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REGULAR ARTICLES

Pages 45-53

Survival and growth of juvenile mussels in an outdoor pond after 28-day laboratory exposure to aqueous zinc Jeffery Steevens, James Kunz, Ning Wang, Chris Barnhart, and Serena Ciparis

Pages 54-68

Applying environmental DNA methods to inform detection of *Simpsonaias ambigua* under varying water velocities in a river **Isabel Porto-Hannes**, Lauren M. Sassoubre, Brandon J. Sansom, and Todd J. Morris

Pages 69-77

Mussels propagated from a single broodstock female retain most population-level genetic variation but have altered genetic structure **Kentaro Inoue, Jillian M. Snow, Kristine M. Schoenecker, and Jessi DeMartini**

NOTES

Pages 78-82

Freshwater mussels in the bycatch of a snail fishery in the Poyang Lake region, China: a potential conservation opportunity **Noé Ferreira-Rodríguez, Liu Xiongjun, Wu Xiaoping, Caryn C. Vaughn, and Isabel Pardo**



REGULAR ARTICLE

SURVIVAL AND GROWTH OF JUVENILE MUSSELS IN AN OUTDOOR POND AFTER 28-DAY LABORATORY EXPOSURE TO AQUEOUS ZINC

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ABSTRACT

The extent to which effects seen in chronic toxicity studies in the laboratory affect mussel fitness later in life is poorly known. We examined juvenile Fatmucket (*Lampsilis siliquoidea*) survival and growth for 56 d following exposure to Zn in a laboratory bioassay. We conducted a 28-d chronic toxicity bioassay with 6-wk-old juvenile mussels exposed to a control and two Zn treatments (120 and 240 μ g/L). We then transferred surviving mussels into a grow-out pond and monitored their survival and growth for 56 d. Survival and shell length were lower in both Zn treatments than in the control after the 28-d toxicity bioassay. After the 56-d grow-out period, survival did not differ among treatments, but shell length was lower in the 240- μ g/L treatment than in the control and mass was lower in both Zn treatments than in the control. Mussel length was lower throughout the experiment in both Zn treatments than in the control, but there was weak support for a difference in the slopes, suggesting that Zn-exposed mussels may fall farther behind in size over time. Persistence of reduced size following Zn exposure could result in delayed sexual maturation and lowered fecundity, which could have long-term population-level effects.

KEY WORDS: freshwater mussel, metal toxicity, risk assessment, growth, recovery

INTRODUCTION

Freshwater mussels (order Unionida) are considered at risk of decline in part because of exposure to environmental contaminants (FMCS 2016). In toxicological studies, mussels are among the most sensitive freshwater species to a variety of contaminants, including ammonia, metals, and road salts (Wang et al. 2007, 2017; Gillis 2011). Resource agencies have prioritized mussel conservation (USFWS 2023) and included them as receptors within ecological risk assessment and natural resource damage assessment and restoration injury assessment (Besser et al. 2009, 2015, 2018). Assessing ecological risk or injury within these frameworks requires data on the toxicological effects of the chemical of concern in controlled, spiked laboratory waters and sediment or, alternatively, by directly measuring the toxicity of field-collected contaminated water or sediment. Standardized laboratory toxicity bioassays determine toxicity thresholds (e.g., 20% effect concentration) by using endpoints such as survival, growth, or reproduction tested under feeding, temperature, and other conditions that optimize control survival (ASTM International 2019). Duration of exposure, life stage, and endpoints all influence the outcomes of the bioassays. The sensitivity of organisms may increase with increasing exposure duration, and juveniles are considered the most sensitive mussel life stage (Wang et al. 2010). Chronic toxicity studies show that mussel growth is a more sensitive endpoint than survival in 28-d exposures to several toxicants (Wang et al. 2018, 2010, 2020). Consequently, the standard laboratory bioassay method for mussel chronic toxicity bioassays stipulates measurement of juvenile growth over a 28-d exposure (ASTM International 2019).

The extent to which sublethal effects seen in chronic toxicity studies in the laboratory affect mussel fitness later in life is poorly known (Newton and Cope 2007). Growth reduction

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from 84-d exposures to NaCl, Ni, and Zn was similar or greater in magnitude than in standard 28-d exposures (Wang et al. 2018, 2020). Mussel fecundity is primarily a function of body size, and a 10% reduction in length is predicted to result in a 19–44% reduction in fecundity (Hanson et al. 1989; Haag and Staton 2003). For other invertebrates, statistical or modeled relationships between laboratory bioassay endpoints and population responses have been derived based on field experiments (Moore et al. 2019). However, uncertainty remains about how growth reduction from standard 28-d exposures affects survival, growth, age at maturity, fecundity, and other fitness components over longer time periods.

Quantitative relationships between reduced growth in bioassays and long-term population outcomes are necessary to extrapolate laboratory-derived data to contaminant risk or injury of mussels in natural systems. We examined juvenile Fatmucket (Lampsilis siliquoidea) survival and growth for 56 d following exposure to Zn. We conducted a 28-d laboratory chronic Zn toxicity bioassay with juvenile mussels with endpoints of survival and length following standard methods (ASTM International 2019). At the end of the 28-d bioassay, we transferred surviving mussels into a grow-out pond and monitored their survival and growth for an additional 56 d. We chose Fatmucket because it is commonly used in aquatic toxicity testing, its growth rates in culture are well studied, and it is a useful surrogate species for inferring the sensitivity of other mussel species to a wide variety of toxicants with different modes of action (Raimondo et al. 2016; Wang et al. 2017). We used Zn as a model toxicant because mussels, including Fatmucket, are highly sensitive to Zn in acute and chronic exposures and exhibit significant reductions in growth during bioassays (Wang et al. 2020).

METHODS

Juvenile Culture

We collected female Fatmucket brooding mature larvae (glochidia) from the Bourbeuse River, Gasconade County, Missouri, USA, and held them at the U.S. Geological Survey (USGS) Columbia Environmental Research Center (CERC), Columbia, Missouri. We held adult female Fatmucket at 10–12°C (to prevent release of glochidia) in a 600-L recirculating tank with pond water (hardness 260 mg/L as CaCO₃, alkalinity 180 mg/L as CaCO₃, pH 7.8) at a flow rate of approximately 2 L/min. We fed mussels twice daily approximately 20 mL of a commercial nonviable microalgal concentrate (*Nannochloropsis*, Nanno 3600TM) and 20 mL of a mixture of six microalgae (Shellfish Diet 1800TM; both from Reed Mariculture, Campbell, CA, USA).

We removed approximately equal numbers of glochidia from each of three adult mussels by gently flushing the mussel marsupium with a syringe filled with culture water. We pooled the glochidia and inoculated them on laboratoryreared Largemouth Bass (*Micropterus salmoides*), which we maintained at 22° C in a recirculating, flow-through water system composed of Zebrafish tanks (Aquaneering Incorporated, San Diego, CA, USA) and designed to collect transformed juvenile mussels. We collected newly metamorphosed juveniles during the peak drop-off days (14-20 d after inoculation) and cultured them at 23°C in 300-mL lipless beakers with sand substrate and well water (hardness ca. 300 mg/L as CaCO₃) diluted with deionized water to a hardness of approximately 100 mg/L. Beakers had a 2.5-cm hole in the side covered with 50-mesh (279-µm-width opening) stainless steel screen to allow for water to overflow during renewal. We used an automated flow-through proportional diluter, typically used in toxicology studies, to deliver water and food every hour (Kunz et al. 2020). During culture, we replaced the sand and inspected the mussels weekly; we aimed for a relatively uniform juvenile size by discarding mussels that failed to grow and were noticeably smaller than other mussels (Barnhart 2006; Kunz et al. 2020).

Twenty-Eight-Day Chronic Toxicity Bioassay

Using our cultured juvenile Fatmuckets, we conducted a standard 28-d toxicity bioassay with three treatments (control water and 120 and 240 µg Zn/L) and eight replicates per treatment. We selected the Zn exposure concentrations based on a previous study with Fatmucket in which survival was high in all treatments, but growth was lower at the two high treatments and the 20% effect level was 66 µg Zn/L (Wang et al. 2020). For context, the 120-µg Zn/L level is approximate to the hardness adjusted chronic water quality criteria for Zn (122-127 µg Zn/L at 104-110 mg/L CaCO₃ hardness), which is intended to be protective of 95% of aquatic life (USEPA 1980). For each replicate exposure chamber, we placed 10 mussels (2.42 \pm 1.6 mm, mean \pm SD) and approximately 5 mL of silica sand into a 300-mL lipless glass culture beaker with 200 mL of water. We prepared silica sand ($<500 \mu m$; Granusil #5020, Unimin Corporation, New Canaan, CT, USA) by washing it in a container overnight with flow-through well water, rinsing it with deionized water for 5 min, and holding it in control water for 24 h before placing it in the beakers.

We used an intermittent proportional diluter to renew exposure water, maintain desired Zn concentrations, and deliver food throughout the bioassay. We prepared stock solutions of ZnCl (>98% purity; Sigma-Aldrich, St. Louis, MO, USA), and 125 mL of solution was delivered to each replicate beaker via a syringe pump (Hamilton, Reno, NV, USA), with each cycle of the diluter (once per hour, 15 times/d). Each day, we prepared a stock algal food mixture consisting of 1 mL of Nanno 3600 and 2 mL of Shellfish Diet 1800 (Reed Mariculture\) in 1.8 L of water (algal concentration ~510 nL cell volume/mL), maintained in aerated containers at <12°C in a cooler with ice packs (Wang et al. 2018). We provided 2 mL of the algal mixture per hour to each replicate beaker by using a peristaltic pump (Masterflex L/S model 07522-20 with 7535-08 multichannel head, Cole-Parmer Instrument Company, Vernon Hills, IL, USA) calibrated to automatically deliver the volume to each of six mixing cells in the diluter following each diluter



Figure 1. Floating upweller system (FLUPSY) used to hold juvenile mussels in the grow-out pond. (Left) Interior of FLUPSY with cover removed showing polyvinyl chloride holding chamber and water pump. (Right) FLUPSYs deployed in the grow-out pond. Inset shows anchor and line used to hold FLUPSYs in place. Photographs by J. Kunz, U.S. Geological Survey.

cycle (Kunz et al. 2020). We conducted the bioassay at 23° C in a temperature-controlled water bath and ambient laboratory light (~500 lux) with 16:8-h light:dark photoperiod.

We measured water quality variables (dissolved oxygen, pH, conductivity, hardness, alkalinity, and ammonia) in each treatment weekly. We measured dissolved oxygen with an HQ30d meter, pH with an HQ440d meter, and conductivity with an HQ40d meter with a CDC401 probe (Hach, Loveland, CO, USA). We measured hardness and alkalinity using the colorimetric burette method (ASTM International 2016, 2017). We measured total ammonia as nitrogen in water by using the titration method (ASTM International 2021). We collected water samples at the beginning and end of the bioassay to confirm Zn concentrations in all three treatments. Zn concentrations were measured by the USGS-CERC Environmental Chemistry Branch by using an inductively coupled plasma mass spectrometry (ICP-MS; NexION 2000 spectrometer, PerkinElmer, Waltham, MA, USA) following U.S. Environmental Protection Agency method 6020B (USEPA 2014). Before analysis, samples were filtered using a 0.45-µm polyethylsulfone membrane (Whatman Puradisc PES, GE Healthcare Bio-Sciences, Chicago, IL, USA) and preserved by adding house-distilled nitric acid to a final concentration of 2% (v/v). Chemical analysis followed established quality management system procedures including laboratory reference control samples, analysis duplicates, and analysis spikes. Percent recovery of spiked samples was 101.2%. Two National Institutes of Standards and Technology laboratory control samples (1640 and 1643) were used to confirm the accuracy of the ICP-MS calibration and were within 3% of the target values. The limit of detection and limit of quantitation was 0.1 and 1 µg/L, respectively.

We replaced bioassay beakers and sand at 14 d. We first rinsed mussels from each replicate beaker into a 200-mL glass dish with the exposure water for survival determination. We classified mussels with empty or gaping shells containing decomposed tissue as dead and removed them from the beakers. We transferred surviving mussels to a new beaker and sand with fresh solution. After 28 d, we removed and counted surviving mussels in each beaker. We measured shell length of each surviving mussel to the nearest 0.001 mm by using digital images captured with an SMZ 1270 stereo microscope and NIS Elements imaging software (Nikon Industries Inc., Melville, NY, USA).

Post-toxicity Bioassay Grow-Out in a Pond

We concluded the bioassay on August 23, 2019. We immediately transferred surviving mussels into a pond on August 23, 2019 (day 1), and monitored their survival and growth for 56-d grow-out until October 18, 2019. The pond was 290 m² and approximately 1 m in depth. It received well water at a rate of approximately 5 L/min via a 7.6-cm inlet pipe, and water exited the pond via an outlet weir at the end opposite the inlet. We transferred mussels from each beaker into separate holding chambers that were placed inside a six floating upweller systems (FLUPSY; Fig. 1); the FLUPSY is frequently used to rear bivalves from the juvenile-to-adult stage (Mair 2018). Our FLUPSY was 40×60 cm and 25 cm in depth and constructed of high-density polyethylene with foam on the upper edge for floatation. We drilled 11.4-cmdiameter holes in the bottom of each FLUPSY to accommodate four holding chambers. We fabricated the holding chambers from a 10-cm-diameter polyvinyl chloride pipe 16.5 cm in height with 1-mm mesh Nitex screen on top and bottom caps. We placed a model 7 magnetic drive utility pump (Danner Pondmaster, Islandia, NY, USA) in each FLUPSY to create an upward flow through the holding chambers. Before placing mussels in ponds, we acclimated them for approximately 1 h by gradually adding pond water to the holding chambers. We randomly assigned holding chambers across six FLUPSY systems.

We recorded water temperature in each FLUPSY every 30 min throughout the grow-out period by using three data

Table 1. Survival and length (N = 8/treatment) of juvenile Fatmucket (*Lampsilis siliquoidea*) in the 28-d Zn toxicity bioassay. Within a column, values (mean ± SD) with the same superscripted letter are not significantly different (Dunnett's test: P < 0.05).

Measured Concentration (µg/L)	Survival (%)	Length (mm)
1.9 ± 0.1	95.0 ± 10.7^{a}	4.4 ± 0.3^{a}
147.0 ± 1.4	81.3 ± 11.3^{b}	$3.6\pm0.3^{\mathrm{b}}$
248.0 ± 2.1	$81.3 \pm 12.5^{\mathrm{b}}$	$3.2\pm0.4^{\mathrm{b}}$
	Measured Concentration $(\mu g/L)$ 1.9 ± 0.1 147.0 ± 1.4 248.0 ± 2.1	Measured Concentration $(\mu g/L)$ Survival (%) 1.9 ± 0.1 95.0 ± 10.7^{a} 147.0 ± 1.4 81.3 ± 11.3^{b} 248.0 ± 2.1 81.3 ± 12.5^{b}

loggers. We obtained light condition (solar radiance) and daylight hours from the University of Missouri South Farm weather station (38.906992°, -92.269976°), located approximately 755 m from the pond at CERC (University of Missouri 2023). We measured dissolved oxygen, pH, conductivity, hardness, alkalinity, and ammonia in each FLUPSY weekly, as described previously. Every 14 d, we checked the mussels for survival and photographed them for length measurements. We collected water samples from the pond every 14 d for measurement of metals, total nitrogen, total phosphorus, total particle volume, and dissolved organic carbon (DOC) as general indicators of diet quality in the pond water. We measured metals by ICP-MS following method 6020B, described above. We froze samples for total nitrogen and phosphorus, stored them for 4 mo, and analyzed nitrogen by derivative spectroscopy (APHA 2017a) and phosphorus by using the ascorbic acid method (APHA 2017b). We held samples for particle count at 4°C and measured total particle volume (size fraction = $2-20 \mu m$) within 24 h by using a particle counter (Beckman Coulter, Indianapolis, IN, USA). We vacuum-filtered samples for DOC (0.45-µm PES), acidified them with 9 N highpurity sulfuric acid to pH 2 or lower, refrigerated them for <28 d, and measured DOC by high-temperature catalytic oxidationnondispersive infrared spectroscopy by using a TOC-L analyzer (Shimadzu Scientific, Kyoto, Japan). At the end of the 56-d grow-out period, we collected mussels from each holding chamber to determine survival, length, and dry mass (shell and tissue, 60°C for 48 h).

RESULTS

Twenty-Eight-Day Chronic Toxicity Bioassay

Mean Zn concentration in the control, 120- μ g/L Zn/L treatment, and 240- μ g Zn/L treatment was 1.9, 147.0, and 248.0 μ g/L, respectively (Table 1), representing 103–123% of nominal concentrations. Water quality conditions met performance criteria for standard toxicity bioassays (ASTM International 2019) and were as follows: pH, 8.0–8.4; alkalinity, 90–96 mg/L as CaCO₃; hardness, 104–110 mg/L as CaCO₃; conductivity, 263–269 μ S/cm at 25°C; Ca 25–26 mg/L; Mg, 8.4–9.1 mg/L; K, 0.9–1.0 mg/L; Na, 9.2–10.0

mg/L; Cl, 9.7 mg/L; and SO₄, 21 mg/L. Ammonia concentrations ranged from 0.05 to 0.08 mg N/L.

Mean survival in the control treatment after 28 d was 95.0% (Table 1) and met test acceptability criterion of >80% survival (ASTM International 2019). Mean survival differed among treatments (analysis of variance [ANOVA]: $F_{2,21} = 3.82$, P = 0.039). Survival in both Zn treatments was 81.3%, significantly lower than in the control (Table 1). Mean shell length differed among treatments (ANOVA: $F_{2,21} = 29.55$, P < 0.0001). Mean shell length in the control treatment was 4.4 mm; mean length was 3.6 and 3.2 mm in the 120- and 240- µg Zn/L treatments, respectively; and length in both Zn treatments was significantly lower than in the control (Table 1).

Post-toxicity Bioassay Grow-Out in a Pond

Water quality conditions in the pond throughout the grow-out period were maintained within the range typically considered adequate for mussel culture (Fig. 2; Kunz et al. 2020). Mean temperature in the pond was 23°C (range, 12–28°C). Total nitrogen concentration (mean \pm SD) was 470 µg/L, total phosphorus was 47.6 \pm 21.1 µg/L, and values for both were lowest on day 56. Total particle volume (2–10 µm) was 19.3 µm³/mL on day 1 and 6.1 µm³/mL on day 56. DOC (mean \pm SD) was 2.34 \pm 0.52 mg/L. The apparent decline in nutrients and particles likely was due to the seasonal decline of solar radiance and temperature later in the study.

After 56-d grow-out, survival of mussels from the control treatment was 91%, survival was 79% and 80% for mussels from the 120- and 240-µg Zn/L treatments, respectively, but survival did not differ among treatments (ANOVA: $F_{2,21} =$ 2.23, P = 0.133; Table 2). Mussels from all treatments grew $3.0-3.5 \times$ in length and $27-35 \times$ in mass during grow-out. Final mean shell length of mussels differed among treatments (ANOVA: $F_{2,21} = 7.13$, P = 0.004). Mean shell length in the 240-µg Zn/L treatment (10.9 mm) was significantly lower than in the control and 120-µg Zn/L treatments (13.7 and 12.7 mm, respectively), which did not differ from each other (Table 2). Final dry mass of mussels differed among treatments (ANOVA: $F_{2,21} = 16.58$, P < 0.0001). Mean mass in the 120- and 240-µg Zn/L treatments (0.78 and 0.63 g, respectively) did not differ from each other, but both values were significantly lower than in the control (1.41 g; Table 2).

Mussel length increased approximately linearly over time (analysis of covariance: time: $F_{1,114} = 1185.07$, P < 0.0001), and Zn concentration was a significant factor in predicting length (treatment: $F_{3,114} = 22.8$, P = 0.0001; Fig. 3). The time × treatment interaction was marginally significant ($F_{2,114}$, P = 0.054), and the estimated slope of the regression equation for the 240-µg Zn/L treatment was lower than for the other two treatments. However, 95% confidence intervals around the estimated slopes overlapped among all three treatments (slopes, 95% confidence intervals: control = 0.163, 0.093–0.234; 120 µg Zn/L = 0.167, 0.126–0.209; and



Figure 2. (A) Water temperature, (B) solar radiance, and (C) nutrients over 56 d in the grow-out pond.

240 µg Zn/L = 0.149, 0.0984–0.200). Consequently, we assumed homogeneity of slopes and omitted the interaction term to interpret main effects. Omitting the interaction term, time ($F_{1,116} = 1145.8$, P < 0.0001) and treatment ($F_{3,116} = 39.0$, P < 0.0001) remained significant factors in predicting mussel length. When time was accounted for, mean length differed among all three treatments (Tukey's post hoc test: P < 0.001 for all comparisons). Predicted lengths showed that, on any given day, mussels from the control treatment were 1.4 ± 0.24 mm (mean ± SE) longer than mussels from the 120-µg/L Zn treatment and 2.3 ± 0.24 mm longer than mussels from the 240-µg/L Zn treatment and mussels from the low Zn treatment were 0.9 ± 0.24 mm SE longer than those from the high Zn treatment.

DISCUSSION

Mussels exposed to even a low level of Zn (120 μ g Zn/L) in our study were smaller than control mussels after a 28-d toxicity bioassay. The 18–26% reduction in our Zn treatments compared with the control was similar to the 25–35% reduction observed at the same concentrations in a previous study of chronic Zn toxicity with juvenile mussels (Wang et al. 2020). In our study, this reduced size persisted even after 56 d in a pond uncontaminated by Zn. Persistent stunting after brief exposure to stressors could result in long-term effects on a mussel population. For example, reduced growth can delay sexual maturation and reproduction for ≥ 1 y (Haag and Rypel 2011; Haag 2012), and reduced size can increase vulnerability to predators (Brondel 2010).

Table 2. Survival, length, and dry mass (N = 8 replicates) of juvenile Fatmucket (*Lampsilis siliquoidea*) after 56 d in the grow-out pond. Treatment group refers to conditions to which juvenile mussels were exposed previously in the 28-d toxicity bioassay. Within a column, values (mean \pm SD) with the same superscripted letter are not significantly different (Dunnett's test: P < 0.05).

Treatment Group (µg Zn/L)	Survival (%)	Length (mm)	Dry Mass (g)
Control	91.3 ± 11.3	13.7 ± 1.8^{a}	1.41 ± 0.44^{a}
120	78.8 ± 15.5	12.4 ± 1.8^{a}	$0.78 \pm 0.19^{\rm b}$
240	80.0 ± 12.0	10.9 ± 0.57^{b}	$0.63\pm0.13^{\rm b}$

It is unclear whether exposure to Zn resulted in similar long-term effects among treatments. The slopes of the relationship between shell length and time were similar for the control and 120-µg Zn/L treatments, which suggests that mussels exposed to low levels of Zn grew at a similar rate as unexposed mussels after removal of the stressor. The slope of this relationship appeared to be slightly lower for the 240-µg Zn/L treatment, and the time \times treatment interaction was marginally significant; however, confidence intervals around the slopes overlapped widely for all three treatments, potentially due to low precision of the slope estimates. A lower slope for the 240-µg Zn/L treatment would indicate that exposure to a higher concentration of Zn can reduce growth rates even after removal of the stressor, causing mussels to fall further behind in size over time. Such an effect could compound population-level effects, but additional work is needed to clarify this issue.

In ecological risk assessment and resource injury assessment, the effects of a contaminant in controlled laboratory studies must be related to its effects in natural systems. Data collected in the laboratory are limited in duration and complexity, whereas contaminant effects in natural systems may occur over longer time scales, changing exposure concentrations, and in the presence of other stressors. The effects of contaminant and habitat stressors on freshwater mussel growth in natural systems have been measured directly using in situcaged mussels (Rogers et al. 2018; Haag et al. 2019; Pieri 2022). Relating laboratory bioassay data to such studies is challenging because of the difficulty of establishing relationships between survival and growth effects seen in the laboratory and factors that affect wild populations (Barnthouse and Stahl 2017). Studies that compare results of laboratory bioassays to results of invertebrate surveys, in situ exposures, or colonization of test sediments placed in natural systems can help translate responses seen in the laboratory to those seen in natural systems (Ingersoll et al. 2005; ASTM International 2018; Johnson 2018; Moore et al. 2019; Pieri 2022). Such studies found that endpoints derived from laboratory bioassays were protective of adverse biological effects observed in natural systems, but they did not always accurately predict biological effects observed in wild populations, particularly for sublethal endpoints such as reproduction (Crane et al. 2007). For example,



Figure 3. Mean length (mm) of juvenile Fatmucket (*Lampsilis siliquoidea*) over 56 d in the grow-out pond after previous exposure to three Zn treatments (control, 0 μ g/L; 120 and 240 μ g/L).

mussels exposed to environmental contaminants may experience sublethal effects including reduced energy stores, fecundity, and reproduction (Rajalekshmi and Mohandas 1993; Leonard et al. 2017).

The relationship between responses seen in the laboratory and in natural systems can be inferred by using data from laboratory studies to model longer term outcomes for a mussel population. Energetic and population models can be used to predict adverse biological effects on wild mussel populations based on effects seen in laboratory bioassays (Sherborne and Galic 2020; Accolla et al. 2021; Raimondo et al. 2021). For example, dynamic energy budget (DEB) models, which couple organism energy balance with toxicant exposure, have been used to predict the effect of toxicants on energy reserves and reproduction in aquatic organisms (Kooijman and Metz 1984). The DEB model has been used to interpret growth responses seen in laboratory toxicity bioassays, but it has not been applied to freshwater mussels. Bioenergetic models have been used to predict the energetic costs of environmental and toxicant stressors on marine mussels. For example, DEB modeling predicted reduced gamete production associated with reduced mussel biomass following exposure to oil production water and Zn particles (Muller et al. 2010, 2014). Similarly, environmental stressors such as variable salinity and hypoxia can incur significant energetic costs for marine mussels, a finding that can inform management decisions (Maar et al. 2015; Thomas et al. 2019; Lavaud et al. 2021). Sublethal growth endpoints, such as mass and length, from a toxicity bioassay can be coupled with energetic and population models to predict the consequences of contaminants on wild mussel populations (Widdows and Donkin 1991). In addition, individual-based energetic models can predict contaminant effects on threatened or endangered fishes, and such models may be useful for assessing contaminant effects on endangered freshwater mussels (Petersen et al. 2008; Bartell et al. 2019).

We showed that brief exposure to Zn can have lasting effects on mussel size and perhaps growth. Longer term studies are needed to better understand the persistent effects of brief contaminant exposure and resulting long-term population effects. For example, our study ended near the onset of winter; longer studies would be valuable to determine whether reduced growth due to Zn exposure reduces energy stores needed to overwinter. Furthermore, longer studies can clarify whether persistent effects differ according to initial contaminant concentration or whether exposed mussels eventually catch up to unexposed mussels via compensatory growth or other mechanisms.

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DATA AVAILABILITY STATEMENT

Full data sets for the present study are available through a data repository in ScienceBase at https://doi.org/10.5066/P96RI2Z4.

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REGULAR ARTICLE

APPLYING ENVIRONMENTAL DNA METHODS TO INFORM DETECTION OF *SIMPSONAIAS AMBIGUA* UNDER VARYING WATER VELOCITIES IN A RIVER

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ABSTRACT

Conventional survey methods to find rare and endangered aquatic species can be time consuming, expensive, destructive to habitat, and limited by the physical conditions of a site. Sampling for environmental DNA (eDNA) shed by organisms into their environments can overcome these limitations, maximizing conservation resources. However, the optimal spatial sampling interval for eDNA detection is poorly known. We developed and assessed eDNA methods for application to Simpsonaias ambigua (Salamander Mussel), a unionid mussel that is considered at risk throughout most of its range. We developed a quantitative PCR assay and optimized methods to detect S. ambigua eDNA in water samples, and we experimentally determined eDNA shedding and decay rates. We used these rates to populate a previously published eDNA transport model to estimate the maximum downstream distance from the source (i.e., the location of live mussels) at which eDNA could be detected as a function of environmentally relevant source eDNA concentrations and water velocities. The model predicted that maximum detection distance varied greatly depending on source eDNA concentration and water velocity. At low eDNA concentration and water velocity (1.0 copy/mL and <0.1 m/s, respectively), eDNA will be detected only at the source, requiring spatially intensive eDNA sampling. At higher eDNA concentration and water velocity (5.0 copies/mL and 0.8 m/s, respectively), eDNA can be detected at least 10 km downstream, requiring less intensive sampling. Based on our results, we provide recommendations for the development of optimal eDNA sampling design for detecting rare or endangered species.

KEY WORDS: environmental DNA, rare or endangered mussel species, survey techniques

INTRODUCTION

Conventional survey methods (e.g., hand sampling, sediment excavation, trawling, seining) to find elusive, rare, or threatened aquatic species are limited both by the difficulty in identifying species and by the physical conditions of a site; furthermore, they can be time consuming and can damage or destroy habitats (Jerde et al. 2011; Clark et al. 2015; Andruszkiewicz et al. 2017; Closek et al. 2019). Environmental DNA (eDNA) approaches recover DNA from an environmental sample without disturbing the species of interest or their habitats. Despite the limitations of eDNA sampling (e.g., filter clogging, PCR inhibitors, transportation and preservation of water samples), eDNA methods can be more cost effective and can overcome the limitations of conventional survey methods (Rees et al. 2014; Thomsen and Willerslev 2015; Ruppert et al. 2019).

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eDNA methods have been used to detect and develop multiscale occupancy models for rare and endangered aquatic species (Dorazio and Erickson 2018; Strickland and Roberts 2019; Coghlan et al. 2021). Results from eDNA surveys support those of conventional surveys (Wilson et al. 2014; Hinlo et al. 2017; Cilleros et al. 2019), and in some cases, eDNA methods are more sensitive and effective, especially for rare species (Jerde et al. 2011; McKelvey et al. 2016; Currier et al. 2017). Although eDNA methods provide many advantages, the effective management of rare and threatened species still requires biological data (e.g., population health, sex ratios, size frequency estimates) that can be obtained only through conventional sampling approaches. Thus, a strategy that involves a combination of conventional and eDNA approaches will best achieve most conservation objectives.

Environmental DNA originates from waste products, gametes, shed body parts, or other sources, and its persistence in the environment is controlled by factors such as the rate of shedding from the organism, resuspension, decay, advection, and transport (Barnes et al. 2014; Strickler et al. 2015; Barnes and Turner 2016). Quantification of eDNA shedding and decay rates has proven to be informative when modeling eDNA presence and transport in the environment, and understanding these processes is critical for developing optimal sampling designs (Sassoubre et al. 2016; Sansom and Sassoubre 2017; Andruszkiewicz et al. 2020).

We developed and assessed eDNA methods for detecting Simpsonaias ambigua, the Salamander Mussel (family Unionidae). Sampling for freshwater mussels is time consuming and expensive because their benthic occurrence and burrowing habits make their detection difficult. Simpsonaias ambigua is small (maximum 50 mm shell length), and it occurs almost exclusively beneath large, flat stones or rock ledges, often in deep water or in turbid conditions (Howard 1915), characteristics that make detecting S. ambigua particularly difficult. Simpsonaias ambigua is listed as globally vulnerable by the International Union for Conservation of Nature Red List (Bogan et al. 2017) and endangered under Canada's Species at Risk Act (Morris and Burridge 2006), and it is a candidate for listing under the U.S. Endangered Species Act (USFWS 2011). The imperiled status of this species, along with the difficulty of its detection, provides impetus for development of sensitive, cost-effective survey methods.

Our study goals were to (1) develop a quantitative (q)PCR assay and optimize methods for detection of *S. ambigua* eDNA, (2) experimentally determine eDNA shedding and decay rates, and (3) use these rates to populate a previously published eDNA transport model to estimate the maximum downstream distance from the source (i.e., the location of live mussels) at which eDNA could be detected as a function of environmentally relevant source eDNA concentrations and water velocities in a third-order stream. Based on our results, we provide recommendations for the development of optimal eDNA sampling designs for detecting rare or endangered species.

METHODS

Simpsonaias ambigua Primer and Probe Development and Optimization in the Laboratory

We developed a qPCR assay for S. ambigua following guidelines in Bustin et al. (2009) and Wilcox et al. (2013), with modifications outlined below. Because there were limited sequences available in public databases, we developed primer probes by amplifying and sequencing two mitochondrial genes, cytochrome oxidase subunit I (COI, 622 bp) and NADH dehydrogenase (ND1, 599 bp), from mantle swabs of five S. ambigua collected from the Sydenham River (known as Jongquakamik in Nishnaabemwin [Ojibwe], Lake St. Clair drainage, Ontario, Canada). We extracted genomic DNA from mantle swabs by using the DNeasy Blood and Tissue extraction kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. We amplified COI by using Folmer et al. (1994) primers and ND1 by using Buhay et al. (2002) primers. We amplified each mitochondrial gene via PCR in a 25-µL reaction, with the following concentrations: 2.0 ng/µL of extracted genomic DNA, 0.3 mM dNTPs, 10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.2 µM of each primer, and 1 U of Tag polymerase. We carried out a touchdown PCR for both genes, with the following amplification conditions: initial heating to 94°C for 2 min; 5 cycles of 94°C for 40 s, annealing at 50°C for 40 s, and a 90-s extension time at 72°C; 25 cycles of 94°C for 40 s; annealing at 40°C for 40 s and a 90-s extension time at 72°C; and a final extension of 10 min at 72°C. We screened all PCR products on 2% agarose gel to confirm amplification and targeted sequence size. We sent successfully amplified samples to the Aquatic Research and Monitoring Section, Ontario Ministry of Natural Resources and Forestry, Trent University, for Sanger sequencing. We edited and aligned chromatograph files of COI and ND1 sequences by using Geneious 10 (Kearse et al. 2012). Sequences were translated using the mitochondrial invertebrate genetic code to ensure the absence of stop codons. Although available S. ambigua sequences were limited, we designed primers by using sequences and specimens from different watersheds to ensure that this assay could be used to detect S. ambigua across its distributional range. We used COI sequences from the Monongahela River, Ohio River basin (voucher NCSM30607, GenBank accession number KX822666), and from five individuals from the Sydenham River (GenBank accession number MN920704). ND1 sequences originated from five individuals from the Sydenham River (GenBank accession number MN920703). All five sequenced individuals from the Sydenham River shared the same COI and ND1 haplotypes.

We designed all primers and probes by using Primer3 v.0.4.0 (Koressaar and Remm 2007; Untergasser et al. 2012). We carried out in silico testing of all primer–probe sets for specificity against 35 mussel species present in Ontario (Table 1). Table A1 provides a list and the properties of two

56

PORTO-HANNES ET AL.

Table 2. Mussel species tested for cross-amplification of the cytochrome oxidase subunit I gene (COI) (SamCOI_1) and the NADH dehydrogenase gene (ND1) (SamND_1) by using primers developed for *Simpsonaias ambigua*. Cycle quantification value (C_q) is presented for each species that yielded amplification after 40 cycles; a dash (—) indicates no amplification. See Table A1 for additional information about the primers. All tissue samples were collected from Ontario by the Great Lakes Laboratory for Fisheries and Aquatic Sciences, Fisheries and Oceans Canada.

		C	9	
Species	Common name	SamCOI_1	SamND1	
Alasmidonta marginata	Elktoe	_	_	
Alasmidonta undulata	Triangle Floater	37.35	_	
Alasmidonta viridis	Slippershell Mussel	_	_	
Amblema plicata	Threeridge	39.15	_	
Anodontoides ferussacianus	Cylindrical Papershell	_	_	
Cambarunio iris	Rainbow	_	_	
Cyclonaias pustulosa	Pimpleback	_	_	
Cyclonaias tuberculata	Purple Wartyback	—		
Elliptio complanata	Eastern Elliptio	_	_	
Epioblasma rangiana	Northern Riffleshell	_	_	
Epioblasma triquetra	Snuffbox	—		
Eurynia dilatata	Spike	_	_	
Fusconaia flava	Wabash Pigtoe	_	_	
Lampsilis cardium	Plain Pocketbook	38.34		
Lampsilis fasciola	Wavyrayed Lampmussel	_	_	
Lasmigona complanata	White Heelsplitter	—		
Lasmigona compressa	Creek Heelsplitter	—	—	
Lasmigona costata	Fluttedshell	—		
Ligumia recta	Black Sandshell	—		
Obliquaria reflexa	Threehorn Wartyback	38.24	39.79	
Obovaria subrotunda	Round Hickorynut	—	—	
Actinonaias ligamentina	Mucket	—		
Pleurobema sintoxia	Round Pigtoe	38.37		
Potamilus alatus	Pink Heelsplitter	—	—	
Potamilus fragilis	Fragile Papershell	38.81		
Ptychobranchus fasciolaris	Kidneshell	—		
Pyganodon grandis	Giant Floater	—	_	
Quadrula quadrula	Mapleleaf	—	_	
Sagittunio nasutus	Eastern Pondmussel	37.00	39.66	
Simpsonaias ambigua	Salamander Mussel	20.10	25.42	
Strophitus undulatus	Creeper	—		
Toxolasma parvus	Liliput	—	_	
Truncilla donaciformis	Fawnsfoot	—		
Truncilla truncata	Deertoe	38.48	_	
Utterbackia imbecillis	Paper Pondshell	—		
Paetulunio fabalis	Rayed Bean	—		

COI and two ND1 primer-probe sets that we designed and tested.

To determine the most sensitive primer–probe combination, we optimized the assays by testing final primer concentrations of 0.3, 0.6, and 0.9 μ M per reaction and final probe concentrations of 0.15 and 0.25 μ M. Throughout this study, we set up all qPCRs in an isolated UV workstation with a set of dedicated pipettes. Before setting up reactions, we decontaminated the workstation with hydrogen peroxide and 15 min of ultraviolet (UV) light exposure. The qPCRs for

both genes were carried out using 2 μ L of extracted genomic DNA in 20- μ L reactions containing the following final concentrations: 1× TaqManTM Environmental Master Mix 2.0 (Applied BiosystemsTM, Waltham, MA, USA), 0.3–0.9 μ M of each primer, and 0.15–0.25 μ M of probe with a ZEN/Iowa Black FQ quencher (IDT, Coralville, IA, USA). Two no template controls (NTCs) were run for each qPCR plate by using 2 μ L of molecular grade water (Sigma-Aldrich, St. Louis, MO, USA) instead of genomic DNA. The amplification conditions were as follows: 95°C for 10 min followed by 40 cycles of 95°C for 10 s and annealing at 60°C for 1 min.

Subsequently, we performed in vitro testing of the COI and ND1 primer–probe sets that had the greatest DNA sequence mismatches with nontarget species (SamND_1 and SamCOI_1, see Table A1) against the same 35 mussel species found in Ontario (Table 1). We used approximately 2 ng/ μ L of genomic DNA of each species to carry out the qPCR reactions.

We determined the limit of detection (LOD, the minimum number of copies in a sample that can be detected accurately) following Hunter et al. (2017) to provide a conservative estimation of LOD. The limit of quantification (LOQ) determines the ability of an assay to precisely quantify the number of DNA copies. In this study, the LOQ was defined as the lowest standard concentration with a coefficient of variation below 35% (Klymus et al. 2020). To calculate LOD and LOQ, and to determine eDNA concentrations from environmental samples, we prepared standard curves consisting of 1:10 serial dilutions of the gBlock oligo from 1 to 1×10^7 copies per reaction. The gBlock Gene Fragments (IDT) consisted of a 471-bp sequence that started with a 40-bp sequence of randomly chosen nucleotides, followed by a 150-bp COI sequence, a 20-bp sequence of randomly chosen nucleotides, and 261-bp ND1 sequence; therefore, the same gBlock was used with all COI and ND1 primers. For LOD and LOO calculation, we ran each standard 12 times in the same plate.

The primer-probes SamND_FWD1: 5'-ACTAGGGCTT-AGTGGCATTCC, SamND_RVS1: 5'-AGGGCGAGTATA-GTTATTGGGG, and SamND Probe1: 5'-AACCCGCAGC-AGACGCCTTG showed the highest specificity of all tested primer-probe sets (Table 1), with S. ambigua DNA being detected at quantification cycle (Cq) = 25.42. Cross-amplification was observed for nontarget species Obliquaria reflexa (Cq = 39.79) and Sagittunio nasutus (Cq = 39.66); however, this was above the Cq threshold (Cq = 38; see below) despite 2 ng/µL of template DNA, which is a high concentration of nontarget DNA to test for cross-reactivity. This ND1 assay also showed good efficiency across six standard curves, with an average efficiency of 94% and $R^2 > 0.99$. Therefore, we used this primer-probe set in all subsequent eDNA qPCR assays. We tested a temperature gradient between 55 and 62°C for annealing temperature, and the optimal temperature was 60°C. The optimized primer and probe concentrations for SamND1 were 0.9 and 0.25 µM, respectively.

Optimization and Testing of eDNA Detection in the Field

We optimized filter pore size and the volume of water filtered in the field by collecting water samples from a site on the Sydenham River that supports a population of S. ambigua (site LSC-SRY-05 in Fig. 1). We collected and filtered water samples with an OSMOS eDNA backpack sampler (Halltech, Guelph, ON, Canada) during two consecutive days in October 2019 (mean water depth, 3.4 m; mean discharge, 5.97 m³/s; real-time hydrometric data for Florence Station; wateroffice. ec.gc.ca). Filtering in the field instead of in the laboratory allowed us to filter larger volumes (1-10 L in the field; <500 m)mL in the laboratory) and to store, refrigerate, and transport filters instead of large volumes of water. We tested three different cellulose nitrate filter pore sizes (0.45, 0.80, and 1.00 µm) and two water volumes (1 and 10 L) to determine which pore size-volume combination was optimal for eDNA capture in the field. We collected water samples at the river surface (Currier et al. 2017) from the bank or by wading in the midchannel, depending on the width and depth of the river. When sampling by wading, we placed the filter housing upstream from the surveyors to avoid contamination. We decontaminated reusable filter housings by soaking them for 10 min in a 10% bleach solution and thoroughly rinsing them with water between samples. We discarded nitrile gloves and decontaminated the forceps after collecting each sample. We collected two field replicate samples for each pore size-volume combination. We did not take field blanks because all samples were taken at the same location and the main goal was to test the volume of water that we were able to filter by using different pore sizes before the filters clogged. After filtration, we placed all filters in 5-mL transport polypropylene tubes (Thermo Fisher Scientific, Waltham, MA, USA), stored them in a cooler with ice, and froze them at -20° C within 12 h. We stored filters at -80° C and conducted DNA extraction within a week of collection.

We extracted DNA from filters by using the DNeasy Blood and Tissue extraction kit (Qiagen) following the manufacturer's protocol, with the following modifications. We completed DNA extractions in a separate room from the qPCR instrument and cleaned bench surfaces with hydrogen peroxide. We placed all pipettes under UV light for 3 min before extractions. We doubled the volume of buffer ATL and proteinase K, and we extended the incubation in buffer ATL and proteinase K to 16-24 h at 56°C. After incubation, we added 400 µL of buffer AL and 400 µL of 100% molecular grade ethanol to obtain a 1:1:1 volume ratio (buffer ATL plus proteinase K:buffer AL:ethanol). The final elution volume with buffer AE was 100 µL. We extracted a DNA extraction blank with each set of samples to check for contamination during the extraction process. Inhibition of qPCR is common in eDNA detection from environmental samples (for review, see Goldberg et al. 2016); therefore, we tested for inhibition by diluting samples 1:10 and 1:100. An increase in eDNA concentration with an increase in the



Figure 1. Map of sites sampled for *Simpsonaias ambigua* environmental DNA (eDNA) in the Sydenham River. Sample site numbers increase in an upstream direction. Inset map shows the location of the Sydenham River in Ontario, Canada.

dilution factor would indicate inhibition affected eDNA detection and quantification. We carried out all qPCR reactions as described previously.

We field tested the primer–probe sets by collecting three field replicate 2-L water samples with an OSMOS eDNA sampler (Halltech) as described previously at four sites along a 45-river kilometer (rkm) reach of the Sydenham River (Fig. 1) during two consecutive days in March 2020 (mean water depth, 3.95 m; mean discharge, 14.93 m³/s; real-time hydrometric data for Florence Station; wateroffice.ec.gc.ca). Although 1 L was the optimal volume (see previous text and Results), we collected 2-L samples to maximize detection probabilities. The distance between adjacent sites ranged from 7 to 25 rkm. A qualitative survey conducted in 2018 and 2019 in this reach detected 43 live S. ambigua within a 12-rkm reach between sites LSC-SYR-29 and LSC-SYR-05 (I. Porto-Hannes, unpublished data). No live S. ambigua were found at LSC-SYR-44, one live individual was found at LSC-SYR-33, and no live individuals were reported upstream of LSC-SYR-33 (LGLUD 2020). We filtered water samples through a 0.8µm cellulose nitrate filter (see previous text and Results), and we stored and extracted all filters and subjected DNA to qPCR as described previously.

We tested for PCR inhibition in field samples in two ways. First, we diluted extracted DNA 1:1, 1:2, and 1:10 and quantified DNA concentration by qPCR with and without the addition of 0.4 mg/mL (final concentration) bovine serum albumin (BSA), which can overcome inhibition in environmental samples (Kreader 1996). Second, we spiked extractions with a known concentration of DNA. We prepared spiked replicates of six samples by adding to each sample 2.0 µL of a 10,000 copies/ml DNA standard to 2.0 µl of each sample's eluate. We then compared DNA concentrations from qPCR reactions against expected DNA concentrations based on spiking. A decrease in DNA detection was observed in only one of the spiked samples; therefore, we ran each environmental sample six times using 5 µl of 1:1 extracted DNA and adding 0.4 µl of BSA per reaction (final concentration, 0.4 mg/mL) to increase the probability of S. ambigua eDNA detection. We ran a standard curve and NTC as described previously for each plate of samples. We pooled standard curves with efficiency >90% across plates to calculate DNA concentrations in unknown samples. We considered a sample quantifiable if at least three of six qPCR replicates amplified at a $Cq \leq 35$ cycles (LOQ).

Estimation of eDNA Shedding and Decay Rates

We performed an experiment to estimate eDNA shedding and decay rates for *S. ambigua* in tap water. Because eDNA decay is influenced by many environmental variables and differs between environmental water and tap water (Sassoubre et al. 2016; Sansom and Sassoubre 2017), we also estimated eDNA decay in environmental water from the Sydenham River. We used eDNA decay rates determined from environmental water in the model for eDNA downstream transport (see subsequent text).

We acquired 60 juvenile S. ambigua (mean shell length, 12.56 ± 3.00 mm; mean wet mass, 0.19 ± 0.10 g) from the Genoa National Fish Hatchery, U.S. Fish and Wildlife Service, Genoa, Wisconsin, USA; juveniles were raised from brood stock from the Chippewa River, Wisconsin. Mussels were shipped to our laboratory, and upon arrival, we placed them in a continuously aerated 40-L tank with gravel substrate (median diameter D50 of 0.01 m) and filled with tap water treated with AmQuel (number-31261, Kordon, Hayward, CA, USA) to neutralize chlorine, chloramine, and ammonia. The tank was continuously aerated with air stones $(5 \text{ cm} \times 10 \text{ cm})$ connected to an air pump (model AAPA15L, ActiveAQUA, Petaluma, CA, USA). We maintained the tank at room temperature ($22 \pm 1^{\circ}$ C) for the duration of the acclimation and experimental periods. We exposed tanks to indirect sunlight through a window and artificial lights in the laboratory. We fed mussels by adding 2.0 mL of algae to the tank (Shellfish Diet 1800, Reed Mariculture, Campbell, CA, USA) every 2 d. We allowed mussels to acclimate for 4 wk before the experiments.

Determination of experimental mussel density and sample volume.- We conducted a pilot study to determine the optimal number of mussels and sample volume needed to detect eDNA with our SamND1 assay in the experiments. We established six 20-L tanks, three containing 15 L of environmental water (Sydenham River) and three containing 15 L of tap water treated as described previously. We collected environmental water from the Sydenham River in 3.78-L acidwashed plastic containers and stored them on ice in coolers during transportation to the laboratory. Each set of three tanks included one tank with two S. ambigua, one tank with 18 S. ambigua, and one control tank with no mussels. From each tank containing mussels, we collected water samples of 100, 500, 1,000, and 3,000 mL 48 h after the initiation of the experiment. We collected replicate samples of each volume in 1-L polycarbonate bottles that previously were acid washed (10% HCl), neutralized in NaHCO₃, and rinsed with deionized water. We filtered samples in the laboratory over 47-mm-diameter polycarbonate filters (EMD, Millipore, Germany) with a pore size of 0.40 μ m for 100-, 500-, and 1,000mL samples and a pore size of 1.2 µm for the 3,000-mL samples. We also collected and filtered 500 mL of water from the control tanks and a filtration control consisting of 200 mL of molecular grade water (Sigma-Aldrich). We placed all filters in 5-mL transport polypropylene tubes (Thermo Fisher



Figure 2. *Simpsonaias ambigua* environmental DNA (eDNA) concentration over time in tap water at (A) low mussel density (4 mussels [0.76 g wet mass]/15 L) and (B) high mussel density (13 mussels [2.47 g wet mass]/15 L). Solid and filled symbols represent two replicate tanks within each density treatment. The vertical dashed line indicates the time at which mussels were removed from the tanks. The horizontal dot-dashed line represents DNA limit of detection (LOD; 2.15 copies/mL in 500-mL sample or 10.76 copies/ μ L).

Scientific) and stored them at -80° C until DNA extraction. We extracted DNA from the filters and subjected DNA to PCR as described previously.

Shedding and decay rates in treated tap water.- We established five 20-L tanks, each containing 15 L of tap water treated as described previously. We established two high-density tanks, each containing 13 mussels (approximate total wet mass, 2.47 g); two low-density tanks, each containing four mussels (approximate total wet mass, 0.76 g); and one control tank containing no mussels. We placed an air stone in each tank to provide mixing. Mussels were not fed for 6 h before, and for the duration of, the experiment, and they were allowed to acclimate for 18 h before beginning the experiment. We collected duplicate 500-mL water samples from each tank at the beginning of the experiment (T_0) and every 6–7 h for the next 26 h (N = 4 after T_0 ; Fig. 2). We collected samples in 1-L polycarbonate bottles cleaned as described previously. From these samples, we determined whether eDNA concentration reached a steady state where eDNA concentration did not change over two consecutive time periods. To estimate eDNA decay rate, we removed mussels from the tanks after 26 h and collected duplicate water samples every 3–4 h within two 12-h periods over the next 2 d (i.e., 30–60 h after T_0 , N = 7; Figs. 2, 3), and at three time points over the following 2 d (i.e., 73–95 h after T_0 ; Fig. 2).

Immediately after collection, we filtered water samples through a 0.45- μ m cellulose nitrate filter (WhatmanTM type WCN cellulose nitrate membranes, GE Healthcare, Chicago, IL, USA) by using 47-mm magnetic funnels (magnetic filter funnels, Pall Corporation, Port Washington, NY, USA). We also filtered a control consisting of 200 mL of molecular grade water coincident with filtering of samples at each time point. We placed filters in 5-mL transport polypropylene tubes and stored them immediately at -80° C until DNA extraction. We extracted DNA from the filters and subjected DNA to PCR as described previously.

Decay rates in environmental water.— We established a 37-L tank containing 19 L of environmental water from the Sydenham River. We collected environmental water using 3.78-L plastic containers that were previously acid washed. All water samples were kept in coolers with ice until arrival at the laboratory. We placed eight S. ambigua (mean individual wet mass, 0.19 ± 0.10 g) in the tank when water reached room temperature (22°C). We used air stones to completely mix the water; we did not add substrate to this tank to avoid potential eDNA capture by sediments. We left mussels in the tank for 24 h to allow the eDNA concentration to reach a steady state then removed all mussels from the tank. Three of the eight mussels died during the first 24 h; however, because this experiment was designed to estimate eDNA decay rates only, death of the mussels is not expected to influence our estimates. We collected duplicate water samples from the tank immediately after the mussels were removed from the tank (T_0) , every 3–4 h within two 12-h periods over the next 2 d (1.5–34.5 h after T_0 ; N = 8), two times per day for 1 d (47.0–52.5 h after T_0 ; N = 2), one sample every 24 h for 2 d $(71.5-95.5 \text{ h after } T_0)$, and once 11 d after T_0 (263.5 h; Fig. 4a,b).

Immediately after collection, we filtered water samples, including filtration controls, over a 47-mm-diameter 0.45-µm cellulose nitrate filter as described previously. We stored and extracted all filters and subjected DNA to PCR as described previously.

Data analysis.— We calculated eDNA shedding and decay rates based on a completely mixed batch reactor model:

$$V\frac{dC}{dt} = S - kCV$$

where V is the volume of the tank (mL), C is the eDNA concentration (copies/mL), t is the time since the start of the experiment (h), S is the eDNA shedding rate (copies/h), and k is the first-order decay-rate constant (/h) (Sassoubre et al. 2016; see subsequent for k calculation). This model assumes that the tank is well mixed and that the decay is first order (linear decay over time). At steady state, dC/dt = 0, therefore



Figure 3. Linear decay of DNA concentration (ln (C/C_0)) over time in tap water at (A) low mussel density (4 mussels [0.76 g wet mass]/15 L) and (B) high mussel density (13 mussels [2.47 g wet mass]/15 L). Solid and filled symbols represent two replicate tanks within each density treatment.

S = kCV. We used a *t*-test to determine whether there was a difference in shedding rates between replicates and experimental tanks with tap water.

We calculated the k value after removal of the mussels, when S = 0 and therefore dC/dt = -kC. We determined k by fitting the data to a linear decay on a plot of $\ln(C/C_0)$ versus time (t) (Fig. 4c). In tap water, C_0 was the mean eDNA concentration until reaching steady state $(T_0 - T_{26})$. In environmental water, C_0 was the eDNA concentration at the time mussels were removed from the tank, because the aim was to calculate only the decay rate. We modeled eDNA decay in environmental water with nine regression models (Table 2) by using GInaFiT (Geeraerd et al. 2005), a software package designed to model the decay of bacteria over time and has also been used to model eDNA decay (Andruszkiewicz et al. 2020). We tested all models from T_0 until the end of the experiment (263.5 h). We chose the best-fit model based on the greatest R^2 and adjusted R^2 .

We compared k values (i.e., the slope representing eDNA decay over time) among different experimental treatments in



Figure 4. (A) *Simpsonaias ambigua* environmental DNA (eDNA) concentration over time in environmental water. The horizontal dot-dashed line represents the DNA limit of detection (LOD; 2.15 copies/mL in 500-mL sample or 10.76 copies/ μ L). (B) Linear decay of eDNA concentration (ln (*C*/*C*₀)) for the duration of the experiment and (C) during the first 28.5 h, which were used to calculate the decay-rate constant (*k*).

tap water (low density vs. high density) with analysis of covariance (ANCOVA) by using R v. 3.6.2 (R Core Team 2011). Before conducting the ANCOVA, we confirmed that the data met the assumptions of linearity, homogeneity of regression slopes, normality of residuals, and homogeneity of variance.

eDNA Transport Model

To evaluate eDNA sampling intervals, we modeled downstream transport of eDNA for a range of realistic source eDNA concentrations and water velocities by using the onedimensional plug-flow reactor model of Sansom and Sassoubre (2017):

$$C = C_{bed} e^{\frac{-kx}{u}}$$

where *C* is eDNA concentration (copies/mL) at a given distance downstream from the source, C_{bed} is a hypothetical value based on lab and field observations and represents the expected eDNA concentration originating from the source, *k* is the first-order decay-rate constant (/h), *x* is the downstream distance (km) from the source, and *u* is the water velocity (km/h). We populated the model as follows. For C_{bed} , we modeled two hypothetical eDNA concentrations: 1.0 and 5.0 copies/mL. These values are based on reported eDNA concentrations for other unionid species in Ontario (<0.5–10 copies/mL; *Quadrula quadrula*,

Table 2. Regression models evaluated to describe environmental DNA decay in environmental water.

Model	R^2	R^2 adjusted
Double Weibull (Coroller et al. 2006)	0.967	0.957
Biphasic + Shoulder (Geeraerd et al. 2005)	0.966	0.951
Biphasic (Cerf 1977)	0.964	0.953
Weibul + Tail (Albert and Mafart 2005)	0.901	0.871
Log-linear + Shoulder + Tail (Geeraerd et al. 2005)	0.901	0.871
Log-linear + Tail (Geeraerd et al. 2005)	0.899	0.881
Weibul (Mafart et al. 2002)	0.839	0.81
Weibul Fixed parameter (0.5) (Mafart et al. 2002)	0.768	0.726
Log-linear + Shoulder (Geeraerd et al. 2005)	0.723	0.672
Log-linear Regression (Bigelow and Esty 1920)	0.487	0.444

Ptychobranchus fasciolaris, and Lampsilis fasciola; Currier et al. 2017); however, given S. ambigua's LOD, shedding rates (see Results), and low population densities in the Sydenham River, we used lower values of eDNA concentration. For k, we used the value 0.164/h, as estimated in environmental water (see Results). For x, we used values from 0 to 10 km, and for u we used values from 0 to 3 km/h (0.00–0.83 m/s), which are within the range of observed water velocity in the Sydenham River (I. Porto-Hannes, unpublished data). The model of Sansom and Sassoubre (2017) assumes no additional eDNA inputs downstream of the hypothetical initial source.

RESULTS

Simpsonaias ambigua Primer and Probe Development and Optimization in the Laboratory

Amplification efficiency was >90% for all the COI and ND1 primers developed for *S. ambigua*, but the specificity of primer pair SamND_1 was highest (Table 1). Cross-amplification was observed for eight nontarget species for SamCOI_I and two nontarget species for SamND1, but all values for nontarget species were above the *Cq* threshold (*Cq* = 38), indicating no significant cross-reactivity with other mussel species (Table 1). All primer pairs amplified *S. ambigua* DNA from individuals from Wisconsin (juveniles used in experiments) and Ontario, suggesting that these assays can be used to detect *S. ambigua* across its distributional range. The LOD and LOQ of primer pair SamND1 was 10.76 copies/µL (95% confidence interval: 7.47–15.51 copies/µL; *Cq* ≤ 38) and 50 copies/µL (*Cq* ≤ 35), respectively.

Optimization and Testing of eDNA Detection in the Field

We determined the optimal filter size was $0.80 \ \mu m$, based on detection of *S. ambigua* eDNA in one of two field replicates and three of four qPCR replicates for each sample volume (Table 3). One field replicate of a 0.45-µm filter and sample volume of 10 L resulted in eDNA detection in two of four qPCR replicates, but no DNA was detected when the sample volume was 1 L. There was no detection of eDNA with a 1.0-µm filter for either sample volume. There was no evidence of contamination in any field, filtration, or extraction blanks, and all qPCR NTCs showed no amplification.

Most detections of *S. ambigua* eDNA were observed at site LSC-SYR-05, which is downstream of the reach of the Sydenham River that appears to support the largest populations of the species. However, we detected eDNA in only two of three field replicates and two to three qPCR replicates at this site. At sites LSC-SYR-29 and LSC-SYR-44, we detected eDNA in only one of three field replicates and one qPCR replicate. We did not detect eDNA at site LSC-SYR-33, which appears to support only small populations of *S. ambigua* and may be near the upstream limit of the species in the river (see previous text).

Estimation of eDNA Shedding and Decay Rates

Determination of experimental mussel density and sample volume.— We detected eDNA in all tanks with mussels (2 and 18 *S. ambigua*) and all sample volumes (100–3,000 mL). No DNA was detected in control tanks with no mussels.

Shedding and decay rates in treated tap water.— Shedding rate was significantly higher in the high-density tanks than in the low-density tanks ($t_{7.74} = -2.59$, P = 0.033; Fig. 2 and Table 4). However, there was considerable variation among replicates, particularly in the low-density tanks, where shedding rate differed significantly between tanks ($t_3 = -5.90$,

Table 2. Results from the October 2019 field experiment to test the effects of filter pore size and water volume on detection of *Simpsonaias ambigua* eDNA. Amplification is the number of qPCR replicates within each field replicate in which *S. ambigua* environmental DNA was detected. *Cq* is the quantification cycle. NA = not applicable.

Filter size (µm)	Volume (L)	Field replicate	Amplification	Mean (range) Cq
0.45	1	1	0/4	NA
		2	0/4	NA
	10	1	2/4	38.10 (36.47–39.72)
		2	0/4	NA
0.80	1	1	0/4	NA
		2	3/4	37.87 (36.23–39.21)
	10	1	3/4	38.12 (37.13-38.75)
		2	0/4	NA
1.00	1	1	0/4	NA
		2	0/4	NA
	10	1	0/4	NA
		2	0/4	NA

Table 2. Environmental DNA shedding and decay-rate constants (*k*) for *Simpsonaias ambigua* over time in tap water at low mussel density [LD: 4 mussels [0.76 g wet mass]/15 L) and high mussel density [HD: 13 mussels [2.47 g wet mass]/15 L). Propagated error for shedding rate was calculated for each tank over four time points from T_0 to T = 26 h. Standard error for *k* was calculated over five time points from T = 30 h to T = 52 h. Within a column, values with different lowercase letters are significantly different (shedding rate, copies/h/mussel: *t*-test; *k*: analysis of covariance). Asterisk (*) indicates LD tank 1 did not follow first-order kinetics

	DNA			
Treatment	Copies/h \pm propagated error	Copies/h/mussel	Copies/h/g	$k \pm SE$
LD (tank 1)	$3.73 \times 10^4 \pm 7.36 \times 10^4$	9.33×10^3 a	4.91×10^{4}	$8.36 \times 10^{-3} \pm 1.64 \times 10^{2*}$
LD (tank 2)	$5.49 \times 10^5 \pm 2.38 \times 10^5$	1.37×10^{5} b	7.22×10^{5}	$7.68 \times 10^{-2} \pm 2.37 \times 10^{2}$ a
HD (tank 1)	$7.49 \times 10^6 \pm 5.42 \times 10^6$	5.76×10^{5} c	3.03×10^{6}	$1.34 \times 10^{-1} \pm 2.53 \times 10^{2}$ a
HD (tank 2)	$2.25 \times 10^6 \pm 3.89 \times 10^5$	$1.73 \times 10^{5} c$	9.11×10^{5}	$1.44 \times 10^{-1} \pm 1.47 \times 10^{2} \mathrm{a}$

P = 0.009); shedding rate did not differ significantly between tanks in the high-density treatment ($t_3 = 2.21$, P = 0.113). eDNA concentration increased in all tanks after 57 h because we tipped the tanks to obtain samples, resulting in unintended resuspension of eDNA from the substrate (Fig. 2). For this reason, we did not consider data points beyond 57 h.

The eDNA k value in both high-density tanks and one low-density tank appeared to follow first-order kinetics (Fig. 3). The eDNA decay in the other low-density tank did not follow first-order kinetics, so we excluded this tank from further statistical analysis. The eDNA k did not differ between treatments (ANCOVA: $F_{1,2} = 2.398$, P = 0.137), indicating that there was no effect of mussel density on the k. The mean value of k across all three tanks was 0.12 ± 0.06 /h. There was no evidence of contamination in any control tanks or filtration and extraction blanks, and all qPCR NTCs showed no amplification.

Decay rates in environmental water.— Throughout the duration of the experiment (T_0 to 263.5 h), eDNA decay in environmental water was best described by a double Weibull model (Table 2). DNA continued to be detected 10 d (263.5 h) after mussels were removed from the tank (Fig. 4). Between T_0 and 28.5 h, eDNA decay followed first-order kinetics (i.e., linear decay over time) (Fig. 4b, c). An increase in eDNA copies/mL was observed at 48 h (Fig. 4a); however, we did not include this point in the *k* calculations because it does not fall within the linear decay period. From T_0 to 28.5 h, the *k* in environmental water was 0.164 ± 0.0124/h. There was no evidence of contamination in any control tanks or filtration and extraction blanks, and all qPCR NTCs showed no amplification.

eDNA Transport Model

The maximum predicted downstream distance at which eDNA could be detected (LOD = $10.76 \text{ copies/}\mu\text{L}$ or 0.54 copies/mL from a 2-L water sample) varied greatly depending on the source eDNA concentration and water velocity (Fig. 5). When source eDNA concentration was 1.0 copy/mL, detection was predicted at 10 km only at high water velocity (>0.6 m/s), and higher detection (i.e., approaching 1.0 copy/mL) was predicted

only at distances less than ~ 2.0 km. By contrast, when source eDNA concentration was 5.0 copies/mL, detection was predicted at 10 km at lower velocity (~ 0.2 m/s) and higher detection (greater than ~ 1.0 copy/mL) was predicted across a much wider range of distance and velocity.

DISCUSSION

Effective use of eDNA methods requires pilot studies that can help optimize the assay and eDNA capture methods (Goldberg et al. 2016). In our study, filter pore size was an important factor that influenced eDNA detection. Detection was greatest with a 0.8-µm filter. This is consistent with other studies that found this pore size to be optimal (Deiner et al. 2018; Li et al. 2018). We filtered larger volumes of water (e.g., 2 L) in the field than in the laboratory to increase detection probabilities; however, given the sediment loads present in the Sydenham during the spring (e.g., LSC-SYR-05, total suspended solids [TSS] for March of 76 mg/L), filtering was challenging (see subsequent text). In other systems, increasing the volume of water may not be possible, because this would likely lead to increased PCR inhibition; therefore, we recommend that the sample volume be optimized for each aquatic system.

We observed greater eDNA detection in the fall (October 2019, 16.67% amplification rate) than in the spring (March 2020, 9.72% amplification rate; see Tables 3 and 5), as has been noted by others (Troth et al. 2021). Our sample volumes differed between seasons, so it is difficult to directly compare eDNA detection. However, lower detection in spring may be expected for several reasons. The suspended sediment load was higher in the spring than in the fall (mean TSS for the Sydenham River in spring of 56.7 mg/L; fall, 14.87 mg/L), which limited the amount of water that we could filter in the spring. Higher discharge in the spring (spring, 14.93 m³/s; fall, 5.97 m³/s) also could have contributed to a diluted eDNA signal, as reported in other studies (Balasingham et al. 2017; Curtis et al. 2021; Gasparini et al. 2020). Lastly, because S. ambigua is gravid and releases glochidia in the fall (I. Porto-Hannes, unpublished data), release of glochidia may



Figure 5. Color gradient graphs showing predictions of the environmental DNA (eDNA) transport model of eDNA concentration as a function of distance from the source and water velocity. Darker colors indicate higher eDNA concentration, and the gray area indicates eDNA concentrations below the limit of detection (LOD; 0.54 copies/mL in 2-L sample or 10.76 copies/ μ L). (A) Source eDNA concentration of 1.0 copy/mL. (B) Source eDNA concentration of 5.0 copies/mL.

increase the probability of eDNA detection, as observed for *Nodularia nipponensis* (Sugawara et al. 2022).

Our detection of *S. ambigua* eDNA in the wild was lower than expected given that we surveyed at three sites where *S. ambigua* is known to occur. Low detection may be due in part to factors associated with spring sampling as discussed previously. However, the unique habitat use of *S. ambigua* also may contribute to lower eDNA concentrations in the water than for other mussel species. Because *S. ambigua* typically occurs in cavities under large rocks, a large proportion of eDNA produced by individuals may remain in those cavities

Table 2. Detection of *Simpsonaias ambigua* environmental DNA at four sites in the Sydenham River, Ontario, Canada, in March 2020. Sites are arranged from upstream to downstream. Amplification is the number of quantitative PCR replicates within each field replicate in which *S. ambigua* eDNA was detected. *Cq* is the quantification cycle. NA = not applicable.

Site	Field Replicate	Amplification	Mean (range) <i>Cq</i>
LSC-SYR-33	1	0/6	NA
	2	0/6	NA
	3	0/6	NA
LSC-SYR-44	1	0/6	NA
	2	0/6	NA
	3	1/6	38.49
LSC-SYR-29	1	0/6	NA
	2	1/6	38.69
	3	0/6	NA
LSC-SYR-05	1	3/6	38.34 (37.57–38.85)
	2	2/6	37.96 (37.46–28.39)
	3	0/6	NA

where it is not readily suspended in the water column or readily detected by conventional sampling.

Our estimates of shedding rate for S. ambigua were comparable to shedding rate of Lampsilis siliquoidea (5.4×10^4 – 2.4×10^6 copies/h/mussel; Sansom and Sassoubre 2017), but they are higher than shedding rates reported for N. nipponensis (0.0066 and 0.33 \times 10⁶ copies/h/individual; Sugawara et al. 2022). The similarity between S. ambigua and L. siliquoidea is surprising because S. ambigua is much smaller and eDNA shedding rates tend to increase with biomass (Takahara et al. 2012; Maruyama et al. 2014). However, in wild Brook Trout (Salvelinus fontinalis), eDNA shedding rates scaled nonlinearly and allometrically with biomass (Yates et al. 2020a, 2020b). Apart from biomass, shedding rate may be related to behavior and metabolism (Maruyama et al. 2014; Klymus et al. 2015). The juvenile S. ambigua used in our study were more active than adult L. siliquoidea used in the Sansom and Sassoubre (2017) study (I. Porto-Hannes, unpublished data; B. Sansom, personal communication). Simpsonaias ambigua juveniles constantly moved vertically and horizontally within the substrate and sometimes crawled up the tank sides or onto the air stones. This behavior may have resulted in greater shedding rates than expected given their small size. In addition, biomass may be a more important determinant of shedding rate in animals that shed skin or scales, which are proportional to biomass. Soft tissues of mussels are enclosed in a hard shell that does not decay readily (Gutiérrez et al. 2003; Strayer and Malcom 2007); consequently, for these animals, activity and filtering rate may be more important determinants of shedding rate than biomass. Further studies are needed to understand how biomass, habitat conditions, and behavior affect shedding rates within and among freshwater mussel species.

Higher concentrations of eDNA can occur in sediment than in the water column because of settling or direct deposition of feces and pseudofeces (Turner et al. 2015). We observed an unexpected increase in eDNA concentration late in our laboratory experiments that coincided with tilting the tanks to obtain a water sample, which probably resuspended eDNA in the substrate. In the wild, eDNA in sediments will not be detected in water samples unless it is resuspended by high flows or other factors (Jerde et al. 2016; Shogren et al. 2017, 2019). Resuspension of eDNA in sediment is an important factor affecting eDNA detection, especially for benthic organisms such as mussels, and this factor needs further investigation. The detection of Margaritifera monodonta eDNA was higher in benthic samples than in water column samples (Lor et al. 2020). However, Currier et al. (2017) found no differences in mussel eDNA detection between surface and subsurface water samples in lotic habitats.

The eDNA decay rate can be influenced by factors such as enzymatic breakdown, microbial grazing, and UV light (Andruszkiewicz et al. 2020). In our study, *k* values were similar between tap water (from 0.077 \pm 0.024 to 0.144 \pm 0.015/h) and environmental water (0.164 \pm 0.012/h). Our *k* values also were similar to decay rates reported for *N. nipponensis* (0.074 \pm 0.021/h; Sugawara et al. 2022) and *Cumberlandia monodonta* (0.067/h; K. Klymus, personal communication), but they were an order of magnitude larger than for *L. siliquoidea* (0.0097–0.053/h; Sansom and Sassoubre 2017) and *Actinonaias ligamentina* (0.045/h; K. Klymus, personal communication).

Our predictions from the eDNA transport model should be viewed with at least two caveats. First, this model is onedimensional: it considers downstream dispersion of eDNA, but not lateral dispersion or settling of eDNA into the substrate. River hydro-geomorphological features have been incorporated in a framework that reconstructs upstream distribution and abundance of a target species across a river network, based on observed eDNA concentration (Carraro et al. 2018). This framework assumes a homogenous distribution of the target species and eDNA production within a river channel. Although the distribution of *S. ambigua* is highly heterogeneous, this framework can be used as a null model. Second, the model is based on decay rates of eDNA present in the water column, but it does not consider settling of eDNA.

Despite the caveats inherent in the eDNA transport model, our model results provide recommendations about optimal sampling designs for eDNA detection. When expected eDNA concentration is low (e.g., 1.0 copy/mL), sampling sites should be spaced at intervals of <2.0 rkm if flow velocity is low. Even if flows are higher, sites should be spaced <5.0 rkm apart to ensure consistent detection. When expected eDNA is higher (e.g., 5.0 copies/mL), sites can be spaced up to 10 rkm apart at moderate to high flow velocity, and ~5 rkm apart at all but the lowest flow conditions. The potential for seasonal variation in eDNA concentration also should be considered, and, if possible, sites should be resampled in different seasons. It is important to consider whether eDNA is present near the LOD, the ability to consistently detect eDNA, and that nondetection may represent Type II error and should not necessarily be interpreted as evidence that the species is absent (Klymus et al. 2020). Incorporating in a sampling design the effects of source eDNA concentration, flow velocity, seasonality, target species habitat use, and other factors can minimize Type II error. In addition, systematic sampling throughout a watershed can reveal consistent, largescale patterns that more accurately indicate the distribution of a species.

The eDNA detection of a target species is a cost-effective way to provide information necessary to prioritize sites for more time-consuming conventional sampling. However, for rare and threatened species, such as *S. ambigua*, management decisions should not be made based solely on the detection of eDNA. Although considered part of the standard fisheries and wildlife management toolkit for population detection, assessment, and monitoring (Klymus et al. 2020, and references therein), eDNA methods cannot replace conventional methods and population monitoring but they can complement and augment them.

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PORTO-HANNES ET AL.

APPENDIX

Table 2. *Simpsonaias ambigua* species-specific primer–probe sets designed from mitochondrial DNA sequences from the cytochrome oxidase subunit I gene (COI) and NADH dehydrogenase gene (ND1). Parameters were developed based on the following criteria: (1) DNA fragment size range 80-150 bp, (2) GC content 35-65% for both primer and probe, (3) primer annealing temperature range $58-63^{\circ}$ C, (4) probe annealing temperature range $68-73^{\circ}$ C, (5) maximum difference in annealing temperature between primer and probe of 10° C, and (6) primer pair specificity. Cross-amplification refers to nontarget species that amplified (see also Table 1). Minimum DNA sequences mismatches with nontarget species refers to the minimum number of nucleotides mismatches between the primer–probe and the nontarget species sequence.

Primer	Gene	Sequence 5'-3'	Primer Length (bp)	Fragment Size (bp)	GC Content	Annealing Temp. (°C)	Cross-Amplification (In Vitro Testing)	Minimum DNA Sequence Mismatches with Nontarget Species
SamCOI_Probe1	COI	TGAGGTCTTCGTTG GTGGAAAGAGGT	26	125	50	62	Obliquaria reflexa, Amblema plicata,	1<
SamCOI_FWD1	COI	ATCGGTGCTCCT GATATGGC	20		55	57	Truncilla truncata, Sagittunio nasutus,	2<
SamCOI_RVS1	COI	ACCGTTCAACCAG TACCCAC	20		55	57	Potamilus fragilis, Lampsilis cardium, Alasmidonta undulata, Pleurobema sintoxia	3<
SamCOI_Probe2	COI	CGGTGCTCCTGATATG GCTTTTCCTCG	27	123	56	63	Not tested	2<
SamCOI_FWD2	COI	TGGTAATTGGCT TGTTCCCT	20		45	54		1<
SamCOI_RVS2	COI	TCCACCAACGAA GACCTCAA	20		50	56		2<
SamND_Probe1	ND1	AACCCGCAGCAGA CGCCTTG	20	125	65	63	Sagittunio nasutus, Obliquaria reflexa	3<
SamND_FWD1	ND1	ACTAGGGCTTAGT GGCATTCC	21		52	57		4<
SamND_RVS1	ND1	AGGGCGAGTATAG TTATTGGGG	22		50	56		4<
SamND_Probe2	ND1	TGGCTACTTTCAAATTC GAAAAGGCCC	27	105	44	70	Not tested	3<
SamND_FWD2	ND1	TGGCTGTAGCATTTT TCACCC	21		48	60		1<
SamND_RVS2	ND1	TGGAATGCCACTA AGCCCTA	20		50	60		3<

REGULAR ARTICLE

MUSSELS PROPAGATED FROM A SINGLE BROODSTOCK FEMALE RETAIN MOST POPULATION-LEVEL GENETIC VARIATION BUT HAVE ALTERED GENETIC STRUCTURE

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ABSTRACT

Captive propagation followed by release to natural habitats has become a common conservation practice to restore and augment mussel populations, but the genetic effects of these efforts remain poorly studied. We examined genetic variation and genetic structure in 2- to 3-yr-old subadults of *Lampsilis cardium* and *L. siliquoidea* that each was propagated from a single broodstock female and subsequently used to augment existing wild populations. We compared genetic variation and structure of the propagated individuals to that of the wild population, including the broodstock females. Using microsatellite markers, we found that propagated subadults retained levels of heterozygosity comparable to the wild population and showed no sign of genetic bottlenecks. This is likely due to high levels of multiple paternity in both species, with the single broodstock females of *L. cardium* and *L. siliquoidea* mating with an estimated 13 and 25 sires, respectively. However, propagated subadults had significantly fewer alleles and lower allelic richness and altered allele frequencies compared with wild adults, and genetic structure of propagated individuals was distinct from the wild population. Our results show that propagation from even a single broodstock female can result in offspring that retain most population-level genetic variation. However, the reduced allelic richness and altered genetic structure we observed in propagated individuals underscore the need for future studies to investigate the ecological and evolutionary impacts of propagated individuals on wild populations.

KEY WORDS: captive breeding, multiple paternity, microsatellites, parentage analyses, propagation, augmentation, genetic variability

INTRODUCTION

Freshwater mussels (Bivalvia: Unionida) are among the most endangered organisms on Earth (Haag and Williams 2014; Pereira et al. 2014; Lopes-Lima et al. 2021). Captive propagation followed by release to natural habitats has become a common conservation practice to restore and augment mussel populations (McMurray and Roe 2017; Patterson et al. 2018; Rytwinski et al. 2021). Mussels typically are

propagated by harvesting parasitic larvae from gravid female broodstock, inoculating host fishes with larvae, and harvesting juveniles that metamorphose on fishes (Patterson et al. 2018). Captively propagated juveniles often are raised to the subadult stage before release, which can reduce the high mortality characteristic of the juvenile stage in the wild (McMurray and Roe 2017). Captive propagation initially was used mainly for imperiled species, but it is now used widely for a variety of species and conservation goals (Patterson et al. 2018; Strayer et al. 2019).

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Despite the benefits of captive propagation, it has potential negative ecological and genetic consequences (McMurray and Roe 2017; Strayer et al. 2019; Rytwinski et al. 2021). One of the primary concerns is preservation of maximum genetic variability within species and populations, which is important for maximizing evolutionary potential and the ability to adapt to environmental change (Pelletier et al. 2009). In some cases, mussel propagation programs use only one or a few broodstock females to produce large numbers of juveniles, which has the potential to capture only a small portion of genetic variation present in the wild population (Hoftyzer et al. 2008). However, freshwater mussels are spermcasters (Bishop and Pemberton 2006), in which spermatozoa are released into the water column by males and captured by females to fertilize their eggs. Consequently, the brood of individual females can be fertilized by multiple males, resulting in multiple paternity within the brood (Christian et al. 2007; Wacker et al. 2018; Garrison et al. 2021). Multiple paternity can increase genetic diversity within the brood of a single female, thereby reducing chances for potential negative effects from using a small number of broodstock females (Jennions and Petrie 2000). Nevertheless, few programs currently quantify genetic diversity of propagated mussels or compare it to that of source or recipient populations (Rytwinski et al. 2021). A better understanding of genetic characteristics of captively propagated mussels is needed to avoid negative consequences potentially associated with stocking those animals into the wild.

We examined genetic variation and genetic structure in 2to 3-yr-old subadults of *Lampsilis cardium* and *L. siliquoidea* that each was propagated from a single broodstock female and subsequently used to augment existing wild populations. We compared genetic variation of the propagated individuals to that of the wild population, including the broodstock females. We also estimated the number of paternal contributions present within each brood used to produce the propagated individuals. We discuss how our results inform the development of captive propagation programs that can reduce the potential for negative genetic effects.

METHODS

Propagation of L. cardium and L. siliquoidea was conducted by the Forest Preserve District of DuPage County at the Urban Stream Research Center in Warrenville, Illinois, USA, as part of a mussel conservation program in the West Branch DuPage River. A single broodstock female of each species was collected from the West Branch DuPage River in January 2016 for L. cardium and February 2017 for L. siliquoidea. Glochidia were extracted from the marsupial gills of the broodstock, and their viability was checked by exposing them to a droplet of saturated NaCl solution. The viable glochidia were inoculated on Largemouth Bass (Micropterus nigricans). The infested fish were held in flow-through tanks until the encysted glochidia metamorphosed into juveniles and dropped off the hosts. Tissue-swab genetic samples from each broodstock female were taken before releasing them into the natural population. Genetic samples were preserved in 95% ethanol and stored at -20° C. However, the *L. siliquoidea* sample became desiccated and thus unusable for DNA extraction; therefore, we estimated microsatellite genotypes of the *L. siliquoidea* broodstock from the offspring genotypes (see below).

Juveniles were reared in the laboratory until they reached approximately 3.5 mm shell length, after which they were moved to floating baskets in a pond on the Forest Preserve District property and reared for 9 mo (*L. siliquoidea*) or 21 mo (*L. cardium*) until they reached the subadult stage (25–40 mm shell length). Subadults were tagged with passive integrated transponders (PIT tags), vinyl shellfish tags, or glitter dots. The tagged subadults were released at multiple sites in the West Branch DuPage River in July 2017 (*L. cardium*) and October 2017 (*L. siliquoidea*).

In summer 2019, we conducted postrelease monitoring for the propagated subadults at all sites. During monitoring, we collected tissue-swab genetic samples from 18 subadults for *L. cardium* and 37 subadults for *L. siliquoidea*, preserved them in 95% ethanol, then stored them at -20° C. In summer 2020, we collected tissue-swab genetic samples of 31 wild adult *L. cardium* and 24 wild adult *L. siliquoidea* at a location near where the broodstock females were collected previously.

We extracted total DNA from all samples using cetyltrimethylammonium bromide (CTAB)-chloroform extraction followed by ethanol precipitation. We diluted the extracted DNA to a concentration of 10 ng/ μ L and used it as a template in polymerase chain reaction (PCR) amplification of microsatellite loci. For amplification, we used primers developed for Lampsilis abrupta (Eackles and King 2002) and Venustaconcha ellipsiformis (Inoue et al. 2021). Prior to genotyping, we screened a subset of microsatellite loci for each species for PCR success and polymorphisms. We selected a total of 10 loci for L. cardium and 11 loci for L. siliquoidea for study (Table 1). We performed PCR reactions in 10 µL volume, including 5 µL of GoTaq[®] G2 Master Mix (Promega Corp., Madison, WI, USA), 0.25 µM of universal fluorescently labeled primer and nontailed primer, 0.05 µM of tailed primer, and 10 ng of DNA. We used the following PCR conditions: initial denaturing at 95°C for 2 min, followed by 40 cycles at 95°C for 30 s, annealing at 61°C for 45 s, extension at 72°C for 45 s, and final extension at 72°C for 30 min (Inoue et al. 2021). We conducted fragment analyses on a 3730xl DNA Analyzer (ThermoFisher Scientific, Inc., Waltham, MA, USA) at the Field Museum (Chicago, IL, USA) with Orange DNA Size Standard (MCLAB, South San Francisco, CA, USA). We verified peak calling using Geneious Prime v2020.1.2 (https://www.geneious.com, accessed September 16, 2023) with the microsatellite plugin based on the microsatellite motifs, and we assigned integer numbers to DNA fragment sizes. Briefly, in Geneious, we created locus information for each locus (e.g., diploid, repeat unit, expected range of fragment sizes) and used Third-Order Least Squares as a sizing method. For each species, we included all individuals to verify size standard and microsatellite peaks, create fragment size bins based on

Table 1. Descriptive statistics of 10 microsatellite loci for *Lampsilis cardium* and 11 loci for *L. siliquoidea* from the West Branch DuPage River, Illinois, USA. Propagated subadults were collected 2 yr after release in the stream. Wild adults were collected from the same sites where subadults were released and include a single broodstock female for each species.

	Lampsilis cardium											
			Propaga	ted subadu	ılts				Wild	l adults		
Locus	$N_{\rm A}$	$A_{\rm R}$	$P_{\rm A}$	$H_{\rm O}$	$H_{\rm E}$	$F_{\rm IS}$	$N_{\rm A}$	$A_{\rm R}$	$P_{\rm A}$	H _O	$H_{\rm E}$	$F_{\rm IS}$
LabC2	3	3.0	0	0.89	0.61	-0.45	4	3.8	1	0.59	0.61	0.02
LabC23	3	3.0	0	0.94	0.52‡	-0.81	4	3.5	1	0.28	0.30	0.06
LabC24	2	2.0	0	0.72	0.46	-0.57	2	2.0	0	0.34	0.39	0.12
LabD213	7	7.0	1	0.67	0.53	-0.25	21	16.3	15	0.91	0.91	0.00
Ve008	5	5.0	1	0.61	0.48	-0.27	4	3.8	0	0.44	0.52	0.16
Ve010	5	5.0	0	0.39	0.34	-0.15	7	6.5	2	0.59	0.79†	0.25
Ve015	4	4.0	0	0.56	0.61	0.09	4	4.0	0	0.59	0.60	0.01
Ve025	6	6.0	0	0.56	0.66	0.16	8	7.7	2	0.63	0.82†	0.24
Ve078	10	10.0	1	0.94	0.79	-0.19	13	11.8	4	0.88	0.90	0.03
Ve169	6	6.0	0	0.61	0.49	-0.25	11	9.0	5	0.69	0.78	0.11
Mean values	5.1	5.1	0.3	0.69	0.55	-0.27	7.8	6.8	3.0	0.59	0.66	0.10

Lampsilis siliquoidea

	Propagated subadults						Wild adults					
Locus	N _A	$A_{\rm R}$	$P_{\rm A}$	H _O	$H_{\rm E}$	F _{IS}	N _A	$A_{\rm R}$	$P_{\rm A}$	H _O	$H_{\rm E}$	$F_{\rm IS}$
LabC23	3	3.0	0	0.38	0.32	-0.17	4	4.0	1	0.64	0.53	-0.21
LabD187	13	11.3	1	0.89	0.80	-0.11	18	18.0	6	0.76	0.93†‡	0.19
LabD213	13	10.8	2	0.68	0.70	0.04	17	17.0	6	0.80	0.87	0.08
Ve001	4	3.4	1	0.22	0.47†‡	0.54	6	6.0	3	0.40	0.64†	0.37
Ve008	9	7.7	0	0.73	0.68	-0.07	9	9.0	0	0.76	0.80	0.06
Ve015	4	3.6	2	0.51	0.45	-0.14	7	7.0	5	0.44	0.46	0.05
Ve025	7	6.8	0	0.73	0.73	0.00	10	10.0	3	0.72	0.87†	0.17
Ve050	7	6.8	1	0.78	0.73	-0.08	7	7.0	1	0.84	0.83	-0.01
Ve058	5	4.8	0	0.32	0.58†‡	0.44	7	7.0	2	0.44	0.68†‡	0.35
Ve138	9	8.0	1	0.51	0.68†	0.25	11	11.0	3	0.88	0.86	-0.02
Ve169	7	6.4	0	0.76	0.70	-0.09	9	9.0	2	0.72	0.69	-0.05
Mean values	7.4	6.6	0.7	0.59	0.62	0.06	9.5	9.5	2.9	0.67	0.74	0.09

 N_A , number of alleles; A_B , rarefied allelic richness; P_A , number of private alleles; H_O , observed heterozygosity; H_E , expected heterozygosity; F_{IS} , inbreeding coefficient. \dagger indicates potential null allele presence. \ddagger indicates deviation from Hardy-Weinberg proportion.

the size of the observed peaks, and assign fragment sizes. When there were no peaks, or when the observed peaks were weak, we repeated PCR amplifications to ensure the correct peak calling.

The sample from the broodstock female *L. siliquoidea* became desiccated and was unusable for DNA extraction. Consequently, we estimated maternal microsatellite genotypes of the *L. siliquoidea* broodstock female COLONY v2.0.6.5 (Jones and Wang 2010) based on the offspring genotypes. We used default input parameters except that the mating system was set to female polygamy (i.e., maternal half-sibs exist) and male

monogamy (i.e., no paternal half-sibs exist because the offspring was derived from a single female), and the length of run was set to "long." We assigned all propagated subadults of *L. siliquoidea* as the offspring of the same female. We included all microsatellite loci in the analyses with an allele dropout rate of 0 and a genotyping error of 0.0001. The genotype of the *L. siliquoidea* broodstock was confirmed with 100% probability at all loci, except for the locus Ve015, which had 99.7% probability. Therefore, we included the estimated genotype of the broodstock female in subsequent analyses. For all subsequent analyses, we included observed or estimated genetic data for the two broodstock females within the wild individuals for each species. We did this because we were interested mainly in the proportion of genetic variation present in the entire wild population that was preserved in propagated subadults; we were less interested in the proportion of genetic variation in the individual broodstock females that was preserved in their offspring. Consequently, we evaluated genetic variation and structure in two sample groups: propagated subadults and wild individuals (including broodstock).

We assessed the utility of each locus by testing for the presence of null alleles using Micro-Checker v2.2.3 (van Oosterhout et al. 2004). We performed exact tests of pairwise linkage disequilibrium (LD) and deviation from Hardy-Weinberg proportion (HWP) for each sample group within each species (i.e., propagated subadults and wild adults) using GenePop v4.7 (Raymond and Rousset 1995; Rousset 2008). We applied sequential-comparison Bonferroni correction for multiple comparisons of LD and HWP (i.e., locus-by-group) (Lessios 1992). We estimated population genetic indices (number of alleles, $N_{\rm A}$; observed and expected heterozygosity, $H_{\rm O}$ and $H_{\rm E}$; and inbreeding coefficient, F_{IS}) for each locus and sample group using GenAlEx v6.5 (Peakall and Smouse 2006, 2012). We estimated rarefied allelic richness (A_R) using FSTAT v2.9.4 (Goudet 1995) to correct for sample-size biases. We used Wilcoxon signed-rank tests to assess statistical differences in the genetic indices between propagated subadults and wild adults for each species.

Based on the allele frequencies calculated by GenAlEx, we calculated the proportion of alleles retained in the propagated subadults relative to the wild adults in both species. We categorized each allele as a rare allele (allele frequency < 0.05), intermediate frequency allele (0.05 < allele frequency > 0.25), or high-frequency allele (allele frequency > 0.25) based on the wild populations. Additionally, we counted the number of private alleles/locus (i.e., alleles observed in only one group) in GenAlEx.

To assess population genetic structure, we estimated Weir and Cockerham's θ (Weir and Cockerham 1984) (equivalent to Wright's F_{ST}) between propagated subadults and wild adults for each species using GENETIX v4.05 (Belkhir et al. 2004). To test whether θ differed significantly from zero (i.e., no population genetic substructure), we calculated 95% confidence intervals around the estimate of θ based on 1,000 bootstraps. Additionally, we used a non-negative matrix factorization algorithm using the *snmf* function implemented in the R package LEA v.3.10.2 (Frichot et al. 2015) to estimate the optimal number of genetic clusters within the samples. Unlike the ΔK method used for STRUCTURE analysis (Pritchard et al. 2000; Evanno et al. 2005), the entropy criterion method allows evaluating K = 1 (Frichot et al. 2014). We ran the *snmf* function for K = 1-10 with 100 replicates for each value of K, and we estimated the optimal number of genetic populations based on the cross-entropy criterion.

To detect recent population bottlenecks within groups, we tested for deviations from mutation-drift equilibrium with BOTTLENECK v1.2.02 (Piry et al. 1999). This method assumes that recently bottlenecked population would exhibit reductions in allelic diversity faster than reductions in heterozygosity, resulting in heterozygote excess expected under mutation-drift equilibrium. We evaluated three mutation models: infinite allele (IAM), two-phase mutation (TPM), and stepwise mutation (SMM). We modeled TPM with a combination of 70% single-step mutations and 30% multistep mutations. We used Wilcoxon tests to test for significant heterozygote excess.

We estimated the most likely number of sires/brood and potential family structure within offspring for each species using COLONY v2.0.6.5 (Jones and Wang 2010). We used the same input parameters described previously.

RESULTS

Ten loci for *L. cardium* and 11 loci for *L. siliquoidea* were successfully amplified, and all showed polymorphism (Table 1). After Bonferroni correction, we found no evidence of LD in 200 locus-by-group pairs. However, deviations from HWP and potential null alleles were found in some loci (Table 1). The number of alleles ranged from two to 21/locus (a total of 81 different alleles over 10 loci for *L. cardium* and 113 alleles over 11 loci for *L. siliquoidea*). Mean rarefied allelic richness ranged from 5.1 alleles/locus for the propagated subadults of *L. cardium* to 9.5 alleles/locus for the wild adults of *L. siliquoidea*. Observed and expected heterozygosity values ranged from 0.55 for the propagated subadults of *L. siliquoidea*.

Propagated subadults had significantly fewer alleles and lower rarefied allelic richness than wild adults in both species (Fig. 1; Table 1). Observed heterozygosity did not differ between propagated and wild individuals for either species; expected heterozygosity differed between the groups for *L. siliquoidea* but not for *L. cardium*. The inbreeding coefficient was significantly lower in propagated subadults than wild adults for *L. cardium*, but it did not differ between groups for *L. siliquoidea*.

The propagated subadults of both species retained over half of the alleles present in the wild adults (*L. cardium*, 61.5%; *L. siliquoidea*, 69.5% (Fig. 2). However, the retention rates decreased as the alleles became rarer. While the propagated subadults retained all high-frequency alleles, they retained, on average, 82.6% of intermediate-frequency alleles and only 37.2% of rare-frequency alleles (intermediate alleles: 80.0% in *L. cardium*, 85.2% in *L. siliquoidea*; rare alleles: 30.3% in *L. cardium*, 44.2% in *L. siliquoidea*]. Additionally, in both species, a higher number of private alleles were found in the wild adults (P_A : 30 in *L. cardium*; 32 in *L. siliquoidea*) than in the propagated subadults (P_A : three in *L. cardium*; eight in *L. siliquoidea*; Table 1).

The mean pairwise θ values between propagated subadults and wild adults were 0.097 for *L. cardium* and 0.071 for *L. siliquoidea*. The 95% confidence intervals did



Figure 1. Violin plots of genetic diversity measures for propagated subadult and wild adult *Lampsilis cardium* and *L. siliquoidea* from the West Branch DuPage River, Illinois, USA. The bold horizontal line is the median value, boxes are the interquartile range, vertical lines are $1.5 \times$ interquartile range and violin shapes indicate kernel density, representing the probability of observations for a given value. Results of Wilcoxon signed-rank tests for differences between subadults and wild adults are given for each measure. *Lampsilis cardium* and *L. siliquoidea*. Significant test results are bolded. N_A , number of alleles; A_R , rarefied allelic richness; H_O , observed heterozygosity; H_E , expected heterozygosity; F_{IS} , inbreeding coefficient.

not include zero for either species (0.043-0.152 for L. cardium; 0.046-0.102 for L. siliquoidea), indicating significant genetic differentiation between groups. Furthermore,the LEA analysis recovered two distinct genetic clusters for bothspecies (<math>K = 2; Fig. 3), and the clusters generally were segregated between the propagated subadults and wild adults (Fig. 4). In *L. cardium*, the propagated subadults grouped exclusively into cluster 1 with the broodstock female, while most of the wild adults grouped into cluster 2 with some admixture with cluster 1. In *L. siliquoidea*, the broodstock female was assigned to both clusters 1 and 2, and a majority of the propagated subadults grouped into cluster 1.

None of the population groups exhibited heterozygote excess (IAM: P = 0.139-0.652; SMM: P = 0.688-1.000; TPM: P = 0.246-0.997), except for the wild adults of *L. cardium* under the IAM (P = 0.009). These results indicate no recent population bottlenecks in most groups and only a small population bottleneck within the wild adults of *L. cardium*.

A high level of multiple paternity was estimated for both species. The COLONY analyses showed that the most likely number of sires/brood was 13 for *L. cardium* and 25 for *L. siliquoidea*, indicating that most of the propagated subadults were half-siblings. Among the 13 families in *L. cardium*, five contained two full siblings. While most families had high probabilities of being true families (0.75 to 0.94), one family had a probability of 0.27, meaning that the family can be split into two families. Similarly, among the 25 families in *L. siliquoidea*, four families contained two full siblings and four families contained three full siblings. However, the probabilities were rather low in seven families (<0.01 to 0.60), indicating that the sibship family structure was statistically unresolved within *L. siliquoidea*.

DISCUSSION

Propagated subadults of *L. cardium* and *L. siliquoidea* derived from a single broodstock female and released into the



Figure 2. Stacked bar plots of allele frequencies for microsatellite loci in propagated subadult and wild adult *Lampsilis cardium* and *L. siliquoidea* from the West Branch DuPage River, Illinois, USA. Colors represent unique alleles at each locus.

wild retained levels of heterozygosity comparable to the wild source and recipient populations. The propagated subadults maintained all the common alleles that were present in the wild populations and even possessed a few private alleles not observed in the wild. High heterozygosity and allele retention in the propagated subadults likely were facilitated by multiple paternity within broods, with less than one-third of the offspring sharing the same father.

The degree of multiple paternity in mussels can vary widely. Our estimates of 13 and 25 sires in each brood are similar to those observed on *Margaritifera margaritifera* in Norway, where up to 32 sires contributed to a single brood (Wacker et al. 2018). However, other studies reported only

two to six sires/brood, including three sires in broods of *L. cardium* in Ohio (Bai et al. 2011; Ferguson et al. 2013). Factors such as the abundance and density of reproductively active males, their position relative to females, timing of sperm release, and the females' ability to capture sperm can influence the levels of multiple paternity. Environmental factors, including flow velocity and hydrodynamics, also may play a role, but the influence of such factors in multiple paternity has not been examined. Further research is needed to investigate variability in multiple paternity among species and to determine the optimal number of offspring required to estimate total paternal contributions within a single female.



Figure 3. Cross-entropy plots for estimating the optimal number of genetic clusters (K) within samples of propagated subadult and wild adult *Lampsilis cardium* and *L. siliquoidea* from the West Branch DuPage River, Illinois, USA, based on the R package LEA (see text). Each value of K was assessed for 100 replicates. Lower values of cross-entropy represent greater support for a specific value of K.

Κ

While the propagated subadults retained all the common alleles, we observed a significant loss of alleles with rare and intermediate frequencies as well as a higher number of private alleles in the wild adults. This led to reductions in overall allelic richness and changes in allele frequencies within the subadult gene pool. Notably, some alleles that were rare in the wild populations became more common among the propagated subadults, likely due to the over-representation of maternal (broodstock female) genotypes within the brood. These changes in allele frequencies resulted in altered population genetic structures of propagated subadults compared to wild population in both species. Although the current study focused on neutral genetic markers, the observed alterations in allele frequencies and population genetic structures have potential implications for genes under selection. Captive breeding programs can affect genes under selection by relaxing selection pressures found in the wild or artificially selecting traits that are advantageous in the captive environment (i.e., domestication) (Frankham 2008; Christie et al. 2012). Modification of genetic structure within and among populations are documented in other species, such as salmonid fishes (Perrier et al. 2013). Because mussel populations often are locally adapted and genetically structured (Riusech and Barnhart 2000; Barnhart et al. 2008; Inoue et al. 2015), altering genetic diversity and genetic admixture between wild and captive-reared individuals may lead to a loss of local adaptation and reduced fitness in wild populations (Araki et al. 2007).

The alteration of genetic diversity and structure that we observed probably was largely due to the production of subadults from a single broodstock female, which underscores the importance of using multiple females in propagation programs (Jones et al. 2006). A previous study found no significant alternation of population genetic structure when juveniles were propagated from multiple broodstock females (VanTassel et al. 2021), but that study evaluated only three to six juveniles/ female. Future research is needed to better understand the effect of the number of broodstock females on population genetic structure of propagated juveniles.

The subadults we studied were released into the wild 2 yr prior to genetic sampling, and we were unable to sample the individuals after metamorphosis or prior to release. During 2 yr in the wild, genetic structure of the subadults may have been influenced by natural selection or stochastic factors, and it would be informative to study how genetic structure changes after release to the wild. However, our results depict the functional genetic variability and structure of propagated cohorts near the time they may begin to interbreed with and influence the genetic structure of natural populations.

As captive propagation techniques for freshwater mussels have advanced, captive propagation and release programs have become widely used in conservation and restoration projects (Patterson et al. 2018). Although previous studies have provided guidance for genetic management in propagation programs (Jones et al. 2006; Hoftyzer et al. 2008; McMurray and Roe 2017), many programs still do not evaluate the genetic characteristics of broodstock, propagated individuals, or recipient populations, and they lack postrelease genetic monitoring (Rytwinski et al. 2021). Given that large numbers of propagated mussels often are released to natural habitats (>10,000 propagated individuals; Bishop et al. 2006), captive propagation and release programs have the potential to significantly alter existing genetic variability and disrupt evolutionary processes necessary for species' adaptation to environmental changes. It is crucial to incorporate strategic genetic management and monitoring into captive propagation and release programs to maximize species recovery success while minimizing negative genetic impact on natural populations.



Figure 4. Stacked bar plots showing the probability of individuals belonging to two genetic clusters identified within propagated subadult and wild adult *Lampsilis cardium* and *L. siliquoidea* from the West Branch DuPage River, Illinois, USA, based on the R package LEA (see text).

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NOTE

FRESHWATER MUSSELS IN THE BYCATCH OF A SNAIL FISHERY IN THE POYANG LAKE REGION, CHINA: A POTENTIAL CONSERVATION OPPORTUNITY

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ABSTRACT

Poyang Lake, China's largest freshwater lake, has high ecological and economic value. The area is a global hotspot of freshwater mussel diversity, and it supports an important dredge fishery for snails, which results in substantial mussel bycatch. The mussel fauna changed dramatically in the last two decades, and many large species disappeared from the lake. We hypothesize that snail dredging may be a factor threatening mussel populations in the area. We describe the snail fishery and its associated mussel bycatch in Qinglan Lake, a satellite of Poyang Lake. We evaluate the potential impact of unselective harvest on the mussel fauna, and we estimate the value of mussels as a commodity for local fishers. Fishers harvested an average of 859 mussels per trip, with 17 mussel species present in the bycatch. We estimated that annual mortality from bycatch represented about 5% of the total mussel standing stock in the lake. The market price for mussels was low compared to target snails. This low value provides a potential conservation opportunity of providing financial incentives to fishers for returning mussels to the lake.

KEY WORDS: Unionoida, Asia, bycatch mitigation, fisheries management, mussel conservation

INTRODUCTION

Freshwater mussels (Mollusca: Bivalvia: Unionoida) are highly imperiled worldwide, and they provide important

ecosystem and cultural services (Vaughn 2018). Mussels have a long history of harvest by humans for pearls, for nacre and craft industries, as a protein source, as calcium for livestock, and as fertilizer in agriculture. Overexploitation for pearls and nacre products decimated mussel populations in Europe and the Americas, but harvest declined after the Second World War, when nacre products were mainly replaced by synthetic materials (Anthony and Downing 2001; Haag 2012; Clavijo 2017). Freshwater mollusk harvest remains of great importance in China (ca. 20,000 tons harvested in 2000; FAO 2023), but many species are facing extinction from overexploitation (Do et al. 2018; Liu et al. 2020b). Most research on mussel diversity and distribution has focused on Europe and North America (Lopes-Lima et al. 2018), but recent research has begun to examine these aspects of the Asian mussel fauna (e.g., Bolotov et al. 2017; Zieritz et al. 2016, 2018). However, the factors important in mussel declines and conservation in Asia remain poorly studied.

Poyang Lake (Jiangxi Province) is China's largest freshwater lake and, along with a series of satellite lakes, is connected to the Yangtze River. Poyang Lake and its satellite lakes are well known for their ecological and economic importance (Leeuw et al. 2010; Xia et al. 2010; Zhong and Lu 2011; Huang et al. 2013). The area is home to more than 35 mussel species (Xiong et al. 2010, 2012; Zieritz et al. 2018), making it a global hotspot for mussel diversity. Two gastropod species, *Rivularia auriculata* and *Bellamya aeruginosa*, dominate the benthos and support a fishery based on traditional fishing craft and gear and labor-intensive methods (Cai et al. 2014). Fishers collect snails using small boats equipped with hydraulic dredges that pump sediments

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(including mollusks) from the lake bottom onto a conveyor. Sediments are screened to remove mud and sand, and mollusks are deposited in sacks for transport to market.

Snails harvested by this fishery are an important source of food for human consumption and an important commodity for the local community (Ma et al. 2010; N. Ferreira-Rodríguez, unpublished observations). Dredging is unselective and results in substantial mussel bycatch, which is sorted from target snail species at lakeside villages and sold as a secondary commodity for human consumption. We describe the snail fishery, and its associated mussel bycatch, in Qinglan Lake, a satellite lake of Poyang Lake. We evaluate the potential impact of unselective harvest on the mussel fauna, and we estimate the value of mussels as a commodity for local fishers. We discuss how mussel bycatch from the snail fishery could be considered in mussel conservation plans in the region.

METHODS

Study Area and Description of the Snail Fishery

Qinglan Lake has an area of 15 km² and is a satellite of Poyang Lake. Its mean depth is 3.5 m \pm 1.2 (SD; Qiu et al. 2022). The water sources of Qinglan Lake are its main tributary (Fuhe River) and local precipitation, which fluctuates between 20 and 35 cm/month (Zeng et al. 2017). The snail fishery in Qinglan Lake included 80-100 boats more than 10 years ago, but the fishery now includes only 20-30 boats. Existing management measures are restricted to closing the fishery from March to June each year. Currently, it is estimated that 500-1,000 kg of snails are collected per boat per trip, with one trip per day (N. Ferreira-Rodríguez, interview with local fishers), and the annual catch reported by the local community is 240-480 metric tons (based on an estimated 480 trips per year by the entire community). The price for snails is \$0.18-0.30/kg (2018 \$US; all dollar amounts will be in \$US), which represents \$90-300 per trip and annual receipts for the local community (10 to 15 families with two dredging boats each) of \$43,200-144,000/year (i.e., wholesale price paid to the local community).

Bycatch Assessment

We obtained bycatch data (where bycatch is defined as non-target mussel species) from two opportunistic encounters with local fishers, one each at two locations: Qinglan Lake (28°33'32.85″N, 116°11'7.26″E) on July 11, 2018, and Tachengxiang village (28°32'19.33″N, 116°7'47.27″E) on July 15, 2018 (Fig. 1). On Qinglan Lake, we intercepted a fisher after conclusion of a fishing trip and purchased mussels before they landed. At Tachengxiang village, we purchased mussels from a different fisher after they docked at the village. We purchased all mussels in possession of each fisher and considered these numbers to represent total mussel bycatch per trip. We summed the number of each mussel species in the bycatch from both encounters to obtain the relative abundance of each species in the bycatch. We transported mussels purchased from fishers to the Conchology Laboratory at Nanchang University, where we preserved them in 70% ethanol and later identified and counted them.

Market Price

We estimated the market price of mussels separately for Qinglan Lake and Tachengxiang village. Although mussels can be bought directly from fishers, there is not an established market for them. Therefore, we used fair valuation to estimate the market price. Fair value is the estimated price at which an asset (here, freshwater mussels) is bought when both the buyer (here, researchers) and the seller (here, the fisher) freely agree on a price. We made no distinction among sizes or species, and we estimated market price in \$US/100 mussels after negotiations with fishers.

RESULTS

The mean number of mussels harvested/trip was 859 (701 at Qinglan Lake and 1,017 at Tachengxiang village). We were unable to measure the size of the mesh used by fishers to screen mollusks from dredged sediments. However, all mussels in our samples were >44 mm long (maximum anterior-posterior dimension), suggesting that the mesh allowed mussels smaller than that size to escape. A total of 17 mussel species were represented in both bycatch samples (Table 1). The most common species were Acuticosta chinensis followed by A. ovata and Nodularia douglasiae. Other frequent species were Schistodesmus lampreyanus, Lamprotula caveata, and S. spinosus, while the remaining species each represented <5% of the bycatch. All species were present in both bycatch samples, except Sinohyriopsis cumingii, which was present only in the sample from Qinglan Lake.

The mean market price was \$0.66/100 mussels (\$0.42 at Qinglan Lake and \$0.90 at Tachengxiang village). Based on these market prices, the mean estimated income per trip provided by mussel bycatch was \$6.05 (\$2.94 at Qinglan Lake and \$9.15 at Tachengxiang village), and estimated mean annual receipts to the communities are \$2,904 (\$1,411–4,392).

Mean mussel density reported in Qinglan Lake was 0.59/ $m^2 \pm 0.21$ SE (Xiong et al. 2010), resulting in an estimated lake-wide standing stock of 8,850,000 mussels (based on 15 km² lake area). Based on the mean number of mussels harvested per trip (859) and the estimated number of trips per year (480), annual mussel mortality from harvest is 412,320, representing about 5% of the standing stock each year.

DISCUSSION

Bycatch from the snail fishery may represent a substantial source of mortality for mussel populations in Qinglan Lake and elsewhere in the Poyang Lake region. Our mortality estimate is based on only two observations of bycatch from



Figure 1. Map showing locations where mussels were purchased from local fishers in the Qinglan Lake (Poyang Lake area, China). Inset maps show the location of the Poyang Lake area in China and the location of Qinglan Lake in relation to Poyang Lake.

two dates, and many factors could influence the extent to which bycatch affects mussel populations. The apparently large mesh size used in the fishery allows escapement of juvenile individuals, which could lessen effects on population growth. However, spatial and temporal variation in harvest, as well as potential underreporting of harvests by fishers, could greatly influence mortality estimates. Nevertheless, our study is the first to quantify mussel mortality from bycatch, and our results suggest that bycatch may exacerbate other potential threats to mussel populations, including water pollution, habitat alteration, and increased drought from climate change (Cai et al. 2014; Zhang et al. 2015; Wang et al. 2019).

Mortality from bycatch may affect mussel species differently, resulting in shifts in community composition. Before 1998, the most common mollusks in the Poyang Lake area included many large unionid species, such as *Lamprotula leaii*, *L. caveata*, *Aculamprotula tientsinensis*, *N. douglasiae*, *Lancelaria* spp., *Cuneopsis pisciculus*, *Arconaia lancelata*, *Sinanodonta woodiana*, *S. cumingii*, and *Cristaria plicata* (Cai et al. 2014). After 1998, many of these large species disappeared from the area or declined in abundance (Xiong et al. 2010). For example, *L. caveata* previously was a dominant species (>15% of the fauna), but it represented only 6.3% of individuals in our samples, and, apart from *N. douglasiae*, other large species were rare or absent. In contrast, our samples were dominated by the smaller species *A. chinensis* and *A. ovata*.

Among the mussel species we found in the snail fishery bycatch, *Cuneopsis rufescens* is the only species of conservation concern. This species is listed as vulnerable at the national and global levels by the IUCN Red List (IUCN 2022; see Table 1), and its current distribution is limited to only two locations in China: Poyang Lake and Dongting Lake (Hunan Province; see Liu et al. 1979). Proposed threats to this species include water pollution and urbanization, but bycatch mortality may represent an additional threat. *Lanceolaria grayii* is listed as "least concern" at the national level, but its conservation status has not been evaluated by the IUCN due to insufficient data; the potential impact of the snail fishery in the Poyang Lake area and elsewhere in its range should be

Species	IUCN Classification	Chinese Conservation Status	Percent	
Aculamprotula tortuosa	Not listed	Vulnerable	2.15	
Acuticosta chinensis	Least concern	Least concern	32.95	
Acuticosta ovata	Least concern	Least concern	19.85	
Anemina arcaeformis	Least concern	Least concern	0.87	
Arconaia lanceolata	Least concern	Not listed	1.16	
Cuneopsis heudei	Least concern	Least concern	0.76	
Cuneopsis pisciculus	Least concern	Least concern	0.29	
Cuneopsis rufescens	Vulnerable	Vulnerable	0.17	
Lamprotula caveata	Least concern	Not threatened	6.34	
Lamprotula leaii	Not listed	Least concern	1.86	
Lanceolaria eucylindrica	Not listed	Vulnerable	0.17	
Lanceolaria grayii	Data deficient	Least concern	0.58	
Nodularia douglasiae	Least concern	Least concern	18.51	
Schistodesmus lampreyanus	Least concern	Least concern	8.73	
Schistodesmus spinosus	Least concern	Near threatened	5.12	
Sinanodonta woodiana	Least concern	Least concern	0.41	
Sinohyriopsis cumingii	Not listed	Least concern	0.06	

Table 1. Representation of mussel species in bycatch of the snail fishery in Qinglan Lake, Jiangxi province, China (N = 1,718 individuals, aggregated from two fishing trips). IUCN classifications are from IUCN (2022), and Chinese conservation status is based on Liu et al. (2020a, 2020b).

considered in future conservation status assessments. Similarly, the impact of the snail fishery on "not listed" species (i.e., *Aculamprotula tortuosa*, *L. leaii*, *Lanceolaria eucylindrica*, *S. cumingii*) should be considered in future IUCN assessments, especially *A. tortuosa* and *L. eucylindrica*, which are listed as "vulnerable" at the national level.

Mussels harvested as bycatch have a low market value (\$3-9 per fishing trip) compared to target snail species (\$90-300 per fishing trip). The low market value may present a conservation opportunity if proper incentives are applied. Specifically, it may be feasible to provide economic compensation to local fishers in exchange for returning mussels to the lake instead of returning them to the village for sale. In Qinglan Lake, such compensation would total \$2,904 per year for the local community. Evaluating costs and benefits of, and building support for, such measures require a better understanding of the threat to mussel populations posed by bycatch mortality and the benefits of reducing this mortality. Additional sampling and development of population models are necessary to quantify the effect of bycatch mortality and how it varies among species based on body size, size at maturity, recruitment rate, and other vital statistics of the fishery. It is also necessary to quantify the value of ecosystem services and other benefits provided by healthy mussel populations in this area. The valuation of mussels in this region and other areas of the world where they are exploited should be a research priority (Strayer 2017).

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