*

Broad-spectrum antifungals often fail to eliminate target organisms (Leifert & Cassells, 2001) and additional research is needed to determine if the same species of fungi are infecting propagation dishes in labs across the country (Ryan, 1994). Identification of fungi can lead to better mitigation strategies and tailored antifungals to target-specific fungi. The prevention of fungal contamination may require experimentation with other drugs not yet tested with freshwater mussel propagation. To prevent an infection by *C. parapsilosis*, three echinocandin drugs—caspofungin, micafungin, and andulafungin—have been recommended as the first line of defense (Tóth et al., 2019).

Fungal contamination can be mitigated beyond the use of antifungals and antibiotics. Good aseptic techniques and good housekeeping include maintaining a clean workspace where culture takes place. Methods may include using a laminar flow hood, a UV light, and 70% isopropyl alcohol for sterilizing the clean bench and equipment. Additionally, because freshwater bivalves are filter feeders, they harbor microorganisms, like fungus and bacteria, so it is important to wash the mussel inside and out (Quinn et al., 2009) before collecting glochidia. Some lab practices call for washing the shell of the gravid mussels with diluted bleach and then placing the mussel in 1–2 changes of sterile filtered (0.45-µg) water for one hour before extracting glochidia (Kern, 2017). Other, more drastic, measures include sacrificing gravid mussels and removing the gills to help promote sterility (Kern, 2017); however, this practice may not be appropriate for rare species. Cleaning the gravid mussel inside and out can help minimize presence of microbial contamination (Ryan, 1994) on the glochidia at the onset for a cleaner culture. Another building block for preventing contamination includes using only high-quality glochidia, which is a practice used in many propagation facilities (Monte McGregor, personal communication, March 15, 2019). If a gravid mussel has low viability, then it may not be used for culture because it may have low-quality glochidia and could cause contamination issues in the dishes.

This research will aid in the improvement of propagation methods by helping in the development of ways to mitigate contamination while maximizing transformation for the propagation of freshwater mussels. Although this research only considered propagation of two *Alasmidonta* species, the information gained will likely offer value for propagation efforts for other freshwater mussel species. Using higher (3–10 µg/ml) concentrations of Amphotericin B than currently recommended (Owen et al., 2010) does not necessarily prevent contamination, and instead infrequent changes of medium (unless significant contamination appears) may promote greater transformation success. With fungal identification, mitigation techniques can more specifically target contaminants using different antifungals. With the establishment of new methods to control fungal contamination, in vitro propagation of freshwater mussels may become more accessible for many labs, thus increasing the potential to restore freshwater mussels.

Acknowledgements This research was funded by the U.S. Geological Survey Science Support Partnership and was carried out in a partnership between the U.S. Fish and Wildlife Service and University of Massachusetts Amherst. We would like to thank Monte McGregor and Julieann Jacobs from the Kentucky Department of Fish and Wildlife Resources for their extensive training of propagation and providing advice.

Funding This work was supported by the U.S. Geological Survey Science Support Partnership and the U.S. Fish and Wildlife Service.

Data availability Datasets generated during and/or analyzed during the current study will be made available at U.S. Geological Survey ScienceBase at https://www.sciencebase.gov/catalog/item/61ddc982d34ed79294021a22 (https://doi.org/10.5066/P90S15LR).

Declarations

Conflict of interest The authors have declared that no competing interests exist.

Ethical approval Section 7 collection permits were obtained from U.S. Fish and Wildlife Service to collect and propagate dwarf wedgemussel *Alasmidonta heterodon*. The triangle floater, *Alasmidonta undulata*, were collected under a Massachusetts state-issued permit.

References

Altschul, S. F., W. Gish, W. Miller, E. W. Myers & D. J. Lipman, 1990. Basic local alignment search tool. Journal of Molecular Biology 215: 403–410.

Barile, M. F., 1973. Mycoplasma contamination of cell cultures: incidence, source, prevention, and problems of elimination. In Kruse, P. F. & M. K. Patterson (eds), Tissue Culture Academic Press, Cambridge: 729–735.

Brooks, M. E., K. Kristensen, K. J. van Benthem, A. Magnusson, C. W. Berg, A. Nielsen, H. J. Skaug, M. Maechler & B. M. Bolker, 2017. glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. The R Journal 9: 378–400.

Colak, E., H. Ozen, B. Emir & S. Oner, 2017. Pairwise multiple comparison adjustment procedure for survival functions with right-censored data. Computational and Mathematical Methods in Medicine 2017: 8.

Cordeiro, J., 2011. Alasmidonta undulata. The IUCN Red List of Threatened Species. https://doi.org/10.2305/IUCN.UK.2011-2.RLTS.T189106A8683096.en.

de Aguiar Cordeiro, R., J. A. Sales, D. D. Castelo, R. S. N. Brilhante, Y. B. de Ponte, G. dos Santos Araújo & J. J. C. Sidrim, 2017. *Candida parapsilosis* complex in veterinary practice: A historical overview, biology, virulence attributes and antifungal susceptibility traits. Veterinary Microbiology 212: 22–30.

Douda, K., W. R. Haag, F. Escobar-Calderón, B. Vodáková, M. Reichard, X. Chen, M. McGregor, J. Yang, & M. Lopes-Lima, 2021. Effects of *in vitro* metamorphosis on survival, growth, and reproductive success of freshwater mussels. Biological Conservation 254: 108964.

Gallis, H. A., R. H. Drew & W. W. Pickard, 1990. Amphotericin B: 30 years of clinical experience. Reviews of Infectious Diseases 12: 308–329.

Gouy, M., S. Guindon & O. Gascuel, 2010. SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Molecular Biology and Evolution 27: 221–224.

Hamill, R. J., 2013. Amphotericin B formulations: a comparative review of efficacy and toxicity. Drugs 73: 919–934.

Hazelton, P. D., W. G. Cope, S. Mosher, T. J. Pandolfo, J. B. Belden, M. C. Barnhart & R. B. Bringolf, 2013. Fluoxetine alters adult freshwater mussel behavior and larval metamorphosis. Science of the Total Environment 445: 94–100.

Keller, A. E. & S. G. Zam, 1991. The acute toxicity of selected metals to the freshwater mussel, *Anodonta imbecilis*. Environmental Toxicology and Chemistry: an International Journal 10: 539–546.

Kern, M. A., 2017. Simplifying methods for *in vitro* metamorphosis of glochidia, Graduate School of Missouri State University, Springfield.

Kovitvadhi, U., P. Pakkong, N. Noparatnaraporn, L. Vilarinho & J. Machado, 2002. Study of a suitable fish plasma for *in vitro* culture of glochidia *Hyriopsis myersiana*. Aquaculture 209: 197–208.

Kurtzman, C., J. W. Fell & T. Boekhout, 2011. The Yeasts: A Taxonomic Study, Elsevier Science, Amsterdam: 1175–1176.

Leifert, C. & A. C. Cassells, 2001. Microbial hazards in plant tissue and cell cultures. In Vitro Cellular and Developmental Biology 37: 133–138.

Lemke, A., A. F. Kiderlen & O. Kayser, 2005. Amphotericin b. Applied Microbiology and Biotechnology 68: 151–162.

Libkind, D., P. Buzzini, B. Turchetti & C. A. Rosa, 2017. Yeasts in continental and seawater. In Buzzini, P., M. A. Lachance & A. Yurkov (eds), Yeasts In Natural Ecosystems: Diversity Springer, Berlin: 1–61.

Lima, P., U. Kovitvadhi, S. Kovitvadhi & J. Machado, 2006. *In vitro* culture of glochidia from the freshwater mussel *Anodonta cygnea*. Invertebrate Biology 125: 34–44.

Lima, P., M. L. Lima, U. Kovitvadhi, S. Kovitvadhi, C. Owen & J. Machado, 2012. A review on the "*in vitro*" culture of freshwater mussels (*Unionoida*). Hydrobiologia 691: 21–33.

Ma, X. Y., H. B. Wen, J. Zou, W. Jin, D. Hua, R. B. Gu & P. Xu, 2018. An improved method for *in vitro* culture of glochidia in freshwater mussel *Cristaria plicata* (*Mollusca, Bivalvia*). Hydrobiologia 810: 133–144.

Macleod, K. G. & S. P. Langdon, 2010. Essential techniques of cancer cell culture. In Langdon, S. P. (ed.), Cancer Cell Culture: Methods and Protocols Humana Press, Totowa: 20.

Madeira, F., Y. M. Park, J. Lee, N. Buso, T. Gur, N. Madhusoodanan, P. Basutkar, A. R. Tivey, S. C. Potter, R. D. Finn & R. Lopez, 2019. The EMBL-EBI search and sequence analysis tools APIs in 2019. Nucleic Acids Research 47: 636–641.

Maideros, A. O., B. S. Missagia, L. R. Brandão, M. Callisto, F. A. Barbosa & C. A. Rosa, 2012. Water quality and diversity of yeasts from tropical lakes and rivers from the Rio Doce basin in Southeastern Brazil. Brazilian Journal of Microbiology 43: 1582–1594.

Mazerolle M. J., 2020. AICcmodavg: model selection and multimodel inference based on (Q)AIC(c). R package version 2.3–1.

McGregor M. (Kentucky Department of Fish and Wildlife Resources, Frankfort, Kentucky, United States). Personal communication, 2019.

McLain, D. & M. Ross, 2005. Reproduction based on local patch size of *Alasmidonta heterodon* and dispersal by its darter host in the Mill River, Massachusetts. USA Journal of North American Benthological Society 24: 139–147.

Michaelson, D. L. & R. J. Neves, 1995. Life history and habitat of the endangered dwarf wedgemussel *Alasmidonta heterodon* (Bivalvia: *Unionidae*). Journal of the North American Benthological Society 14: 324–340.

Nedeau, E. J., 2008. Freshwater mussels and the Connecticut River watershed Connecticut River Watershed Council, Greenfield.

Németh, T., A. Tóth, J. Szenzenstein, P. Horváth, J. D. Nosanchuk, Z. Grózer & A. Gácser, 2013. Characterization of virulence properties in the *C. parapsilosis* sensu lato species. PLoS ONE 8: e68704.

Nett, J. E., 2016. The host's reply to *Candida* biofilm. Pathogens 5: 33.

Neves, R. J., L. R. Wever & A. V. Zale, 1985. An evaluation of host fish suitability for glochidia of *Villosa vanuemi* and *V. nebulosi* (*Pelecypoda*: *Unionidae*). The American Midland Naturalist 113: 13–19.

Newbound, M., M. A. Mccarthy & T. Lebel, 2010. Fungi and the urban environment: A review. Landscape and Urban Planning 96: 138–145.

Owen, C. T., 2009. Investigations for the conservation and propagation of freshwater mussels, Graduate School of the University of Louisville, Louisville.

Owen, C. T., J. E. Alexander Jr. & M. McGregor, 2010. Control of microbial contamination during *in vitro* culture of

larval unionid mussels. Invertebrate Reproduction and Development 54: 187–193.

Patterson, M. A., R. A. Mair, N. L. Eckert, C. M. Gatenby, T. Brady, J. W. Jones, B. R. Simmons & J. L. Devers, 2018. Freshwater Mussel Propagation for Restoration, Cambridge University Press, Cambridge.

Pereira, L., S. Silva, B. Ribeiro, M. Henriques & J. Azeredo, 2015. Influence of glucose concentration on the structure and quantity of biofilms formed by *Candida parapsilosis*. FEMS Yeast Research, 15: fov043.

Popp, A., W. G. Cope, M. A. McGregor, T. J. Kwak, T. Augspurger, J. F. Levine & L. Koch, 2018. A comparison of the chemical sensitivities between *in vitro* and *in vivo* propagated juvenile freshwater mussels: implications for standard toxicity testing. Environmental Toxicology and Chemistry 37: 3077–3085.

Quinn, B., M. J. Costello, G. Dorange, J. G. Wilson & C. Mothersill, 2009. Development of an *in vitro* culture method for cells and tissues from the zebra mussel (*Dreissena polymorpha*). Cytotechnology 59: 121–134.

Rich, J., J. Neely, R. Paniello, C. Voelker, B. Nussenbaum & E. Wang, 2010. A practical guide to understanding Kaplan-Meier curves. Otolaryngology - Head and Neck Surgery 143(3): 331–336.

Robideau, G., A. de Cock, M. Coffey, H. Voglmayr, H. Brouwer, K. Bala, D. Chitty, N. Désaulniers, Q. Eggertson, C. Gachon, C. Hu, F. Küpper, T. Rintoul, E. Sarhan, E. Verstappen, Y. Zhang, P. Bonants, J. Ristaino & C. Lévesque, 2011. DNA Barcoding of Oomycetes with cytochrome c oxidase subunit and internal transcribed spacer. Molecular Ecology Resources 11: 1002–1011.

Robledo-Leal, E., M. Elizondo-Zertuche, L. Villarreal-Treviño, R. D. J. Treviño-Rangel, N. García-Maldonado, J. M. Adame-Rodríguez & G. M. González, 2014. Killer behavior within the *Candida parapsilosis* complex. Folia Microbiologica 59: 503–506.

Ryan, J. A. 1994. Understanding and managing cell culture contamination. Corning Incorporated, Corning.

Silva, S., C. F. Rodrigues, D. Araújo, M. E. Rodrigues & M. Henriques, 2017. *Candida* species biofilms' antifungal resistance. Journal of Fungi 3: E8.

Steimbach, L. M., F. S. Tonin, S. Virtuoso, H. H. Borba, A. C. Sanches, A. Wiens & R. Pontarolo, 2017. Efficacy and safety of amphotericin B lipid – based formulations – A systematic review and meta – analysis. Mycoses 60: 146–154.

Thermo Fisher Scientific (Waltham, Massachusetts, United States). Personal communication, 2019.

Therneau, T., 2021. A package for survival analysis in R. R package version 3.2 13. https://CRAN.R-project.org/package=survival.

Tóth, R., J. Nosek, H. M. Mora-Montes, T. Gabaldon, J. M. Bliss, J. D. Nosanchuk, S. A. Turner, G. Butler, C. Vágvölgyi & A. Gácser, 2019. *Candida parapsilosis*: from genes to the bedside. Clinical Microbiology Reviews 32: e00111–e00118.

Trofa, D., A. Gácser & J. D. Nosanchuk, 2008. *Candida parapsilosis*, an emerging fungal pathogen. Clinical Microbiology Reviews 21: 606–625.

US Fish and Wildlife Services (USFWS). 2007. Dwarf wedgemussel (*Alasmidonta heterodon*) 5 year review: summary and evaluation. US Fish and Wildlife Services, New England Field Office, Concord, New Hampshire.

Weber, A. M., J. E. Bauer & G. T. Watters, 2017. Assessment of nutritional subsidies to freshwater mussels using a multiple natural abundance isotope approach. Freshwater Biology 62: 615–629.

Weems, J. J., Jr., 1992. *Candida parapsilosis:* epidemiology, pathogenicity, clinical manifestations, and antimicrobial susceptibility. Clinical Infectious Diseases 14: 756–766.

Zupančič, J., M. N. Babič & N. Gunde-Cimerman. 2018. High Incidence of an Emerging Opportunistic Pathogen *Candida parapsilosis* in Water-Related Domestic Environments. In de Loreto, É. S., J. S. Moraes Tondolo (ed.), Fungal Infection. IntechOpen, London.