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REGULAR ARTICLE

LIFE STAGE SENSITIVITY OF A FRESHWATER SNAIL TO HERBICIDES USED IN INVASIVE AQUATIC WEED CONTROL

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ABSTRACT

Invasive aquatic plants like hydrilla (*Hydrilla verticillata*) threaten native species in many ways, ultimately degrading overall habitat quality and quantity. Aquatic herbicides are often chosen as a control and management strategy, but few peer-reviewed studies address their effects on non-target organisms, especially native freshwater mussels and snails. The aim of this study was to assess the life stage sensitivity of a rare snail, *Somatogyrus virginicus* (Lithoglyphidae), to two aquatic herbicides (dipotassium salt of endothall and fluridone). We collected adult snails, cultured their eggs on a vinyl card substrate, exposed adults and eggs in 96-h static-renewal experiments, and monitored eggs through hatching. Because fluridone is typically applied for $\geq$ 60 d, an additional treatment was exposed in static-renewal through hatching (30 d total) to improve environmental relevance. Eggs present on the shells of adult snails were also monitored. Endpoints were adult survival and egg hatching success. Fluridone did not affect adult snail survival at concentrations up to 1500 µg/L, and in the test with eggs on vinyl cards, fluridone did not significantly delay ($p = 0.12$) or influence overall hatching success ($p = 0.22$), including in the 30-d exposure (Dunnett's $p = 0.09$). However, fluridone significantly delayed hatching of eggs on adult shells ($p < 0.01$) and reduced their overall hatching success ($p < 0.01$). The 96-h median effect concentration (EC50) for fluridone on hatching success of eggs on adults was 1334 µg/L (95% CI, 1215 – 1466 µg/L). For endothall, the adult 96-h median lethal concentration (LC50) was 223 mg/L (157 – 318 mg/L). Endothall negatively affected hatching success in both egg tests by delaying hatching ($p < 0.01$ in both tests) and by reducing overall hatching success ($p = 0.04$ for eggs on cards, and $p < 0.01$ for eggs on adults). The endothall 96-h EC50s for egg hatching success were 54.1 mg/L (95% CI, 35.6 – 82.2 mg/L; eggs on adults) and 83.4 mg/L (95% CI, 60.4 – 115.2 mg/L; eggs on cards). Neither herbicide had toxic effects to either life stage at concentrations typically prescribed for control of hydrilla (5 – 15 µg/L fluridone and 1 – 5 mg/L endothall). However, applying the minimum amount of herbicide needed for effective weed control is recommended for ensuring safety of non-target organisms.

KEYWORDS - Gastropoda, prosobranch snail, hatching success, Panhandle Pebblesnail, dipotassium salt of endothall (Aquathol-K®), fluridone (Sonar-Genesis®)

INTRODUCTION

Understanding the effects of toxicants on rare and imperiled species in environments laden with contaminants is as critical to achieving conservation goals as is understanding life history and habitat requirements. Toxicological and other studies on freshwater mollusks (mainly freshwater mussels) have increased over the past ~20 years (Cope et al. 2008; FMCS 2016), but they still number far fewer than studies of other taxa (e.g., fishes, insects, and other invertebrates). Gastropods – especially gill-breathing species in the clades Caenogastropoda and Neritimorpha (formerly known from the subclass Prosobranchia) – are represented by just a few recent studies (Besser et al. 2009, 2016; Archambault et al. 2015; Poznanska et al. 2015; Gibson et al. 2016) despite their high imperilment rates and importance to the functional ecology of freshwater systems (Johnson et al. 2013).

Invasive plants and animals are another credible and widely documented threat to freshwater mollusks, and resource managers must often balance their control with

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conserving native species. For example, researchers have long worked to identify chemicals to combat invasive mollusks (e.g., Zebra Mussels (*Dreissena polymorpha*)) without harming non-target species, including native mussels (e.g., Waller et al. 1993; Cope et al. 1997; Meehan et al. 2014). The effects of herbicides used to combat invasive aquatic plants, such as hydrilla (*Hydrilla verticillata,* Hydrocharitaceae), on non-target organisms has also been investigated (Hamelink et al. 1986; Keller 1993; Paul et al. 1994; Yi et al. 2011), including the most recent study on freshwater mussels and snails (Archambault et al. 2015). Hydrilla is an aquatic invasive weed non-native to the United States (US), and is included on the Federal Noxious Weed List (USDA APHIS 2012). It can form vast monocultures, shade out native vegetation (FWC 2013), alter water quality parameters including dissolved oxygen (Pesacreta 1988), and can serve as a vector for a neurotoxic cyanobacteria that affects waterfowl and their predators (Wiley et al. 2008; Williams et al. 2009). Hydrilla has been frequently dispersed anthropogenically via boat motors, trailers, and angling gear, and eradication or long-term maintenance control is difficult (Langeland 1996).

The most common hydrilla control methods include application of aquatic herbicides, introduction of non-native (to the US) Grass Carp (*Ctenopharyngodon idella*), and mechanical removal (Langeland 1996). Fluridone (market name Sonar®; CAS number 59756-60-4), typically prescribed for one to four months, and the dipotassium salt of endothall (market name Aquathol®; CAS number 2164-07-0), typically prescribed two to three times during the growing season, each for a period of days, are among the most commonly used aquatic herbicides for control of hydrilla (Archambault et al. 2015). The impetus for this study was the persistence of hydrilla in the Eno River, located in the Piedmont region (Durham and Orange Counties), North Carolina, USA – a river with high biodiversity, high rates of endemism, and the presence of threatened and endangered species (Smith et al. 2002; NCWRC 2015; LeGrand et al. 2013; NatureServe 2013) – where the targeted use of herbicides has been recommended as the most appropriate hydrilla control method. However, more information on the potential effects to non-target organisms was needed, especially for the Panhandle Pebblesnail (*Somatogyrus virginicus*), whose habitat has been invaded by hydrilla and where herbicide applications would occur.

Somatogyrus virginicus (Lithoglyphidae) is a rare, non-pulmonate snail in the clade Caenogastropoda; species in this genus have an annual reproductive ecology in which most adults die soon after breeding (Johnson et al. 2013). *Somatogyrus virginicus* has a limited and patchy distribution in Atlantic Slope streams of Virginia, North Carolina, and South Carolina (USA; NatureServe 2013), and the Eno River has the only confirmed population in North Carolina (LeGrand et al. 2013), where it has been identified as a species of greatest conservation need in the North Carolina Wildlife Action Plan (NCWRC 2015). The Eno River, which is also culturally important as a recreational destination and municipal drinking water source, supports a variety of other rare species, including the Carolina Madtom (*Noturus furiosus*, state-listed threatened), and one state-threatened (*Lampsilis radiata*) and three state-endangered (*Fusconaia masoni, Lampsilis cariosa, Lasmigona subviridis*) freshwater mussels (LeGrand et al. 2013).

Like other lithoglyphids, *S. virginicus* lays its eggs in spring, with timing of reproduction and development of eggs influenced by stream temperature (P. Johnson, Alabama Aquatic Biodiversity Center, personal communication). Those in the Eno River begin laying eggs in mid- to late-April when the water temperature approaches $\sim$ 17°C and continues through mid-May, often depositing them on a clean surface of silt-free rocks with riffleweed (*Podostemum ceratophyllum*), an aquatic plant that provides habitat for the snails. Eggs are most abundant (e.g., hundreds per rock) within stream riffle habitat and are deposited individually in a clear, hard casing. The duration of development is dependent upon temperature, typically requiring 2 – 4 weeks before hatching (P. Johnson, personal communication, and author personal observations).

Prior to the recent study by Archambault et al. (2015) on the effects fluridone and the dipotassium endothall on freshwater mussels and juvenile *S. virginicus*, peer-reviewed toxicity data were limited to only a few studies of other freshwater invertebrates and fishes (Crosby and Tucker 1966; Hamelink et al. 1986; Paul et al. 1994; Yi et al. 2011). The toxicity thresholds for freshwater mollusks ranked among the lowest (i.e., most sensitive) compared to fishes and other invertebrates, but concentrations associated with acute toxicity were still greater than the concentrations typically prescribed for controlling invasive aquatic weeds ($\sim$10 to 100 times greater; Archambault et al. 2015). The potential risks of such aquatic herbicides to freshwater mollusks should be assessed and balanced appropriately against the significant biological threat posed by invasive aquatic weeds like hydrilla.

Fluridone (market formulation liquid Sonar-Genesis®) and the dipotassium salt of endothall (hereafter, simply 'endothall'; market formulation Aquathol-K®) were considered for management of hydrilla in the Eno River. The complex management situation of snail habitat juxtaposed with dense stands of hydrilla and, therefore, snail reproduction and egg development with timing and location of herbicide applications required a thorough assessment of potential hazards of these herbicides to the life stages of *S. virginicus*. An earlier study reported the acute median lethal concentrations (LC50s) of fluridone to *S. virginicus* juveniles (409 – 500 µg/L, 96 h test to 48 h post-exposure; Archambault et al. 2015), but effects on snail eggs and adults, effects from longer duration exposures, and effects from other chemicals (e.g., endothall) have not been studied. The aims of this study were to determine the effects of two herbicides used for control and management of hydrilla and other aquatic weeds on *S. virginicus* eggs and adults so that the species' sensitivity can be holistically understood with information from multiple life stages; to expand the toxicological data base for gill-breathing snails in Caenogastropoda and related clades; and to assess the

results in the context of typically prescribed invasive plant control methods in high-biodiversity ecosystems.

METHODS

Test Organisms

Adult *S. virginicus* were collected from the Eno River when their eggs were abundant on river rocks to ensure they were reproductively active (120 snails on 7 May 2014 and 255 snails on 13 April 2015). Upon collection, snails were placed in sanitized Naglene® bottles filled with river water, placed in a cooler to maintain the ambient water temperature, and immediately transported (~45 min travel duration) to our laboratory at North Carolina State University (Raleigh, USA). Average shell height, as measured from the apex to the base of the aperture, perpendicular to the spiral axis was 4.45 mm (± 0.43, SD) in 2014 and 4.32 mm (± 0.42 mm) in 2015. Snails were acclimated from river water to the test water by placing them in a 50:50 solution of river/reconstituted water for 2 h, then further diluting the river water to a 25:75 ratio with reconstituted water, and held for an additional 2 h before being placed in 100% reconstituted water (ASTM 2007; 2013). ASTM reconstituted soft water (ASTM 2007) was selected because it most closely approximated the water quality parameters in the native range of *S. virginicus*.

Egg culture.—Ten (in 2014) or 12 (in 2015) snails were placed in each of 12 (in 2014) or 18 (in 2015) beakers containing 300 mL of water and a 5x8-cm card cut from a section of vinyl siding, which was suggested as an appropriate substrate for egg deposition (P. Johnson, Alabama Aquatic Biodiversity Center, personal communication). All vinyl cards were oriented with the rough surface facing downward and the smooth side facing upward. The first study was smaller to minimize collection of animals, given the uncertainty of potential success with culturing eggs of gill-breathing snails in a laboratory setting for the first time. Typically, current culture methods focus on augmenting wild populations, and are accomplished with large numbers of adult snails grown in outdoor pools sourced with food-rich pond water (P. Johnson, Alabama Aquatic Biodiversity Center, personal communication), whereas we needed to produce eggs on discrete units for individual exposure in independent experimental replicates. Water was renewed (100% volume) twice per week during the egg culture phase, and each chamber received a one-time dose of $<$ 1 mL of Instant Algae® Nannochloropsis (Nanno 3600; Reed Mariculture, Campbell, California, USA) concentrate to aid establishment of a biofilm on which the adult snails might feed. Eggs were counted twice per week until it was determined there was a sufficient quantity for testing, which took 7 – 10 d. The initial egg count on vinyl cards at the beginning of the experiments averaged 47 per card in 2014 (range 10 – 91; age $\leq$ 10 d) and 15 per card in 2015 (range 5 – 29; age $\leq$ 5 d).

Experimental Conditions

We selected herbicide treatment concentrations based on recommended application rates for treatment of hydrilla, herbicide label maximum application rates, and acute toxicity data reported for other taxa in the peer-reviewed literature (Crosby and Tucker 1966; Sanders 1969; Hamelink et al. 1986; Paul et al. 1994; SePRO 2010, 2011; Yi et al. 2011; UPI 2011, 2012), including the only known toxicities of fluridone and endothall to other freshwater mollusks (Keller 1993; Archambault et al. 2015). Sonar – Genesis® (fluridone), labeled as 0.5 lb/gal (59,913 mg/L) was provided by the SePRO Corporation Research and Technology Campus (Whitakers, North Carolina, USA) and was stored refrigerated until use in toxicity tests. Before use, the fluridone was diluted to a working stock of 1500 µg/L (parts per billion) active ingredient, as formulated. Acute test concentrations of fluridone ranged from 5 to 1000 µg/L in the exposure of eggs on vinyl cards, with an additional chronic (30-d) test treatment at 5 µg/L. Test concentrations for adult snails ranged from 5 to 1500 µg/L. Endothall (Aquathol-K®; United Phosphorus, Inc., King of Prussia, Pennsylvania, USA), labeled as 4.23 lb/gal (~506,866 mg/L), was obtained from personnel in the Aquatic Plant Management Program in the Department of Crop Science, North Carolina State University, and subsequently diluted to a working stock of 1000 mg/L (parts per million) active ingredient, as formulated. Test concentrations of endothall ranged from 5 to 100 mg/L in the exposure of eggs on vinyl cards, and from 1 to 1000 mg/L in the adult snail test. Composite water samples (10 mL from each of 3 replicates, 30 mL total volume) were collected for herbicide concentration verification prior to placing organisms into the chambers, and again at 48-h; samples were stored at 4°C until they were shipped to the SePRO Corporation analytical laboratory (fluridone quantified via HPLC) or the US Army Engineer Research and Development Center's Environmental Laboratory (endothall quantified via immunoassay; Gainesville, Florida, USA).

As in the culture phase, all experiments were static-renewal tests conducted in reconstituted soft water (ASTM 2007), with 90 – 100% water renewal at 48 h during the tests, and 3x/wk during the observation period following the tests. No formalized guidelines (e.g., ASTM) exist for conducting acute or chronic toxicity tests with freshwater snails, so quality assurance and control were ensured by conducting all tests according to guidelines for other freshwater mollusks (ASTM 2013), as per protocol in other recently published studies (Besser et al. 2009, 2016; Archambault et al. 2015). Tests were conducted in light- and temperature-controlled environmental chambers (Precision Model 818, Thermo Fisher Scientific, Marietta, Ohio, USA), held at 20°C and a light:dark cycle of 16:8 h (3678 lux). During the post-exposure observation period, temperature conditions were adjusted to approximate the natural river conditions, encouraging timely development of eggs. The final temperature was 23.5°C in 2014 and 23°C in 2015. In exposures with adults, six (in 2014) or seven (in

2015) snails were placed in each of three replicates per treatment, including in controls (0 µg/L). Because the adults were carrying embryos on their shells, we used the opportunity to observe them throughout the experiment, and afterward, transferred the adults to untreated reconstituted water to observe the embryos through hatching. Adult snail shells had an average of 9 total embryos per replicate in 2014 (range 1 – 20) and 12 embryos per replicate in 2015 (range 4 – 21). Eggs on adult shells ranged from freshly laid to final developmental stages because many were present when the adult snails were collected from the river and they continued to deposit eggs on shells or beaker surfaces while in the laboratory. In exposures of eggs on vinyl cards, each card was distributed to one of three independent replicates per treatment. Mean water quality conditions among experiments were 30.0 mg $CaCO_3$/L alkalinity, 42.0 mg $CaCO_3$/L hardness, 261 µS/cm conductivity, 7.78 pH, and 8.49 mg/L dissolved oxygen ($n = 4$ for alkalinity and hardness, $n = 15$ for all other variables). After the experiments, each chamber was dosed with < 1 mL of Nanno 3600 concentrate to aid establishment of a biofilm on which the snails and hatchlings might feed.

Data Collection and Statistical Analysis

At the end of each 96-h exposure, survival of adult snails was assessed by viewing them under a stereomicroscope and observing for righting behavior or movement within five minutes, an endpoint used in other studies and similar to assessment guidelines established for other freshwater mollusks (Besser et al. 2009, 2016; ASTM 2013; Archambault et al. 2015). Eggs were assessed after 96 h and three times weekly for viability until hatching senesced in each test (18 – 26 d post-exposure) by observing for vibrant yellow yolks, their characteristic constant rotation, and embryo development. Yolks/embryos that separated, stopped moving, lost color (turned white), stopped developing, or aborted were documented as non-viable.

The effects of herbicide concentration on survival of adult snails and on hatching success of snail eggs were analyzed by using survival data to generate median lethal/effective concentrations (LC50, EC50) and 95% confidence intervals (CI) via the Trimmed Spearman-Karber method (Comprehensive Environmental Toxicity Information Software (CETIS)™, v1.8.0.12, Tidepool Scientific, LLC, McKinleyville, California, USA). The LC50 or EC50 was defined as the concentration that caused mortality (LC50) or observed effect (i.e., lack of hatching; EC50) in 50% of the individuals in the exposed sample, and the LC05/EC05 was defined as the concentration that caused mortality/effect in 5% of the sample. LC and EC values were considered significantly different when their 95% CIs did not overlap (i.e., $\alpha = 0.05$).

The effect of herbicide concentration on hatching success was further analyzed using a repeated measures analysis of variance (PROC MIXED; SAS version 9.4; SAS Institute, Inc., Cary, North Carolina, USA). Significant effects ($\alpha = 0.05$) of herbicide concentration were further analyzed using a Dunnett's post-hoc test to elucidate toxic effects compared to controls.

RESULTS

Herbicide Concentration Analysis

Exposure accuracy (i.e., measured herbicide concentration compared to target concentration) was calculated as: exposure accuracy $= (P_m)/(P_t) \bullet 100$, where P_m is the measured herbicide concentration and P_t is the target concentration. The mean exposure accuracies are an average among all treatments sampled (all concentrations for fluridone and 0 – 100 mg/L for endothall because samples from the highest concentrations exceeded the dilution curve for analysis). They include sample results from both the test start (time zero) and 48-h time points (prior to solution renewal). The mean exposure accuracy of fluridone in experiments was 114.3% (range 99 – 154%) of target treatment concentrations. The mean exposure accuracy in endothall experiments was 93.6% (range 80 – 108%) of target treatment concentrations. All results were, therefore, expressed based on target concentrations. Post-hatch mortality was minimal in all egg tests and was similar among treatments within a given test; therefore, any post-hatch mortality was considered an effect of holding conditions rather than a treatment effect. Accordingly, the following statistical analyses of treatment effects were based on the ratio of total eggs hatched/initial egg count.

Fluridone

Hatching success.—In the exposure with eggs on vinyl cards, hatchlings began appearing 19 d after exposure, allowing four observation time points to be used in the analysis. Fluridone did not significantly affect hatching success ($p = 0.22$) (Table 1, Figure 1A). While no treatments were significantly different from controls at the $\alpha = 0.05$ level, a comparison of the treatment continuously exposed at 5 µg/L yielded a p-value of 0.09, trending lower than others (all other comparisons had p-values ranging from 0.13 to 0.57) (Table 1, Figure 1A).

In the exposure of eggs laid on adults, hatchlings were present 5 d after exposure and eight time points were used in the analysis. Fluridone significantly decreased hatching success ($F_{5,12} = 15.55$, $p < 0.01$), and its effect was dependent on time ($F_{35,84} = 14.07$, $p < 0.01$) (Table 1, Figure 1B). Hatching was delayed in the 500, 1000, and 1500 µg/L treatments (e.g., significantly lower on day 19 compared to controls). Further, overall hatching success was lower in the 500 and 1500 µg/L treatments compared to controls (Dunnett's $p = 0.05$ and $p < 0.01$, respectively) (Figure 1B).

Median lethal concentrations.—A fluridone 96-h LC50 for adult snails could not be calculated due to lack of mortality; most snails survived in all treatments, including the highest

Table 1. Results of repeated measures analysis of variance of the effects of fluridone on *Somatogyrus virginicus* hatching success.

Effect	Numerator degrees of freedom	Denominator degrees of freedom	F value	p-value
Eggs on cards				
day	3	36	254.59	<0.0001
fluridone	5	12	1.65	0.2198
fluridone*day	15	36	1.61	0.1206
Eggs on adults				
day	7	84	408.23	<0.0001
fluridone	5	12	15.55	<0.0001
fluridone*day	35	84	14.07	<0.0001

Table 2. Results of repeated measures analysis of variance of the effects of endothall on *Somatogyrus virginicus* hatching success.

Effect	Numerator degrees of freedom	Denominator degrees of freedom	F value	p-value
Eggs on cards				
day	3	24	75.27	<0.0001
endothall	3	8	4.29	0.0441
endothall*day	9	24	3.91	0.0036
Eggs on adults				
day	7	112	42.54	<0.0001
endothall	7	16	6.14	0.0013
endothall*day	49	112	3.87	<0.0001

treatment of 1500 µg/L. Likewise, a 96-h EC50 for egg hatching success could not be determined in the exposure of eggs on cards because of high hatching rates in all treatments. The 96-h EC50 for hatching success of eggs on adults was 1334 µg/L (95% CI, 1215 – 1466 µg/L). The only fluridone EC05 derived was for the same test, and was 288 µg/L (95% CI, 0 – 593 µg/L).

Endothall

Hatching success.—Somatogyrus eggs on vinyl cards began hatching 14 d after the end of the exposure, allowing four observation time points to be used in statistical analysis. Endothall had a significant fixed effect on overall hatching success ($F_{3,8} = 4.29$, $p = 0.04$), and the Dunnett's post-hoc analysis showed that hatching success was significantly lower in the 100 mg/L treatment compared to control ($p = 0.02$), but not in other treatments (p-values ≥ 0.11) (Table 2, Figure 2A). In addition to the main effect, the significant treatment-time interaction ($F_{9,24} = 3.91$, $p < 0.01$) provided evidence of a delay in hatching (i.e., eggs took longer to hatch at high concentrations). For example, hatching in the 100 mg/L treatment on day 17 was significantly less than control (Figure 2A).

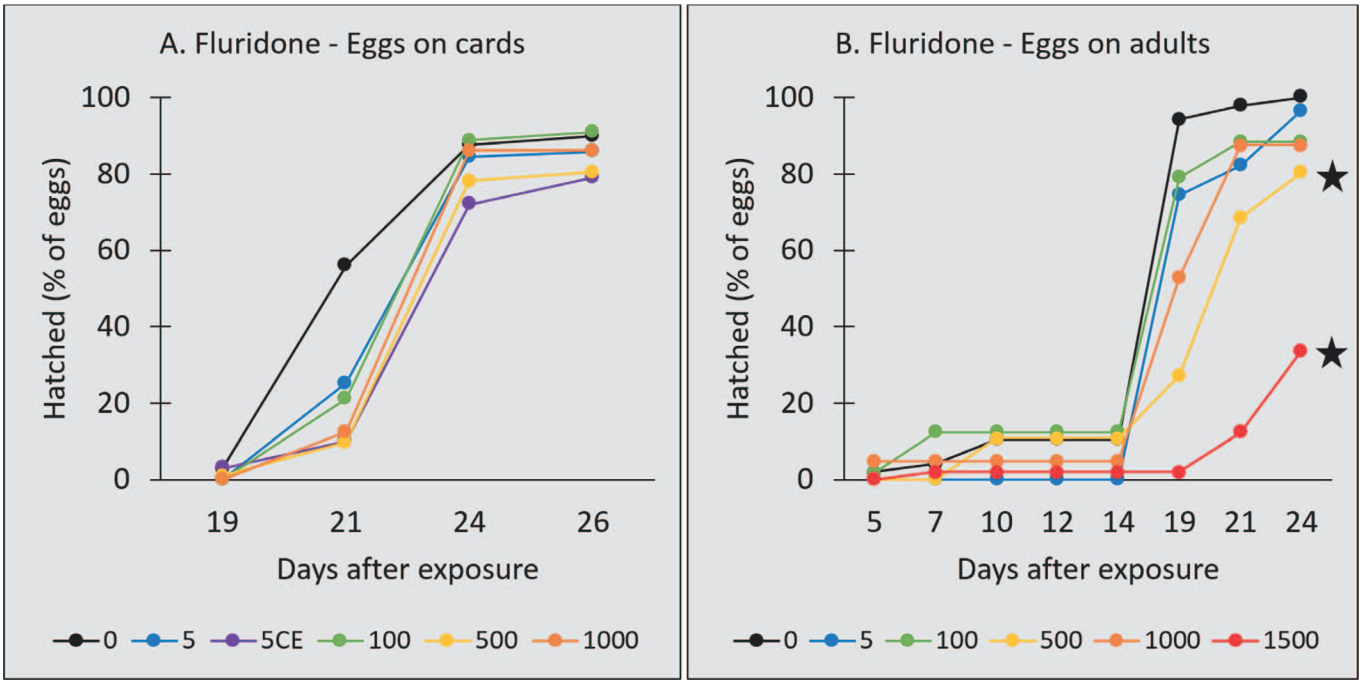

Figure 1. Mean percent of *Somatogyrus virginicus* eggs on vinyl cards (A) and on adult snail shells (B) counted initially in each fluridone treatment that hatched by each observation time point. Warmer colors represent higher concentrations (in µg/L), as legend indicates. Notes: 5CE in panel A denotes the continuously-exposed static-renewal treatment that received fluridone throughout observation period. Black stars indicate significantly lower overall hatching success at final time point, compared to control (Dunnett's $p \leq 0.05$). Standard errors for each data point are listed in the Appendix.

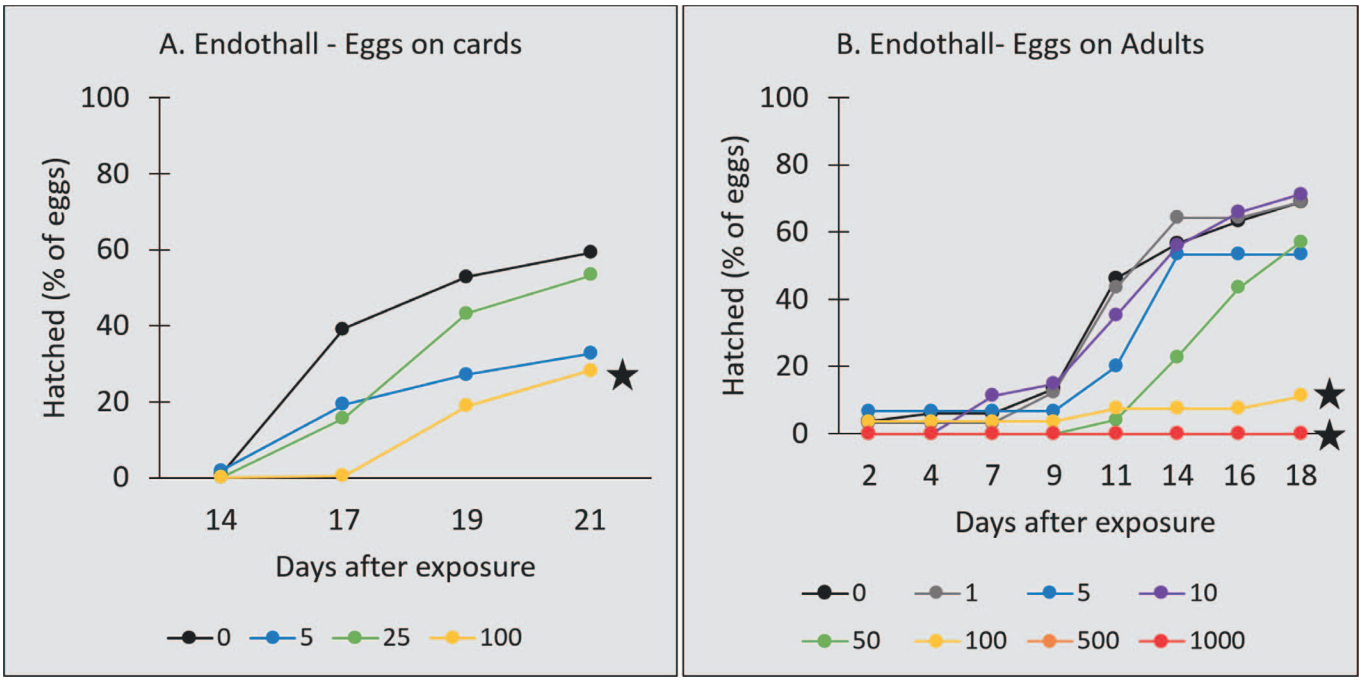

Figure 2. Mean percent of *Somatogyrus virginicus* eggs on vinyl cards (A) and on adult snail shells (B) counted initially in each endothall treatment that hatched by each observation time point. Warmer colors represent higher concentrations (in mg/L), as legend indicates. Black stars indicate significantly lower overall hatching success at final time point, compared to control (Dunnett's $p \leq 0.05$). Responses in the 500 and 1000 mg/L were the same and overlap. Standard errors for each data point are listed in the Appendix.

The first hatchlings from eggs on adults appeared 2 d post-exposure, and eight observation time points were used in analysis. In addition to the significant fixed effect of endothall on overall hatching success ($F_{7,16} = 6.14$, $p < 0.01$), the treatment-time interaction ($F_{49,112} = 3.87$, $p < 0.01$) again provided evidence of a hatching delay at higher concentrations (Table 2, Figure 2B). A Dunnett's post-hoc test of the main effect of treatment showed significantly poorer hatching success in the 100, 500, and 1000 mg/L concentrations ($p = 0.03$, 0.01, and 0.01, respectively), but not in lower concentrations (p-values ≥ 0.24) (Figure 2B).

Median lethal concentrations.—Both the 48-h and 96-h LC50s for adult snails exposed to endothall were 223 mg/L (95% CI, 157 – 318 mg/L). Responses were the same at both time points, because mortality occurred within the first 48 h. LC05s could not be determined for either time point due to lack of partial mortality responses – all snails survived in all treatments from 0 – 100 mg/L endothall, and no snails survived in the 500 and 1000 mg/L treatments. Most surviving snails remained alive and active during the observation of eggs on their shells following the exposure with no differences among treatments, indicating there was no latent effect of the acute duration of endothall exposure on adults. The 96-h EC50s for egg hatching success were 54.1 mg/L (95% CI, 35.6 – 82.2 mg/L; eggs on adults) and 83.4 mg/L (95% CI, 60.4 – 115.2 mg/L; eggs on cards) in the two separate tests. The EC50 results from the two tests were not significantly different, based on comparison of the overlapping 95% confidence intervals.

DISCUSSION

Comparative Toxicity

Based on LC50s and EC50s determined in our study, the *S. virginicus* egg and adult life stages appear less acutely sensitive to fluridone than its previously-tested juvenile life stage (Archambault et al. 2015). Compared to the known acute toxicity of fluridone to other aquatic organisms, the egg and adult life stages of *S. virginicus* are more sensitive to fluridone than most other species (Hamelink et al. 1986; Paul et al. 1994; Yi et al. 2011; Archambault et al. 2015). The greater sensitivity of the snails' egg and adult life stages to fluridone than other species is in agreement with that of other freshwater mollusks (including the *S. virginicus* juvenile life stage), all of which were found to be more sensitive than nearly every other organism for which fluridone toxicity values have been published (LC50 range 1300 – 32,000 µg/L, except *Arrenurus* spp. (10 – 891 µg/L); Archambault et al. 2015).

This study produced the first *Somatogyrus* LC50 and EC50s for endothall. Based on these values, the egg life stage is more sensitive than that of adults, whose LC50 value was 2.7 – 4.1 times greater than the egg EC50s. The sensitivity of juvenile *S. virginicus* to endothall has not yet been determined. If the juvenile life stage is more sensitive, as the fluridone data

indicates (Archambault et al. 2015), determining juvenile sensitivity to endothall may be prudent, especially because they would be present during summer applications of herbicides for aquatic weed control. The adult snail LC50 for endothall is approximately 6 – 7 times greater, and the egg EC50s are approximately 1.6 – 2.7 times greater, than the LC50s reported for the freshwater mussel *Lampsilis siliquoidea* (31 – 34 mg/L), the only freshwater mollusk for which endothall dipotassium salt toxicity data are published (Archambault et al. 2015). Keller (1993) evaluated the toxicity of Hydrothol 191 (CAS number 66330-88-9), a mono-amine salt of endothall to in-vitro propagated *Anodonta* (now *Utterbackia*) *imbecillis* and reported an LC50 of 4.85 mg/L. Another experiment in our laboratory with the dipotassium salt of endothall and in-vitro propagated *Lampsilis cardium* resulted in a 96-h LC50 of 137 mg/L (105 – 178 mg/L) (J. Archambault, unpublished data). Together, these findings indicate that mollusks may exhibit a wide range of tolerance to endothall formulations, even within a genus or among life stages. Compared to the known acute toxicity values of endothall to other non-molluscan aquatic organisms (16 – 130 mg/L (Crosby and Tucker 1966; Sanders 1969; Paul et al. 1994)), the adult life stage of *S. virginicus* is more tolerant of endothall than other species, having the highest acute LC50 value, and *S. virginicus* egg EC50s are in the middle of that range. That contrasts with some of their freshwater mussel counterparts, whose LC50s occur at the sensitive end of the known toxicity range (Archambault et al. 2015).

Relative Risk

Fluridone is typically applied at a rate of 5 to 15 μg/L for hydrilla control, with a maximum allowable application rate of 150 μg/L (SePRO 2010), and its application is most effective once plants are emerging from winter senescence and actively growing (e.g., May for hydrilla in the Eno River) to ensure maximum exposure to the product. Because of the similar spring timing of reproduction in *S. virginicus* and the growth of hydrilla, herbicide application during snail egg development and hatching overlap, and would likely be similar in other locations in the southeastern US where lithoglyphids co-occur with invasive plants. The negative effects of fluridone on *S. virginicus* egg hatching were due to delayed hatching and lower hatching rates in the highest concentrations tested (i.e., $\geq$ 500μg/L, Figure 1), indicating fluridone poses a minimal risk of harm compared to the potentially substantial risk of habitat degradation posed by hydrilla or other invasive aquatic weeds. Negative effects were not observed in the environmentally relevant range of concentrations in either egg test, providing consistent results from both 96-h exposures (Figure 1). Despite the lack of a statistically significant effect on hatching success in the 30-d exposure of 5 μg/L fluridone, the results were lower than the 96-h treatments of all other concentrations in the same test, and may be biologically relevant (Figure 1A). The 30-d exposure was about half to one-third as long as the typical treatment duration for fluridone in flowing waters. Laboratory conditions are vastly different from the natural swift river environment of *S. virginicus*, likely rendering longer duration studies in the laboratory impractical. Fluridone's primary degradation pathway is photolysis, and according to the Sonar Genesis product label, it may be less effective if in contact with highly organic sediments (SePRO 2010); however, water concentrations are typically monitored to maintain the target treatment concentrations during an herbicide application. Other factors that may reduce exposure of non-target organisms like *S. virginicus* include uneven mixing within complex habitats of a river course and proximity of the treatment area to species of concern (if not overlapping, as in our study area).

The acute exposures of endothall to *S. virginicus* eggs had a significant negative effect on hatching success at higher concentrations ($\geq$ 100 mg/L) in both tests (Figure 2), but not at concentrations typically prescribed for invasive aquatic plant control (1 – 5 mg/L). However, rates up to 150 mg/L are authorized for use on the product label (UPI 2011), and such concentrations in high biodiversity ecosystems should be avoided based on our findings, especially because the acute test durations are environmentally relevant for prescribed endothall applications, and endothall application would likely overlap with gastropod egg development when the target plants are actively growing.

Adult snails were unaffected by 96-h exposure to both herbicides in the label recommended application ranges. Further, any latent mortality following the tests and documented during the observation of eggs on their shells was sporadic, minimal, and equivalent in all treatments, including controls. Moreover, the risk of exposure to adult *S. virginicus* is minimized because most adults of this snail species will have already reproduced and are likely to die naturally before, or in the early phase of, any field application of herbicides. The egg and hatchling/juvenile life stages would be the most exposed and potentially vulnerable to any negative effects of herbicides.

At environmentally relevant concentrations (those typically applied to control hydrilla and other aquatic weeds), fluridone and endothall pose a minimal risk to all life stages of *S. virginicus*, compared to the potential risk that hydrilla infestation poses by degrading physical habitat and water quality. In riverine situations, such as in the Eno River of North Carolina, stands of hydrilla often grow directly within, and adjacent to, optimal snail habitat. In the swift-flowing riffles with clean rocks and riffleweed that provide snail habitat, hydrilla may shade out the native preferred vegetation and reduce water velocity, facilitating increased siltation. During our field collections of adult snails– even within an occupied riffle – snails were often more abundant in the swiftest flowing portion of the stream reach, despite available riffleweed habitat throughout the riffle. Snails were often more difficult to find in abundance or seemingly absent in slower portions of the riffle, where the slightest layer of sediment was apparent on rocks. The genus *Somatogyrus* has a strong foot

compared to many other freshwater snails (P. Johnson, Alabama Aquatic Biodiversity Center, personal communication), a possible adaptation for living in clean, swift water habitats where they are most often encountered. Because these and many other snails in the gill-breathing clades, Caenogastropoda and Neritimorpha, are simultaneously imperiled and geographically restricted, conservation of high quality habitat is imperative. We recommend that resource managers apply our findings in protecting freshwater habitats infested by aquatic weeds, while also recognizing their limitations. For example, selecting aquatic herbicide treatment prescriptions that use the minimum necessary concentrations to achieve effective control of invasive aquatic plants would be prudent because detrimental effects on egg hatching success were observed within the application range allowed on existing endothall labels, and because higher fluridone concentrations were not tested over relevant treatment durations (e.g., 30 – 90 d).

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Appendix

Mean percent of snail eggs hatched and associated standard error for each treatment and time point shown in figures.

Table A1. Fluridone eggs on cards; corresponds with Figure 1A.

Treatment (µg/L)	Days after exposure	Mean (% hatched)	Standard Error
0	19	2.90	2.90
5		0.00	0.00
5CE		3.03	3.03
100		0.00	0.00
500		1.15	1.15
1000		0	0
0	21	55.99	7.71
5		25.27	7.06
5CE		9.98	5.32
100		21.07	3.48
500		9.75	5.10
1000		12.50	7.22
0	24	87.58	8.46
5		84.43	13.69
5CE		72.01	15.76
100		88.89	5.56
500		78.25	3.23
1000		86.11	7.35
0	26	89.96	6.22
5		85.71	14.29
5CE		78.97	13.23
100		91.00	14.29
500		80.55	13.23
1000		86.11	5.56

Table A2. Fluridone eggs on adult shells; corresponds with Figure 1B.

Treatment (µg/L)	Days after exposure	Mean (% hatched)	Standard Error
0	5	2.08	2.08
5		0	0
100		1.96	1.96
500		0	0
1000		4.76	4.76
1500		0	0
0	7	4.17	4.17
5		0	0
100		12.25	7.22
500		0	0
1000		4.76	4.76
1500		2.08	2.08
0	10	10.42	5.51
5		0	0
100		12.25	7.22
500		10.82	5.53
1000		4.76	4.76
1500		2.08	2.08
0	12	10.42	5.51
5		0	0
100		12.25	7.22
500		10.82	5.53
1000		4.76	4.76
1500		2.08	2.08
0	14	10.42	5.51
5		0	0
100		12.25	7.22
500		10.82	5.53
1000		4.76	4.76
1500		2.08	2.08
0	19	94.21	3.22
5		74.52	2.49
100		79.12	2.17
500		27.19	7.42
1000		52.98	9.91
1500		2.08	2.08
0	21	97.92	2.08
5		82.06	6.63
100		88.33	7.26
500		68.54	7.18
1000		87.50	7.22
1500		12.42	4.40
0	24	100	0
5		96.67	3.33
100		88.33	7.26
500		80.29	3.65
1000		87.50	7.22
1500		33.71	9.88

Table A3. Endothall eggs on cards; corresponds with Figure 2A.

Treatment (mg/L)	Days after exposure	Mean (% hatched)	Standard Error
0	14	0.93	0.93
5		1.85	1.85
25		0	0
100		0	0
0	17	39.29	7.43
5		19.30	9.68
25		15.72	5.65
100		0.44	0.44
0	19	52.96	8.77
5		27.23	10.99
25		43.19	4.02
100		18.79	1.21
0	21	59.14	8.75
5		32.73	14.07
25		53.13	4.00
100		28.26	1.42

Table A4. Endothall eggs on adults; corresponds with Figure 2B.

Treatment (mg/L)	Days after exposure	Mean (% hatched)	Standard Error
0	2	3.70	3.70
1		3.17	3.17
5		6.67	6.67
10		0.00	0
50		0.00	0
100		3.70	3.70
500		0	0
1000		0	0
0	4	5.93	3.23
1		3.17	3.17
5		6.67	6.67
10		0.00	0
50		0.00	0
100		3.70	3.70
500		0	0
1000		0	0
0	7	5.93	3.23
1		3.17	3.17
5		6.67	6.67
10		11.11	11.11
50		0.00	0
100		3.70	3.70
500		0	0
1000		0	0
0	9	13.33	10.18
1		12.48	3.80
5		6.67	6.67
10		14.81	9.80
50		0.00	0
100		3.70	3.70
500		0	0
1000		0	0
0	11	46.30	8.17
1		43.46	5.14
5		20.00	20.00
10		35.19	8.07
50		4.17	4.17
100		7.41	7.41
500		0	0
1000		0	0
0	14	56.67	13.47
1		64.25	3.74
5		53.33	29.06
10		56.02	3.62
50		22.97	6.01
100		7.41	7.41
500		0	0
1000		0	0
0	16	63.33	6.94
1		64.25	3.74
5		53.33	29.06
10		65.74	5.63
50		43.38	12.28
100		7.41	7.41
500		0	0
1000		0	0
0	18	68.89	5.88
1		68.89	5.88
5		53.33	29.06
10		71.30	8.23
50		57.05	22.33
100		11.11	11.11
500		0	0
1000		0	0