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REGULAR ARTICLE

TRANSGENERATIONAL EFFECTS OF COPPER ON A FRESHWATER GASTROPOD, *PLANORBELLA PILSBRYI*

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ABSTRACT

Although much less common in ecotoxicology than traditional single-generation studies, multigenerational studies may offer a deeper understanding of the chronic and population-level effects caused by contaminants. To evaluate the potential utility of multigenerational contaminant studies and develop a feasible generational test design, we conducted a two-generational toxicity test using the freshwater File Ramshorn snail (*Planorbella pilsbryi*). Adults were exposed to five sublethal concentrations of copper, which resulted in significant delays in reproduction with increasing copper exposure and complete reproductive inhibition at the highest concentration (75.0 µg/L). Mortality and inhibition of reproduction were not observed in the control and three lowest concentrations (4.7, 9.4, and 18.8 µg/L Cu) over the course of the exposure and during recovery in clean water, indicating no lasting adverse effects. However, subsequent exposure of the unexposed juveniles that were produced during the recovery period (i.e. those not directly exposed to copper) showed that juveniles born to copper-exposed parents (LC50: 11.57 µg/L Cu; 95% CI: 3.71–19.43 µg/L Cu) were significantly less tolerant to copper exposure than juveniles born to unexposed parents (LC50: 29.25 µg/L Cu; 95% CI: 22.17–36.32 µg/L Cu). Despite no obvious changes in parental reproductive success, the fitness of unexposed juveniles was compromised due to parental exposure.

KEY WORDS: molluscs, multigenerational, ecotoxicology, copper, snails

INTRODUCTION

In the environment, an organism may be exposed to numerous contaminants over its lifetime. Due to the size and longevity of some organisms, as well as constraints on time and space in laboratory studies, single-generation/single-lifestage toxicity tests are far more common for ecotoxicological risk assessment (Kimberly and Salice 2015). However, it is now understood that exposure to certain stressors can have impacts extending far beyond the generation of exposed individuals. Single-generation/single-lifestage tests may not reveal chronic effects that may occur in the environment. Environmental stressors, both natural and anthropogenic, can be strong drivers for change within a population, and over time, they may trigger acclimation, cause genetic adaption, or weaken the population to the point of extinction (Lagisz and Laskowski 2008). Exposure to contaminants or stressors may lead to changes in an organism's behavior, physiology, or diet or in its interactions with the environment, which may directly alter the organism's fitness and lead to subsequent changes in the fitness of its unexposed offspring (Plautz and Salice 2013). As a result, exposure history may influence the risks posed by a contaminant to the current population. The alteration of risk due to past exposure events may intensify with increasing frequency or duration of exposure, thus limiting the predictive power of a single-generation risk assessment (Kimberly and Salice 2014).

These changes, termed multigenerational effects, are nongenotypic alterations in life history traits that may be passed on as a result of one or more of the following factors: changes in parental diet or environment; transfer of immune system factors, hormones, or organelles; phenotypic plasticity; and epigenetic effects, which are defined as heritable changes in gene function or expression in the absence of changes to the

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Table 1. Summary of a range of observed changes in offspring fitness due to parental exposure as reported in various multigenerational studies.

Species	Parental Stressor	Multigenerational Endpoint	Effect	Source
Increased offspring fitness due to parental exposure to stressor				
Biomphalaria glabrata (freshwater pulmonate snail)	Predation	Cd tolerance	Decreased	Plautz and Salice 2013
Culicoides furens (biting midge)	Metal exposure Zn, Cr, Ag, Ni, Hg, Pb, Cu, Mn, Cd	Metal tolerance	Decreased	Vedamanikam and Shazilli 2008
Lymnaea stagnalis (freshwater pulmonate snail)	Cd exposure	Cd tolerance	Increased	Reategui-Zirena et al. 2017
Protophormia terraenovae (blow fly)	Cu exposure	Innate immunity	Increased	Pölkki et al. 2012
Decreased offspring fitness due to parental exposure to stressor				
Daphnia magna (water flea)	High temperatures	Oxidative stress	Increased	Bae et al. 2016
Daphnia magna (water flea)	Cd/ high temperature	Cd/ high temperature tolerance	Decreased	Kimberly and Salice 2015
Chironomus tepperi (nonbiting midge)	Poor diet	Zn tolerance	Decreased	Colombo et al. 2014
Orizyas latipes (medaka fish)	Bisphenol-A	Reproduction/embryo mortality	Decreased/ Increased	Bhandari et al. 2015
Physa pomilia (freshwater pulmonate snail)	Cd exposure	Reproduction	Decreased	Kimberly and Salice 2014
No change in offspring fitness due to parental exposure to stressor				
Enchytraeus albidus (potworm)	Metal (Zn, Cd, Cu, Pb)	Metal tolerance (Zn, Cd, Cu, Pb)	No change	Lock and Janssen 2002

DNA sequence (Morgan et al. 2007; Plautz and Salice 2013). These changes in life history may be induced by environmental or anthropogenic stressors and can cause secondary phenotypic changes in organisms not directly exposed to the stressor (Anway et al. 2005; Skinner 2014). The resulting transgenerational impacts are variable and may have positive or negative effects on the fitness of subsequent generations depending on the parental stressor and the multigenerational endpoint used (Table 1). Continued investigation into multigeneration toxicity for a variety of chemicals, stressors, species, and endpoints will be critical in ensuring accurate risk assessment of environmental pollutants.

While research regarding the transgenerational impacts of contaminants is limited, there are even fewer transgenerational studies involving molluscs, especially gastropods (Oehlmann 2007). After arthropods, the Molluscan phylum is the second most species-rich taxonomic group, and the most species-rich Molluscan class is the gastropods (Strong 2008). Additionally, freshwater gastropods make up 80% of freshwater molluscs by species (Brown 2001; Strong 2008). Molluscs are often at higher risk of exposure to elevated concentrations of contaminants because of their sedentary lifestyle, resulting in prolonged exposure compared to more motile organisms (Gao et al. 2017). Additionally, pulmonate gastropods are known to be especially susceptible to accumulating metals from the environment relative to other aquatic organisms (Pyatt et al. 2002). Benthic invertebrates, such as gastropods, are abundant in freshwater systems and play an important role in nutrient cycling and microbial activity through the breakdown of organic matter and the mixing of surface sediments (Covich et al. 1999). Gastropods and their eggs also represent an important food source for other aquatic organisms and waterfowl, representing up to 60% of the benthic invertebrate biomass and abundance in some freshwater ecosystems (Habdija et al. 1995). Despite their considerable diversity and importance, gastropods are still underrepresented in ecotoxicological studies, and little is known about the multigenerational impacts of contaminants on gastropod populations or the possible cascading impact that transgenerational effects may have on the greater freshwater ecosystem (Lysne et al. 2008; Tallarico 2016).

The objective of this study was to evaluate possible multigenerational effects of contaminants on freshwater gastropods. We used a two-generation study design to follow the influence of a sublethal copper (Cu) pulse exposure on adult freshwater snails and the subsequent generation of unexposed offspring. Copper is a widespread waterborne contaminant that may enter aquatic systems from a range of anthropogenic sources, including mining, municipal and industrial wastewater effluents, and road runoff (CCME 1999). Copper is also a common ingredient in agricultural fertilizers and pesticides, and it can enter the water column via overland runoff or when applied directly to water bodies as an algaecide or molluscicide. In small concentrations, Cu is

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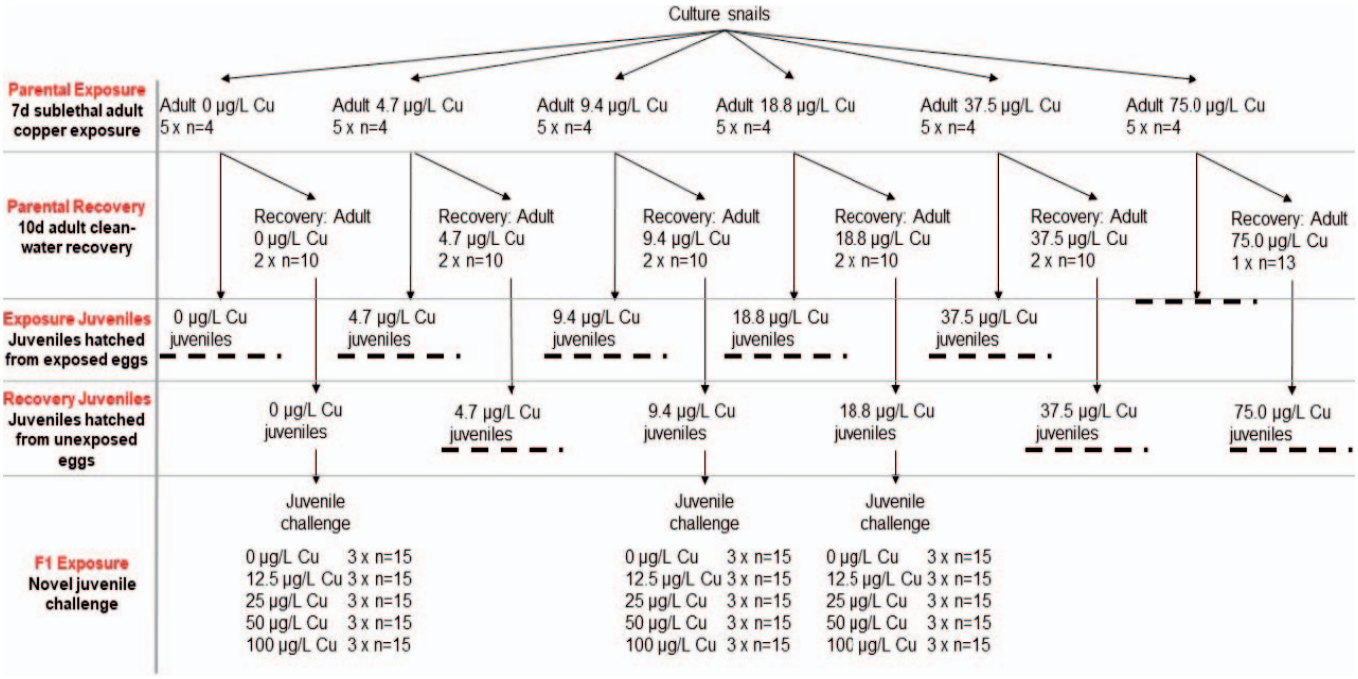

Figure 1. The multigenerational test design used in the present study involved three stages: the Parental Exposure, the Parental Recovery, and the F1 Exposure. The Parental Exposure involved an acute adult copper exposure and resulted in eggs laid during exposure. Following the exposure, adults were moved to clean water for the Parental Recovery phase, which resulted in unexposed offspring born to exposed parents. Finally, these unexposed offspring were subject to a novel juvenile challenge in the F1 Exposure to assess the sensitivity of unexposed juveniles according to their Parental Exposure levels. The dashed lines indicate the termination of a trial. Reproduction was inhibited completely at the highest concentration during the Parental Exposure resulting in no Exposure juveniles. Only juveniles produced during the Recovery stage from the control, 9.4, and 18.8 µg/L Cu treatments were used in the F1 Exposure. The other Recovery juveniles as well as all the Exposure juveniles were omitted due to lack of survivorship and logistical constraints.

essential to metabolic function including mitochondrial activity in most organisms (Gao et al. 2017), and therefore, it must be obtained from the environment. However, above normal background concentrations, copper can cause significant damage to metabolic pathways (Ng et al. 2011). Using Cu as a reference toxicant, this study evaluates whether multigenerational effects can be induced by contaminant exposure and whether fitness and contaminant sensitivity in unexposed offspring are affected by parental exposure.

METHODS

Test Organism

The freshwater gastropod used in this study, *Planorbella pilsbryi*, or File Ramshorn, inhabits lakes, ponds, and slow-moving streams throughout south-central Canada and the northern United States (Clarke 1981; Burch 1982; Johnson et al. 2013). *Planorbella pilsbryi* is pulmonate and hermaphroditic, meaning it has a modified mantle and can breathe air directly and it possesses both male and female genitalia (Clarke 1981). The generation time of *P. pilsbryi* is relatively short (typically 56–63 d), and, under ideal conditions, adults are prolific, making them an excellent model organism for multigenerational studies.

Planorbella pilsbryi were obtained from a culture held at the Canadian Centre for Inland Waters in Burlington, Ontario, Canada, for several years. The culture was maintained at room temperature in a 16:8 light-dark cycle in several large aquaria fitted with circulatory pumps and aeration. Aquaria water was changed weekly, and snails were fed continuously a diet of organic spinach in excess supplemented with dried shrimp pellets (Shrimp Pellets, Cobalt Aquatics, Rock Hill, SC, USA), algae pellets (Algae Grazers, Cobalt Aquatics, Rock Hill, SC, USA), and calcium carbonate (> 99% purity, Fisher Scientific, Ottawa, ON, Canada) for shell health. Similar-sized adults were randomly selected from culture. The shell length of subsampled snails was measured to characterize the adult snail population used in the test (mean shell length of 11.96 ± 1.04 mm [$n = 20$]). Aquaria, or culturing, water was dechlorinated Lake Ontario water and had a hardness of 126 mg $CaCO_3$, dissolved organic carbon (DOC) of 0.9 mg/L, and a pH of 8.13. Full characterization of the physicochemical properties of culture and copper exposure water is provided in the Supplemental Information (Tables S1–S4).

Test Design

A multigenerational test, summarized in Figure 1, comprises three stages: a 7-d adult sublethal Cu exposure (Parental Exposure), a 10-d recovery and egg-laying period in clean water (Parental Recovery), and a 72-h juvenile Cu

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exposure for the F1 offspring (F1 Exposure). At each stage, several endpoints were observed including: qualitative behavior and feeding; reproductive timing including oviposition periodicity and rate over time; reproductive outputs including number of egg masses and egg counts; developmental markers including egg viability and hatch time; and finally, juvenile mortality as a measure of partial-exposure influence.

Incubators were maintained at 21°C and subject to a 16:8 light-dark cycle. Test solutions were made using a concentrated stock solution of copper sulfate ($CuSO_4 \cdot 5H_2O$, Fisher Scientific) diluted with dechlorinated culture water to nominal Cu concentrations of 0, 4.7, 9.4, 18.8, 37.5, and 75.0 μg/L for the 7-d adult exposure and 0, 12.5, 25, 50, and 100 μg/L for the 72-h juvenile Cu challenge. Nominal concentrations were within ±10.8% of measured concentrations, and therefore all concentrations reported in this study are nominal (Table S4 and S5). The Parental Exposure included five replicates of each concentration and four snails per replicate. For the Parental Recovery period, the snails from each parental treatment were randomly reassigned into two replicate test vessels per exposure with 10 snails per replicate for all treatments except the highest (75.0 μg/L). In the 75.0 μg/L treatment, seven snails died during exposure, so the 13 remaining were placed in a single test vessel for the Parental Recovery. Finally, the F1 Exposure was a juvenile Cu challenge that involved three replicates per concentration with five juvenile snails per replicate. This format for the F1 Exposure was repeated for the juveniles born during the Parental Recovery phase under pristine conditions to the control, 9.4, and 18.8 μg/L Cu parental exposure groups. The 37.5 and 75.0 μg/L Cu parental exposure groups did not contain enough juveniles to include them in the F1 Exposure because all concentration exposures were simultaneous. The 4.7 μg/L Cu parental exposure group was omitted as well.

Following the initial 7-d Parental Exposure, the adult snails were removed, rinsed, and placed in new 1-L beakers with clean water according to their original exposure level. Adults were kept in the new beakers for the Parental Recovery, a 10-d recovery period during which they laid eggs under pristine conditions. Adults were then removed, and the eggs were left to develop for 3.5 wk. Both the eggs laid during the Parental Exposure in copper-contaminated water, and those laid during the Parental Recovery were rinsed after the removal of the adults and subsequently maintained in dechlorinated culture water that was changed weekly until they hatched. Resulting juveniles born from adults in the Parental Exposure and Parental Recovery were maintained in the same containers under similar conditions and fed spinach for 4 wk.

The final stage involved a juvenile challenge in which 1-mo-old F1 offspring were exposed to Cu for the first time. The F1 Exposure was limited to juveniles that were laid by adults in clean water during the Parental Recovery phase. The juvenile challenge was designed to assess whether parental Cu exposure influenced offspring sensitivity to Cu. This experiment involved a 72-h acute Cu exposure in 250-mL jars under similar conditions as the Parental Exposure. In contrast to the adult exposure, juveniles in the F1 Exposure were unfed during the exposure as 250-mL beakers were not aerated due to the short duration and lower oxygen demand of the juveniles. This exposure was designed as a factorial mortality test (Fig. 1) in which juveniles born to exposed parents in the Parental Recovery phase were subject to three identical copper toxicity tests in concentrations ranging from 0 to 100 μg/L. For example, juveniles born to parents in the Parental Exposure control treatment were randomly assigned to one of the F1 Exposure treatment levels (0–100 μg/L) and exposed for 72 h. This was done for juveniles born to parents from the control, 9.4 μg/L, and 18.8 μg/L Cu treatments. Juveniles born to the 4.7 μg/L, 37.5 μg/L, and 75.0 μg/L parental treatment groups were omitted due to logistical constraints and few surviving juveniles in the two highest Parental Exposure groups. Mortality data collected from the juvenile challenge was used to calculate an LC50 value for the juveniles born to each of the Parental Exposure groups tested.

Macrophotography Analysis

To assess the impact of copper on reproduction, several endpoints were measured using macrophotography and digital image processing. The number of egg masses, number of eggs, number of eggs per mass, viability, time to oviposition, and time to hatch were quantified by taking photographs of masses at regular intervals throughout development using an Apple iPhone® 6S (Apple Inc., Cupertino, CA, USA) equipped with an Ollo® Clip macro lens (olloclip; Foothill Ranch, CA, USA). Images were analyzed using ImageJ to count and track output and development (Schneider et al. 2012). Photographs and subsequent measurements were recorded for all eggs produced during both Parental Exposure and Recovery trials across all Cu treatments.

Copper Analysis

Exposure solution samples were taken at the start of the Parental Exposure and pooled across replicates and concentration. A subset of samples was taken at the end of the Parental Exposure to characterize any change in Cu concentration (Table S4). Samples were acidified with HNO_3 (Reagent Grade, Fischer Scientific) to approximately a pH of 1, filtered to 0.45 μm using a syringe-tip filter, and stored at 4°C until analyzed. Concentrations of Cu in water samples were measured using inductively coupled plasma mass spectrometry at Environment and Climate Change Canada's National Laboratory for Environmental Testing (ECCC NLET) using methods developed by the ECCC NLET (Environment Canada 2014). The limit of detection for Cu was 0.02 μg/L.

Statistical Analysis

SigmaPlot v14.0 (Systat Software, San Jose, CA, USA) was used to analyze data for distribution, outliers, and

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significant differences between treatments and to generate figures.

Adult survival and qualitative behavior.—Adult survival over time was recorded as well as observations on behavior and feeding during the Parental Exposure and Parental Recovery.

Reproductive timing, output, and quality.—Reproductive endpoints (time to oviposition, oviposition rate over time, number of egg masses, number of eggs, number of eggs/mass, egg viability, and time to hatch) were measured for eggs laid during the Parental Exposure and Recovery. Due to the random reassignment of adults into fewer replicate test vessels for the Recovery, the endpoints for both the Parental Exposure and Recovery trials cannot be compared statistically. For the Parental Exposure endpoints, a one-way ANOVA ($\alpha = 0.05$, d.f. = 5) was conducted to compare endpoints among Cu treatments. When the assumptions for a one-way ANOVA could not be met (normality and homoscedasticity), a one-way ANOVA on ranks (Kruskal-Wallis) was conducted instead ($\alpha = 0.05$, d.f. = 5). Where significant differences between treatments were found, a Dunnett's post-hoc test was used to compare treatments to the control treatment ($\alpha = 0.05$). For the Parental Recovery, there were insufficient replicates to perform statistical analyses (0–37.5 μg/L d.f. = 2; 75.0 μg/L d.f. = 1).

The time to oviposition was measured by recording date of oviposition for each egg mass across all replicates and treatments. To incorporate the replicates in which no egg masses were laid throughout the test, time to oviposition was measured as the delay in oviposition by percent of the duration of the test. For example, replicates in which no egg masses were laid received a value of 100% for time to oviposition as no egg masses were laid for the entire duration of the test. Mean time to oviposition as percent test duration was calculated for all egg masses and replicates.

For the purpose of this test, nonviable eggs were defined as those that showed no sign of development after 10 d. In viable eggs, early signs of development were observed between 3 and 5 d. Typical hatching time for eggs laid by unstressed adults at room temperature (~22°C) is approximately 12 d, and no development after 10 d suggests that hatching success is unlikely. Figure S1 illustrates the development of a viable and a nonviable egg.

Juvenile survival and copper sensitivity.—Juvenile sensitivity was assessed in RStudio v.1.1.456 (Ritz et al. 2015; RStudio Team 2016) using the drc library to generate dose–response curves and to calculate the LC50s and 95% confidence intervals for juveniles born to each Parental Exposure grouping (0, 9.4, and 18.8 μg/L Cu). Sample code used in RStudio is provided in the Supplemental Information (Table S6).

RESULTS

Adult Survival and Qualitative Behavior

Parental exposure survival and behavior.—Adult survival was 100% in each of the 0, 4.7, 9.4, 18.8, and 37.5 μg/L treatments and 65% in the 75.0 μg/L treatment (13/20 individuals survived).

Within minutes of the initiation of the sublethal adult exposure, all individuals within the 75.0 μg/L Cu treatment were rendered immobile as well as most individuals in the 37.5 μg/L exposure. These findings are in contrast with those seen in the individuals exposed to lower concentrations, which were active and began eating immediately. Immobility was observed only in the three highest treatment groups and in the first 24 h was 40%, 65%, and 75% for the 18.8, 37.5, and 75.0 μg/L Cu treatments, respectively. Upon completion of the 7-d exposure, all individuals in the 18.8 μg/L Cu treatment, and 95% of those in the 37.5 μg/L Cu treatment, had recovered and had begun eating and laying eggs. None of the individuals in the highest treatment showed any sign of recovery throughout the 7-d exposure. To determine survival in the highest treatment after the 7-d exposure, the retracted foot of each snail was touched with a probe to gauge response. Of the 20 individuals in the 75.0 μg/L Cu treatment, seven individuals were unresponsive and were removed from the study.

Parental recovery survival and behavior.—All individuals that survived the 7-d exposure also survived the 10-d recovery period. In the first 24 h of the recovery period, all snails had resumed eating and oviposition.

F1 exposure survival.—Survivorship between eggs laid during the Parental Recovery and successfully hatched juveniles was not significantly different between exposures, resulting in a mean hatching success rate of 90.6% after 3 wk across all exposures. The highest hatching success occurred in the 75.0 μg/L Cu parental treatment group with 93.6% success, and the lowest hatching success was 88.1% in the 9.4 μg/L Cu parental treatment level. As juveniles laid during the Parental Exposure were not used in the F1 Exposure, the hatchability and survivorship for that cohort was not assessed.

Reproductive Timing

Parental exposure time to oviposition.—An increasing delay in oviposition was observed with increasing copper concentration, likely a consequence of immobility. Delay in oviposition was measured as the number of days to the first oviposition from the beginning of the exposure. No delay in oviposition in the control treatment was observed, but a 2-d, 4-d, and 7-d delay in oviposition was observed in the 4.7–18.8, 37.5, and 75.0 μg/L Cu treatments, respectively (Fig. 2). The 7-d delay observed in the 75.0 μg/L Cu treatment resulted in complete inhibition of oviposition throughout the 7-d exposure (Fig. 2).

Time to oviposition was measured as the percent delay of the total 7-d adult Cu exposure time to account for replicates with no egg masses. In general, mean time to oviposition increased slightly with increasing Cu exposure concentration for eggs laid during the parental exposure (Fig. 2). No egg masses were laid in any of the replicates of the highest

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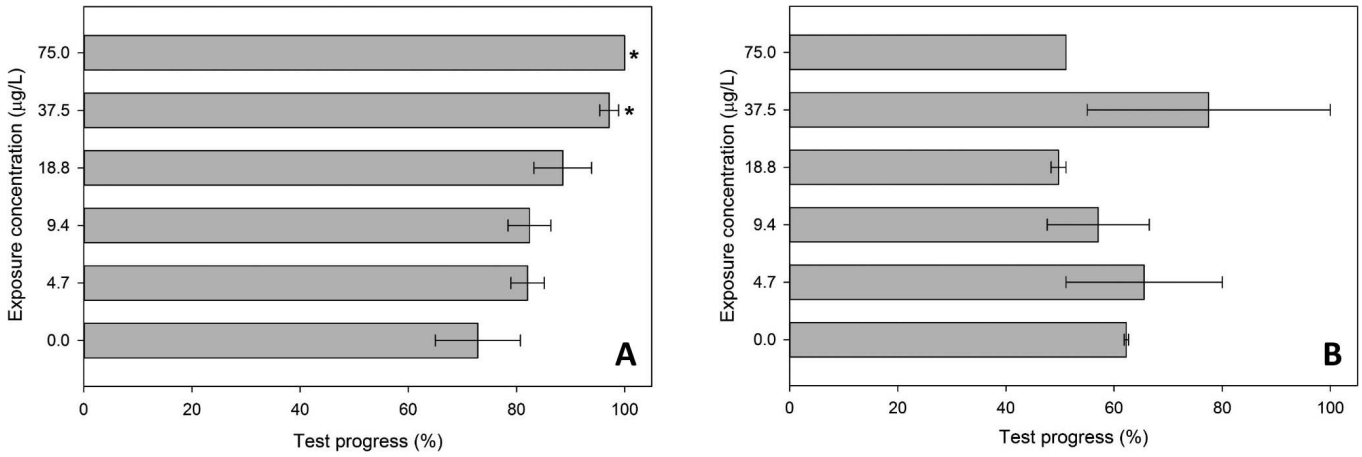

Figure 2. Mean (S1: $n = 4$, S2: $n = 2$) delay in oviposition during the sublethal Parental Exposure (A) and Parental Recovery (B) phases measured as the delay in the percent of the test's progress. Bars represent the mean time along the test progress at which oviposition occurred. Due to complete inhibition of oviposition at the highest treatment level, delay in test progress was used to be able to include these data in our analysis. Since no egg masses were laid in the Parental Exposure 75.0 µg/L (A) treatment, oviposition was delayed by 100% of the test duration. During the Parental Exposure (A), delays in oviposition increase with increasing exposure and statistically significant delays in oviposition were observed at 37.5 µg/L ($P = 0.037$) and 75.0 µg/L ($P = 0.008$). Asterisks indicate statistically significant differences from the control treatment group (Kruskal-Wallis one-way ANOVA on ranks and Dunnett's post-hoc: $\alpha = 0.05$, $H = 17.83$, d.f. = 5).

treatment (75.0 µg/L Cu), and two replicates in the 18.8 and 37.5 µg/L Cu treatments had no egg masses. Time to oviposition was significantly delayed in the 37.5 and 75.0 µg/L Cu treatments relative to the control ($H = 17.83$; $P = 0.037$ and $P = 0.008$, respectively).

Parental recovery time to oviposition.—Within the first 24 h of the recovery phase, snails in all treatments were laying eggs. In contrast to time to oviposition during the Parental Exposure, there was a slight negative correlation between exposure concentration and mean time to oviposition in clean water during the Parental Recovery (Fig. 2). No obvious delays in oviposition occurred, although a slight delay was noted in the 37.5 µg/L Cu treatment, likely due to the influence of a single replicate with no egg masses. Interestingly, after a complete inhibition of egg laying during the Parental Exposure, adults from the 75.0 µg/L Cu treatment had a slightly lower delay in oviposition than the control treatment during the 10-d recovery period (Fig. 2), which could suggest that snails in the highest treatment accelerated output in comparison to the controls once removed from the Cu exposure.

Reproductive Output

Parental exposure reproductive output.—There were no significant differences in the number of egg masses laid between treatments during Cu exposure (Fig. 3). A slight increase in oviposition in the 4.7 µg/L Cu treatment was observed and mean oviposition tended to decrease with increasing Cu concentration (Fig. 3). Complete inhibition of oviposition was observed in the 75.0 µg/L Cu treatment and, although not significant, was very close to significance ($H = 14.033$; $P = 0.055$) (Fig. 3).

The number of eggs laid per snail was considerably more variable than egg masses per snail, and poor correlation was observed with eggs/snail and Cu exposure concentration (Fig. 3). However, a noticeable decrease in egg production per snail was observed in the 37.5 and 75.0 µg/L treatments, with a 63% and 100% reduction in egg production, respectively (Fig. 3).

Within each egg mass, individual eggs were counted and measured (Fig. 3). As no eggs were produced in the 75.0 µg/L treatment, it was omitted from this analysis. Although no statistically significant differences were observed, there was a trend of increasing eggs per egg mass with increasing copper concentration up to the 18.8 µg/L Cu treatment (Fig. 3). Interestingly, despite a reduction in mean egg masses per snail, the 18.8 µg/L Cu had the highest number of eggs/egg mass or the largest egg masses.

Parental recovery reproductive output.—There was no apparent trend in the number of egg masses laid per snail during the recovery, although treatments with higher outputs tended to coincide with faster mean oviposition (Fig. 3). The two treatments with the slowest mean oviposition were 4.7 and 37.5 µg/L Cu, and they also had the fewest mean egg masses/snail (Fig. 3). Similarly, the 9.4 and 18.8 µg/L Cu treatments had the fastest mean time to oviposition and ultimately resulted in the highest mean number of egg masses produced per snail (Figs. 2, 3). However, despite having faster time to oviposition than the control, the 75.0 µg/L Cu treatment resulted in 57% fewer egg masses produced per snail, indicating that gastropods did not compensate for previous reproductive inhibition during exposure by increasing oviposition during the recovery phase (Figs. 2, 3). The same trends observed for egg masses per snail also applied for the number of eggs laid per snail (Fig. 3).

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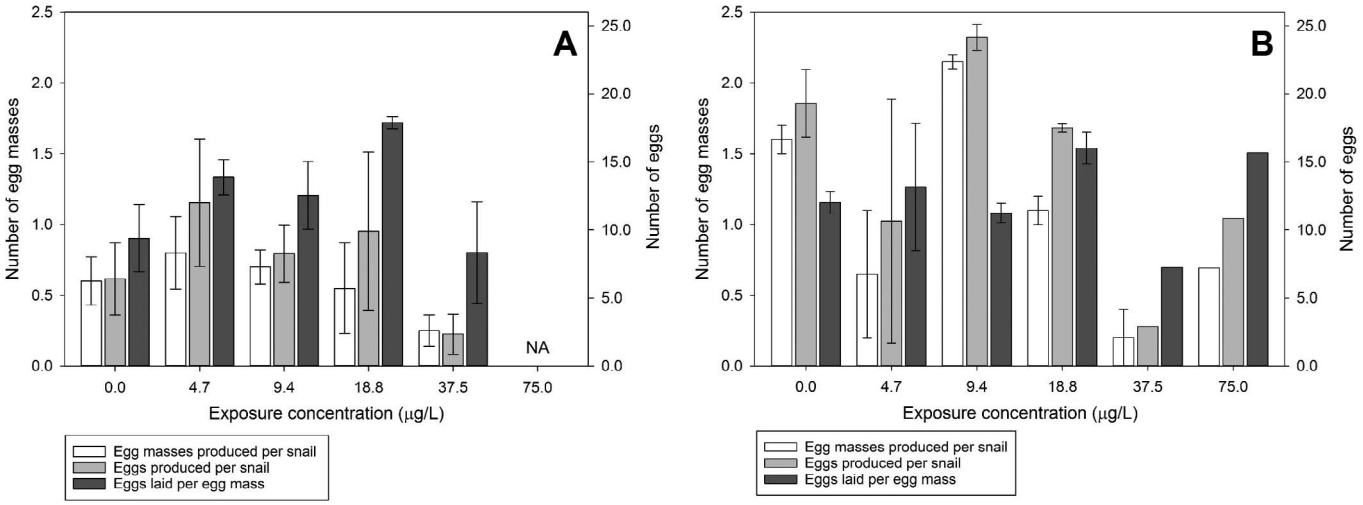

Figure 3. Three measurements of reproductive output for adults during the Parental Exposure (A) and Parental Recovery (B) phases. Mean (Exposure: $n = 4$, Recovery: $n = 2$) number of egg masses laid per snail (white bars) and mean number of eggs laid per snail (gray bars) decreased with increasing Cu exposure in the Parental Exposure with no egg masses or eggs laid at 75.0 μg/L (A) but was not strongly affected once adults recovered (B). The mean (Exposure: $n = 4$, Recovery: $n = 2$) number of eggs laid per egg mass (black bars) was not calculated for the Exposure 75.0 μg/L treatment (A) as no egg masses were laid. In both the Exposure and Recovery reproduction tests, number of eggs per egg mass was not strongly affected by copper exposure. Error bars indicate the standard error of the mean and could not be calculated for the Recovery 37.5 and 75.0 μg/L treatments (B) due to no egg production in some replicates.

The mean number of eggs per mass did not correlate with time to oviposition, unlike the number of egg masses and eggs per snail (Fig. 3). Although a trend was not evident, we observed that the highest treatment (75.0 μg/L Cu) had the highest egg-per-egg-mass ratio (largest egg masses) despite decreased egg production relative to the control and lower treatment groups.

Reproductive Output Quality

Parental exposure viability and time to hatch.—For egg masses laid during the Parental Exposure period, parental exposure, gamete exposure within the parents, and exposure of the egg masses themselves (in ovo exposure) are factors in the health of the F1 juveniles. As the eggs were laid throughout the parental exposure, duration of in ovo Cu exposure depended on time of oviposition and was typically longer at lower treatments due to inhibition of reproduction and delay in oviposition caused at the higher treatments. The maximum in ovo exposure duration for each treatment (Fig. S2) was 4, 5, 4, 1 and 0 d, respectively (Table S5). Mean exposure duration of egg masses was 2 d for the three lower treatments, 1 d for the 37.5 μg/L Cu treatment, and 0 d for the 75.0 μg/L Cu treatment (as no egg masses were laid).

Percent viability remained relatively consistent across all treatments, with greatest variability observed in the control treatment (71.2% ± 18.1) (Fig. 4). The lower mean and greater variability in egg viability in the control treatment are due to there being one replicate with only one egg mass that contained one nonviable egg, resulting in a value of 0% viability for that entire replicate. This anomalous replicate is considered a significant outlier (>3·IQR), and when omitted, the mean percent viability of the control treatment is 89.0% ± 4.1, consistent with the other treatment groups. Time to hatch was calculated for each egg mass as the difference in days between oviposition date and hatch date. No significant differences were observed in time to hatch among the treatments (Fig. 4).

Parental recovery viability and time to hatch.—As the egg masses were laid in clean water during the Parental Recovery phase, parental exposure was the only factor as in ovo exposure did not occur. Viability remained consistent, regardless of parental exposure level for eggs laid during the adult recovery period (Fig. 4).

Time to hatch was relatively consistent and demonstrated no obvious influence of parental Cu exposure level on time to hatch (Fig. S4). However, a slight decrease in time to hatch was observed in the highest concentration (75.0 μg/L Cu) (Fig. 4).

F1 exposure juvenile Cu sensitivity.—Juveniles born to the parental control group, the parental 9.4 μg/L Cu treatment, and the parental 18.8 μg/L Cu treatment were randomly selected and subjected to the same range of Cu concentrations for the F1 Exposure. From these tests, a dose–response curve was created, and LC50s were calculated for juvenile snails based on their parental exposure level. Juvenile Cu sensitivity increased with greater parental exposure. The juvenile LC50 of the highest-tested parental exposure group (18.8 μg/L Cu) was significantly lower (11.57 μg/L Cu; 95% CI: 3.71–19.43 μg/L Cu) than that of the parental control group (29.25 μg/L Cu; 95% CI: 22.17–36.32 μg/L Cu), demonstrating a significant increase in juvenile sensitivity caused by increased parental exposure to Cu (Table 2).

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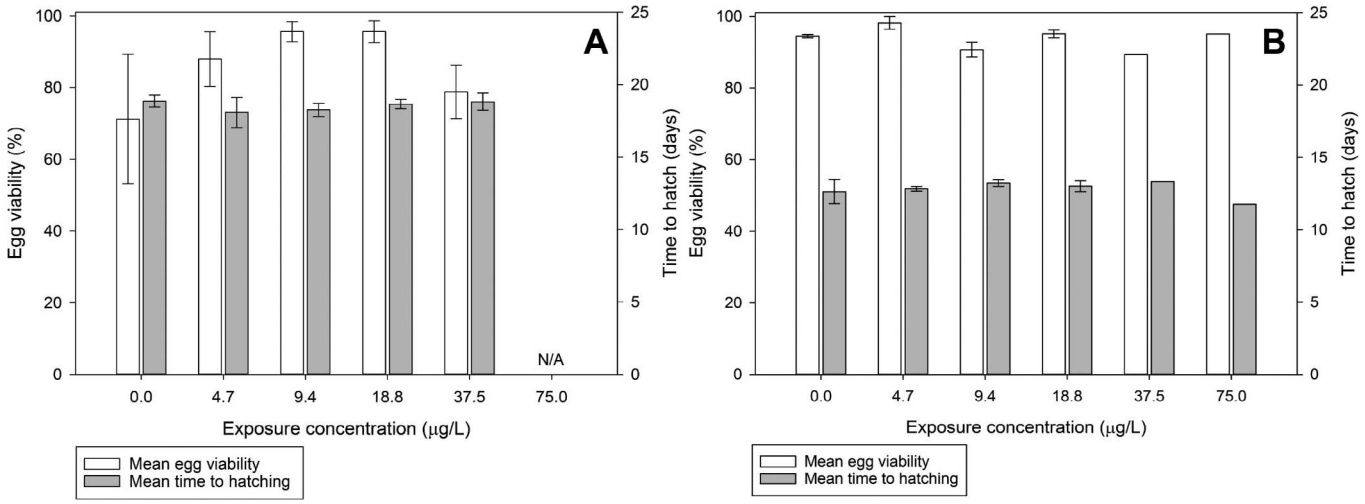

Figure 4. Two measures of reproductive success for juveniles born during the Parental Exposure (A) and Parental Recovery (B) phases. Mean (Exposure: $n = 4$, Recovery: $n = 2$) percent viability of eggs (white bars) was close to 90% across all treatments and was unaffected by the combination of parental exposure and in ovo exposure (A) as well as parental exposure alone (B). Egg viability for this study is defined in Figure S1. Egg viability could not be calculated for the Exposure 75.0 μg/L treatment as no egg masses or eggs were laid. Mean (Exposure: $n = 4$, Recovery: $n = 2$) time to hatch (gray bars) also was unaffected by in ovo and parental Cu exposure for eggs laid during both the Parental Exposure (A) and Parental Recovery (B) phases. Error bars indicate standard error of the mean and could not be calculated for the Recovery 37.5 and 75.0 μg/L treatments (B) due to no egg production in some replicates.

DISCUSSION

Adult Survival and Qualitative Behavior

Despite the inhibition of mobility, feeding, and reproduction in the adults exposed to copper concentrations over 9.4 μg/L, all exposed individuals quickly recovered once removed from Cu exposure, and the initial exposure concentration had no influence on recovery time. While Cu is an essential element at very low concentrations, even slight elevations above background Cu levels can have significant adverse effects on freshwater gastropods (Gao et al. 2017). For example, in *Lymnaea stagnalis* juveniles, exposure to increasing concentrations of Cu for 96 h was proportional to decreases in Na and Ca concentrations in the soft tissues, which was hypothesized to cause adverse effects on the nervous and muscular systems (Ng et al. 2011). This mechanism is consistent with the observed inhibition of mobility, feeding, reproduction, and eventually death seen in the Parental Exposure phase of our study.

Table 2. Estimated LC50 values, standard error, and 95% confidence intervals for the F1 Exposure. Juveniles born during the Parental Recovery to Cu-exposed parents had increased sensitivity to Cu with increasing parental exposure. Parental treatment level is indicated in the first column. As the confidence intervals of the LC50 for juveniles born to the 18.8 μg/L treatment and the control treatment do not overlap, the 18.8 μg/L juveniles are considered to be significantly more sensitive to Cu.

Parental Exposure Level (μg/L Cu)	LC50 (μg/L Cu)	Standard Error	95% CI Lower Boundary	95% CI Upper Boundary
0.0	29.25	3.609	22.17	36.32
9.4	26.65	2.494	21.77	31.55
18.8	11.57	4.011	3.710	19.43

Dissolved organic carbon (DOC) and other sources of organic material can bind to aqueous Cu, reduce its bioavailability, and ameliorate toxicity (Allen et al. 1980; Erickson et al. 1996; Schwartz et al. 2004). Increasing levels of DOC in laboratory exposures have been shown to reduce Cu toxicity to molluscs (Gillis et al. 2008, 2010; Wang et al. 2009). In the Parental Exposure, all replicates were fed identical quantities of spinach and contained the same number of snails, in an effort to limit variation in organic matter and thus variation in bioavailable Cu. The possible sources of organic material included the test organisms, spinach feed, egg masses laid during the test, and feces. However, due to the differing responses to Cu toxicity (inhibition of reproduction and feeding), the organic matter produced and present in each vessel also changed throughout the test. For example, in lower treatments, reproduction was not inhibited, and a number of egg masses were produced which could act as a possible Cu sink. In contrast, reproduction was inhibited in higher concentrations, meaning that there were no egg masses to act as a potential Cu-binding sink. Due to feeding inhibition in higher Cu treatments, uneaten spinach would potentially bind Cu, in contrast to the lower treatments in which snails consumed most of the spinach. Despite possible variations in organic matter present in each vessel and over time, a clear concentration-response relationship was observed for the inhibition of reproduction and feeding. Increasing Cu was bioavailable in proportion to the exposure concentrations and not likely ameliorated in any one treatment due to increased feeding or reproduction.

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Reproductive Timing

Exposure to sublethal concentrations of Cu for 7 d caused significant delays in oviposition and inhibition of reproduction with increasing concentration (Figs. 2, 3). However, once Cu exposure was terminated, these effects dissipated, and oviposition returned to levels within the control range. Similar behavioral responses were seen by Gao et al. (2017) with exposure of the freshwater pulmonate snail *Physella acuta* to Cu. Exposure to 40 μg Cu/L resulted in inhibition of movement and reproduction in the first 24 h of exposure, and complete inhibition was observed at 80 μg Cu/L in water with a hardness of 84.8 mg/L as $CaCO_3$ (Gao et al. 2017).

In the Parental Recovery, snails in the highest treatment (75.0 μg/L Cu) were the first to begin laying egg masses, possibly indicating an overcompensation mechanism after the week-long inhibition of mobility and reproduction. The same species (*P. pilsbryi*) demonstrated recovery with overcompensation in reproduction when exposed to sublethal concentrations of the surfactant MON 0818, resulting in significantly higher egg production in treatment groups exposed to the highest concentrations compared with those that were not exposed (Prosser et al. 2017). This response is similar to what was observed in the current study with exposure to sublethal concentrations of Cu resulting in faster oviposition (Fig. 2; 11% faster in 75.0 μg/L treatment compared with control treatment during the recovery phase) and more eggs produced per mass (Fig. 3; 30% more eggs/mass than control). However, we observed that sublethal Cu exposure (>18.8 μg/L Cu) caused reduced egg production compared with the control even after recovery (Figs. 3, 4; 57% fewer egg masses/snail and 44% fewer eggs/snail).

Additionally, there is possible evidence for an energetic trade-off between number of egg masses laid and the number of eggs per mass. During the Parental Exposure, the minimum egg mass size (measured as number of eggs/mass) increased with increasing Cu concentration until the highest two treatments, in which a significant decrease in egg mass production was observed. For Parental Recovery egg masses, this trend was less pronounced, but elevated egg-to-mass ratios were observed in the highest treatment, despite relatively lower reproductive output compared with control and lower exposures. It is possible that, rather than laying more egg masses (which would require more laying events), individuals instead invested energy toward increasing egg numbers laid during each event to maximize offspring survival under Cu-contaminated conditions. It is widely accepted that body size is the primary driver of reproductive output, with larger individuals tending to produce more eggs (DeWitt 1954; Norton and Bronson 2006). In the closely related freshwater pulmonate snail *Helisoma trivolis*, Norton and Bronson (2006) observed that while there was a significant positive relationship between body size and egg production, body size accounted for only 24% of the variability in eggs laid per mass. The clear growth-reproduction trade-off described by Norton and Bronson (2006) is further supported by Koene and Maat's (2004) calculation of energy use in growth and reproduction in *Lymnaea stagnalis*. Snails reared in isolation had a mean dry weight 11.3% higher than those raised in groups that were actively reproducing, and 9.5% of total energy intake by group-reared animals was allotted to egg production, accounting for observed differences in final average dry weight between the two test groups (Koene and Maat 2004). Additionally, Ng et al. (2011) suggested that the lack of growth in *L. stagnalis* exposed to sublethal concentrations of Cu was due to energy use for detoxification rather than growth. These studies demonstrate that snails in stressful conditions employ energetic trade-offs relating to reproduction and toxicity. It is possible that the variability in egg mass production observed in the current study is controlled by factors related to resource availability rather than body size alone, and that under stressed conditions, resources may be allocated to survival and detoxification rather than reproduction or growth.

Reproductive Output and Quality

Although reproductive output quantity was affected by exposure to Cu both during exposure and in recovery, the viability of eggs laid during both the Parental Exposure and Parental Recovery phases was unaffected by copper concentration (Fig. 4). During the Parental Exposure stage, some eggs also experienced in ovo exposure for a maximum of 5 d, depending on date of oviposition. For example, eggs laid toward the beginning of the Parental Exposure remained in the exposure solutions with the parents for the remainder of the 7-d test. However, viability remained consistently close to 90% for all treatments, with the exception of one anomalous egg mass in the control treatment of the Parental Exposure (Fig. 4). All eggs laid during Parental Recovery were unexposed and influenced only by the treatment of the Parental Exposure. Viability of eggs was constant among all treatment groups whether the eggs were laid during Cu exposure or recovery.

As a measure of developmental delays, time to hatch also appeared to be unaffected by both parental exposure and in ovo exposure, remaining constant across all treatment for eggs laid during both the Parental Exposure and Recovery (Fig. 4). This suggests that for sublethal Cu exposure, if egg masses are successfully laid, there are minimal effects on the hatching success of the embryo due to parental exposure and in ovo exposure. In contrast, Cd exposure in another pulmonate, *L. stagnalis*, resulted in decreased viability and increased time to hatch under exposure and recovery conditions (Reategui-Zirena et al. 2017). Similarly, both parental exposure and in ovo exposure to environmentally relevant concentrations of Cd in *Physa pomilia* influenced offspring tolerance to Cd with in ovo exposure being a stronger driver of change in offspring tolerance (Plautz and Salice 2013). This difference in response to Cd could be influenced by a number of factors, including variability in the sensitivity of the two species or variability in the protection offered by the gelatinous matrix of the egg mass between species and metals (Pechenik 1979; Przeslawski 2004).

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Although time to hatch was relatively consistent within each test stage, hatch time varied slightly for eggs laid during the Parental Exposure and those laid during Parental Recovery (Fig. 4). Mean hatch time across all treatments for the Parental Exposure was 18.3 ± 1.4 d, compared with 12.9 ± 0.6 d for Parental Recovery. While temperature and light regime were the same for both Parental Exposure and Parental Recovery, the two experiments were conducted in different incubators. However, no direct comparisons between hatch time or other endpoints were made between eggs laid in the Parental Exposure and those laid during the Parental Recovery.

Juvenile Survival and Copper Sensitivity

Results from the parental exposure to Cu and recovery alone suggest that waterborne Cu exposure in lab-exposed pulmonate snails does not induce lasting effects on assessed endpoints, if a population survives initial exposure. Survival, feeding, behavioral, and reproductive endpoints measured during the Parental Exposure clearly demonstrate a dose–response relationship with increasing Cu exposure causing increasing adverse effects. These endpoints returned to control levels during the Parental Recovery, and the quality of reproductive output (viability and time to hatch) was unaffected by both parental exposure and in ovo exposure.

Additionally, parental treatment level appears to have minimal impact on hatching success and early juvenile survivorship in the generation laid during the Parental Recovery period. However, the subsequent juvenile Cu challenge clearly demonstrates a reduction in Cu tolerance due to parental Cu exposure (Fig. S3). Copper sensitivity of juveniles raised under pristine conditions increased with increasing parental exposure and was significantly higher in juveniles born to the highest-tested parental treatment group compared with juveniles born to unexposed control parents (Table 2).

While in ovo exposure of the egg masses laid during the Parental Recovery did not occur, it is possible that the gametes experienced copper exposure during the sublethal Parental Exposure before being laid in clean water during the Recovery. Pulmonate snails, including our test species, receive sperm and may store it for long periods of time, with the potential for both eggs and sperm to be exposed to a contaminant within the parent (Norton and Bronson 2006). While fertilization is internal, to the best of our knowledge, egg masses are deposited fairly quickly after fertilization, given that in several developmental studies involving pulmonates, the earliest stages of development can be seen occurring outside of the parent (Brown 2001; Khangarot and Das 2010; Bandow and Weltje 2012). As such, we conclude that negligible in ovo exposure occurs in the developing embryo except when the embryo itself is exposed to a contaminant.

Although the juveniles used in the F1 Exposure likely experienced negligible in ovo exposure before being laid during the Parental Recovery, their gametes were potentially exposed to Cu within the parents during the sublethal exposure. However, since egg viability and time to hatch were unaffected by the Parental Exposure concentration, we can conclude that any internal copper exposure did not prevent the gametes from successfully fertilizing and developing into a viable offspring. This suggests that the significant difference in juvenile Cu sensitivity may be attributed to either latent influence of gamete exposure, indirect effects caused by parental exposure, or a combination of both. Additional studies are needed to discern the possible influence of each of these exposure routes on juvenile fitness and sensitivity, including the use of a depuration period between exposure and recovery to minimize the influence of gamete exposure as well as further toxicity testing using embryos to allow us to better account for effects due to in ovo exposure.

Stream water quality monitoring data collected by the Ontario Provincial Water Quality Monitoring Network (PWQMN) throughout 2016 demonstrate that Cu is ubiquitous in the environment and that concentrations can reach or exceed the reproductive and multigenerational effect concentrations produced in the present study (Ontario Ministry of the Environment 2016). As this is not an exhaustive evaluation of Cu in the environment, it is important to note other modifying factors that can affect bioavailability and thus the toxicity of Cu in natural aquatic systems. For example, Gillis et al. (2010) reported that Cu EC50s were up to three-fold higher when freshwater mussel glochidia were exposed in Cu-augmented natural water compared to Cu-augmented reconstituted laboratory water. While we cannot comment on how frequently Cu concentrations in the environment may exceed the multigenerational effect concentrations reported here without accounting for possible modifying factors, our study demonstrates that even one pulse event at potentially environmentally relevant concentrations can cause significant detriment to the next generation.

Multigenerational Ecotoxicological Studies

Both historical exposure as well as developmental exposure to environmental stressors can cause latent or transgenerational effects later in life or even in future generations (Salice et al. 2010; Kimberly and Salice 2014). Multigenerational studies of these effects are much less common in ecotoxicology than the standard single-generation and single-lifestage tests. One major reason for the lack of multigenerational research is that studies spanning multiple generations can involve substantial logistical challenges especially for larger or long-lived organisms. However, our study, among other recent investigations, demonstrates that transgenerational effects caused by previous contaminant exposure may pose additional risks to future generations of offspring, a reality that adds complexity to environmental risk assessment. There is evidence that gastropods are prone to experiencing transgenerational effects as seen in Cd exposure of *Physella pomilia* and *Biomphalaria glabrata*, as well as increased multigenerational Cu burden in *Pomacea paludosa*

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(Rogevich et al. 2009; Salice 2010; Kimberly and Salice 2014).

Although a number of mechanisms are commonly associated with inducing transgenerational effects, DNA methylation is the most well-studied mechanism and considered to be the most important (Bombail et al. 2004; Vandegehuchte and Janssen 2011). Exposure of adult *P. pomilia* to sublethal concentrations of the pharmaceutical prednisolone resulted in reduced fecundity and increased juvenile developmental abnormalities and mortality of the F1 with increasing exposure concentration and duration (Bal et al. 2017). With subsequent prednisolone exposure in the F2 generation, developmental abnormalities occurred at lower concentrations than in the F1 (Bal et al. 2017). Additionally, this study demonstrated the presence of a relationship between DNA methylation and multigenerational effects, with DNA methylation decreasing with increasing prednisolone concentration in the F1 (Bal et al. 2017). Furthermore, metals such as cadmium have been shown to induce DNA methylation in terrestrial snails, although minimal literature exists linking DNA methylation caused by metal exposure with multigenerational effects (Nica et al. 2017).

While it is possible that the multigenerational effects we observed could be evidence of epigenetics, it is important to note that some authors suggest continuation of induced changes must be observed in the F3 to confirm the role of DNA methylation or epigenetics (Youngson and Whitelaw 2008). We believe that it is more likely that the primary driver of the observed multigenerational effect in our study is related to energetic trade-offs between survival and reproduction under stressed conditions in the parental generation. Gastropods have detoxification, resistance, and repair mechanisms to improve chances of survival under contaminant stress, including the use of metallothioneins (Dallinger and Berger 1993). There is some evidence to suggest that these mechanisms in invertebrates have an associated energetic cost that may impede growth and reproduction to prioritize survival (Walker et al. 2012). Production of gametes, especially eggs, is also an energetically expensive process, and the encapsulation of eggs in a gelatinous matrix has been shown to have considerable energetic costs in gastropods (Stickle 1973). It is possible that under extreme contaminant stress, less energy is allocated to reproduction to improve the individual's chance of survival. Additionally, the cost of producing the gelatinous egg mass may contribute to the trend of increasing number of eggs per egg mass seen during the Parental Exposure at the three lower treatments, which were not experiencing significant reductions in total oviposition due to Cu exposure.

Additionally, it is important to note that there is a potential influence of latent effects due to gamete exposure within the exposed parents in multigenerational studies like this one. However, the potential routes of exposure, both direct and indirect, that were modeled in this study reflect the potential multigenerational hazard posed by certain contaminants, such as copper, in the environment. In a real pulse scenario, Cu concentrations easily may reach levels capable of inducing severe parental effects but not mortality, as seen in our study. While we are not able to distinguish between the direct effect of gamete exposure in the parents and the indirect effects due to parental exposure, both would be present in an actual environmental exposure and, in light of our results, could still potentially cause serious latent changes to the sensitivity of the F1 generation, which ultimately could affect the survival of the entire population in the event of a subsequent exposure.

In conclusion, sublethal Cu exposure to adult *P. pilsbryi* caused significant reduction in reproductive output but did not affect the hatching success of the egg masses that were laid. However, a subsequent novel exposure of juveniles reared under pristine conditions but born to Cu-exposed parents demonstrated that parental Cu exposure decreased juvenile tolerance. A clear dose–response relationship was observed with increasing parental exposure causing significant decreases in juvenile Cu tolerance later in life. Contaminants, in this case Cu, can induce latent effects in freshwater gastropods that manifest a generation after exposure has ended. Multigenerational studies such as this one, reveal the added complexity of the transgenerational risks of contaminants in a population that traditional single-lifestage toxicity tests do not capture. As transgenerational effects may significantly alter the tolerance of subsequent generations to future stressors, multigenerational studies have important implications for accurate and protective risk assessment. Given the abundance and importance of gastropods in freshwater ecosystems and food webs, chronic population-level transgenerational effects in gastropods may have larger impacts on the overall health of the ecosystems they inhabit (Bal et al. 2017). This study demonstrates that sublethal pulse exposures to contaminants such as Cu can induce effects that are not evident in the exposed generation, once recovered, but that continue to negatively impact the health and success of a subsequent unexposed generation and should therefore be considered for their potential to cause additional population-level risk.

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