EMERGING METHODS FOR EMERGING CONTAMINANTS: NOVEL APPROACHES TO FRESHWATER MUSSEL TOXICITY TESTING

by

PETER DONALD HAZELTON

(Under the Direction of Robert B. Bringolf)

ABSTRACT

Freshwater mussels of the order Unionoida (unionids) are among the most imperiled aquatic fauna worldwide. Numerous causes contribute to the loss of diversity and abundance of unionids, including the presence and toxicity of chemical pollutants. Mussels are often the most sensitive animals to contaminants, and routes of exposure differ depending on the stage of their complex lifecycle. Unionid ecotoxicology testing and risk assessment has largely focused on acute and chronic toxicity of metals, pesticides and nutrients on the larval and early juvenile stages. A paucity of data exists on effects of emerging contaminants such as pharmaceuticals and personal care products, and legacy compounds that persist in the environment. I propose a partial-lifecycle assay to be incorporated within a larger lifecycle assessment framework of emerging contaminants on unionids and used a persistent industrial contaminant (perfluoroalkyl sulfonate, PFOS) and an antidepressant pharmaceutical (fluoxetine) as test compounds with the assay. The partial-lifecycle test of PFOS to *Lampsilis siliquoidea* incorporated a 30-d exposure to larvae within the maternal marsupia, and a 24-h exposure to glochidia removed from the mother. Results revealed that exposure *in marsupia* caused significant

reductions in initial larval viability and duration of viability at 4.5 µg/L PFOS, and reduced metamorphosis success at 69.5 µg/L PFOS. Exposure of larval *Lampsilis fasciola* to fluoxetine resulted in increased probability of metamorphosis at 1 and 100 µg/L fluoxetine, suggesting importance of a seratonergic pathway in larval development or in the larvae-host interaction. Adult *L. fasciola* exposed to fluoxetine were more likely to display mantle-flap lures, and had greater occurrences of foot swelling beyond the shell margins. In a follow-up experiment lasting 67 d, fluoxetine was associated with increased movement rates and changes to more diurnal behavior, which could increase susceptibility to predation with *in situ* exposures. The results of these experiments have been successful at identifying chemical threats to unionids at concentrations below those reported in traditional acute and chronic tests. Partial-lifecycle tests may play an important role in better understanding effects of contaminants on unionid biology and may be combined with population modeling to ascertain effects of emerging contaminants on population persistence.

INDEX WORDS: Perfluoroalkyl acids, PFAAs, Selective serotonin reuptake inhibitors, SSRIs, Unionidae, Adverse outcome pathway, ecotoxicology

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A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2013

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August 2013

DEDICATION

To Benjamin and his wonderful mother: I could not have done this without your love and support. Here is to new adventures, and a long awaited return home!

ACKNOWLEDGEMENTS

First, and foremost I would like to thank my family. I would not be the man and scientist that I am if it were not for the love and support of my wife. After 12 years, 5 relocations, countless jobs, and 3 degrees… she has stood beside me and been my best friend and my greatest champion. Thank you to my parents: who have always inspired me to do the best I can and to never give up. To Bill and Elizabeth: for their continued love and support of our family even though we are regretfully moving farther away from Florida. To my brothers Eric and Brian: one for continuously challenging me as an ecologist and colleague, and the other for keeping me grounded in who I am away from my work. I love you all very much and could not have completed this journey without you.

It has been a delight to study under the direction of Robert Bringolf. Robert has been an outstanding mentor, a valuable coauthor and editor, a tremendous advocate of my work, and a wonderful friend. Thank you for your enduring dedication to helping me realize my research interests, gain vital professional development and pursue my ongoing quest for a fulfilling personal and work life balance. I could truly not have achieved the quality of research and publication without input and extraordinary effort of Dr. Bringolf and my other coauthors. Thank you for collaborating with me, and for improving these works.

I could not have chosen a more supportive dissertation committee. Dr.'s Black, Gogal and Rashleigh have been actively involved in my training and research direction since I first assessed their interest in serving as my referees. Thank you for the continued

dedication to my research project and professional development. I also would like to thank faculty and my classmates of the Interdisciplinary Toxicology Program, including our previous and acting directors: Dr.'s Jeff Fisher, Julie Coffield and Brian Cummings. You have truly built a program of which I'm very proud to be an alumnus. And there could be no one more important to thank in the ITP than Joanne Mauro. Joanne, you are the grease-on-the-wheels of this program and none of us would accomplish as much without you!

To my labmates and colleagues: Jennifer Johnson, Andrea Fritts, Kristen Kellock, Colin Shea, Duncan Elkins, Jason Wisniewski and Andrew Gascho-Landis for being a delight to work with and bounce ideas off of for the past few years. I can only hope your contributions were reciprocated. And to Bob Ratajczak, for being the best friend and co-worker I could have ever asked for. You and your family have been a haven and a joy in our lives for the past nine years.

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CHAPTER 1

INTRODUCTION AND REVIEW OF THE CURRENT LITERATURE ON EMERGING CONTAMINANT TOXICITY TO FRESHWATER MUSSELS

1.1. Introduction

The term "emerging contaminants" refers to a group of organic compounds with either recent occurrence in the environment, or those for which little is known about their environmental impact. Many of these compounds have been associated with municipal wastewater and origins range from industrial compounds to pharmaceuticals and natural and synthetic hormones (Chen et al. 2006). Evaluation of how contaminants affect freshwater organisms has been largely focused on acute lethal toxicity. While these tests are important, environmental exposures are likely to be chronic and at relatively low concentrations. Such levels may not cause overt toxicity, but may interfere with behavior and physiological processes such as reproduction, development, metamorphosis, growth and fitness (Chapman 2002). Responses *in situ* may result in reduced reproductive fitness, which leads to reduced recruitment of individuals to a population, ultimately leading to population reductions or even extirpation.

North America has the highest worldwide diversity of freshwater mussels (families Unionidae & Margaritiferidae) worldwide with nearly 300 species (Williams et al. 1993; Haag and Williams 2013). More than one hundred of those species are native to the state of Georgia, incorporating more than 50% of the total genera and the third highest species richness in the United States (Williams et al. 1993). Freshwater mussels are also among

the most imperiled species as well. Approximately 72% of unionid species on the continent are considered at risk (i.e. endangered, threatened, or special concern), and locally more than 14% of the taxa in Georgia are listed as Threatened or Endangered at either state or federal jurisdiction (Williams et al. 1993). The major threats to unionid biodiversity are thought to include introduced species, historic and current over-harvesting, loss of fish hosts, as well as physical and chemical alterations to habitat (Williams et al. 1993; Strayer 2008; Downing et al. 2010). In addition to the list of imperiled mussel species, concern is growing that even more common species are found in lesser abundances than in previous decades (Strayer 2008; Haag and Williams 2013).

Beyond the staggering loss of biodiversity, reductions in unionid populations pose a great threat to function of many freshwater ecosystems because of the loss of services that unionids provide. Mussels can encompass more biomass than all other macroinvertebrates (Layzer et al. 1993), and are often responsible for more nutrient production than all other macroinvertebrates in a riverine system (Strayer et al. 1994; Vaughn et al. 2004; Christian et al. 2008). In dense beds, mussels are able to clear up to 100% of suspended algae, and provide an important nutrient linkage from the pelagic zone to the benthos (Vaughn et al. 2004). Mussel presence has been linked with bioturbation and oxygenation of sediments, as well as adding stability to stream sediments (Vaughn and Hakenkamp 2001; Vaughn et al. 2004; Spooner and Vaughn 2008). Their presence increases habitat for other macroinvertebrates (Vaughn et al. 2008), and may also be important spawning habitat for some fish species (Wisniewski et al. 2013).

The unique life history of the Unionidae is crucial to understanding their sensitivity to changes in environment. Barnhart et al. (2008) and others (Fisher and Dimock 2002; Rogers-Lowery and Dimock 2003) have provided detailed synopses of the freshwater mussel life-cycle and the variation among species. Briefly, adult males broadcast spermatozeugmata ("sperm spheres") into the water column, which are then siphoned by adult females to fertilize eggs internally. Once fertilized, the progeny develop to a larval stage known as glochidia in modified gills (marsupia). Brooding duration is species dependent and ends either through the direct infection of a host fish through the use of a lure-like structure or by broadcasting glochidia into the water column and subsequent infection of a host (Barnhart et al. 2008). Once attached to the host, glochidia become encapsulated by host epithelial cells (typically on the gills or fins), where they undergo further development into juvenile mussels (Fisher and Dimock 2000; Fisher and Dimock 2002; Fisher and Dimock 2002; Rogers-Lowery and Dimock 2006). Following metamorphosis, juvenile mussels excise from epithelial tissue, fall to the substrate and continue to grow to adulthood. Each stage of the life-cycle is critical to the recruitment of adults into the breeding population (Barnhart et al. 2008).

Adding to the complexity of the freshwater mussel lifecycle, is that each stage also has unique exposure routes to chemical contaminants. As filter feeders, adult mussels are exposed to aqueous contaminants through uptake of surface water at the gills, and also through ingestion of contaminants that are bound to organic debris or accumulated in algal or bacterial cells that are consumed (Cope et al. 2008). Both adults and juveniles are infaunal organisms, and burrowing in polluted sediments is likely to increase exposure to contaminants that bind to sediments for exposure durations of years to an

entire lifetime (Cope et al. 2008). Exposure to contaminated sediments is of particular concern to juvenile mussels as their unique pedal feeding strategy includes the ingestion of organic matter from the sediment (Ingersoll et al. 2007; Watters 2007; Cope et al. 2008). The greatest potential routes of exposure to the larval glochidia stage occur while brooding within the female mussels marsupial gills, or while encapsulated on host fish epithelial tissue. At both timepoints this stage is likely exposed to both surface water contaminants and also may be exposed to contaminants within the maternal or host tissue (Cope et al. 2008). Furthermore, larval and juvenile stages of freshwater mussels are among the most sensitive organisms to many environmental contaminants (Cope et al. 2008).

1.2. Ecotoxicology of Freshwater Mussels

1.2.1. A myriad of approaches, exposures, & endpoints

There is not enough space here to critically review the body of work that is freshwater mussel ecotoxicology. Indeed the topic has already warranted books (Farris and Van Hassel 2007), semiannual symposia and workshops at international conferences (Society of Environmental Toxicology & Chemistry), invited reviews in special journal volumes not normally associated with toxicological studies (Christian and Harris 2008; Cope et al. 2008), and special journal volumes solely devoted to Unionid ecotoxicology (Augspurger et al. 2007). Thus, the concern for contaminant effects on freshwater mussels is well established and documented. In addition to the threat of biodiversity loss from contaminant exposure, mussels are often cited as ideal candidates for biomonitoring

of environmental health and toxicity testing because (adapted fromVan Hassel and Farris 2007):

1. Most species inhabit productive habitats that are most susceptible to pollution.
2. They are largely sedentary and therefore represent continuous exposures in one locale.
3. They are long-lived, and shells can provide a record of contaminant history.
4. They can be abundant and easy to sample.
5. They contain sufficient soft tissue mass for contaminant analysis, and they accumulate contaminants to concentrations that correlate well with exposure concentration and duration.
6. Many species are among the most sensitive to some contaminants, while other species may be very tolerant and accumulate contaminants.

Investigations of chemical contaminant exposure and effects on unionids have varied from field surveys and exposures (Armstead and Yeager 2007), and biomonitoring efforts including contaminant accumulation (Salazar and Salazar 2007; Van Hassel and Farris 2007; Bringolf et al. 2010); acute and chronic exposures in the laboratory (ASTM 2006; Ingersoll et al. 2007; Keller et al. 2007); and investigations into physiological and genetic markers of exposure and effect (Black et al. 1996; Gagne et al. 2001; Conners and Black 2004; Gagne et al. 2005; Newton and Cope 2007; Van Hassel and Farris 2007). Certainly the breadth of topics covered in unionid toxicology is nearly as diverse as those of other aquatic species, even if we have not collected data on as many

contaminants. However, the relative lack of understanding of the physiology and ecology of freshwater mussels (compared with vertebrates), along with unique challenges to their culture (Bishop et al. 2007) and a complex lifecycle, has left ample room for further research into methodology in unionid ecotoxicology testing.

1.2.2. Development and use of standard methods

Prior to the development of standard toxicity testing methods for freshwater mussels, many studies with lethal endpoints were conducted using adult animals (ASTM 2006; Keller et al. 2007; Van Hassel and Farris 2007). A paradigm shift to the use of glochidia and juvenile mussels for toxicity testing was based on a number of factors: (1) many species of unionids are in decline, so use of wild caught adults was perhaps not a sustainable strategy for conservation research, (2) earlier lifestages (glochidia & juveniles) were more sensitive to many contaminants than adults (Ingersoll et al. 2007; Keller et al. 2007) as these lifestages are also the most sensitive organisms tested to some metals (March et al. 2007) and ammonia (Newton et al. 2003) toxicity; (3) glochidia and cultured juveniles are more plentiful and represent a smaller sacrifice of adults removed from the population (Keller and Zam 1990; ASTM 2006; Ingersoll et al. 2007; Valenti et al. 2007; Cope et al. 2008); (4) glochidia and juvenile mussels are less able to avoid contamination through movement or gape closure (Van Hassel and Farris 2007).

The need for a standard method for toxicity testing has been presented more than once (Johnson et al. 1990; ASTM 2006). Guidelines were finally published in 2006 after a consensus was established from previous research, the scientific community and regulatory agencies (ASTM 2006; Ingersoll et al. 2007). Guidelines were established for

acute tests for both glochidial (24 & 48 h) and juvenile stages (48 & 96 h), and chronic (> 28 d) tests for juveniles (ASTM 2006). Juvenile tests utilize the absence of heartbeat, ciliary, valve or foot movement as a sign of mortality, and chronic tests often incorporate a metric for growth (ASTM 2006; Ingersoll et al. 2007). Chronic juvenile tests occur as water-only exposures, pore-water exposures in the presence of uncontaminated sediment, and tests assessing the toxicity of sediment bound contaminants (ASTM 2006; Ingersoll et al. 2007). In glochidia toxicity tests, mortality is assumed through an inverse relationship to viability, which is a proportion of glochidia that show a closure response when exposed to saturated NaCl solution (ASTM 2006). To meet standard toxicity guidelines, initial survival (viability) and that of the control should remain above 90% for acute tests, and control juvenile survival in chronic tests should remain above 80% (ASTM 2006). Interlaboratory comparisons for these methods have resulted in variances no greater than intralaboratory comparisons (Wang et al. 2007), thus validating the performance of the standard methods.

Though the standard methods provide rigid research requirements, and thus may be conservative estimates of toxicity, conflict has still ensued regarding the validity of these tests to establishing water quality criteria (USEPA 2009). First, concerns exist whether glochidia viability is an adequate endpoint for toxicity testing because of the species specific variation in viability duration, and duration at which glochidia are exposed outside of maternal marsupia or encapsulation by fish epithelium. Also, there was concern that the relationship between viability and glochidia metamorphosis was not well established (Ingersoll et al. 2007; USEPA 2009). Recently, Fritts et al. (*in review*) used two methods to assess viability and the corresponding metamorphosis success of

seven unionid species:1) aging glochidia outside of the female marsupia and inoculating host fish every 24 h until viability reached zero, and 2) exposing glochidia to reference toxicants (NaCl and $CuSO_4$) for 24 h, then inoculating host fish. In the aging exposure, metamorphosis success did not differ from viability at hour 0 or hour 24 for all species tested, and one species (*Lampsilis dolabraeformis*) maintained high viability and metamorphosis at 48 h post-extraction from the female. Once viability began to drop, the decrease in metamorphosis success was greater than the decrease in viability. In the toxicant exposures, the inoculations were adjusted to maintain a consistent number of viable glochidia (i.e. 4000 viable glochidia/L). Metamorphosis success did not differ among the toxicant concentrations, indicating that the reduction in viability is attributed to a smaller number of glochidia being open and available to attach to a host fish. Combined, these results suggest that both 24 and 48 h test durations are environmentally relevant and defensible durations for toxicity testing as long as control viability remains > 90%.

1.2.3. Need for a lifecycle toxicity approach

Though data generated through toxicity tests with unionids has become more reliable through the standardization of methods, Unionid ecotoxicology now mirrors questions heard throughout the larger field of ecotoxicology: Should we base ecological risk assessment on standardized acute and chronic lethality using assays that lack ecological realism? Indeed mortality events at environmentally relevant exposures will likely cause significant harm to a population. However, with the exception of spills, contaminants are typically found at lower environmental concentrations than those

known to cause overt lethality. Thus it is far more important to assess the effects of chemicals at concentrations found in the environment (Chapman 2002), and to understand how effects at various levels of biological organization will result in adverse consequences on populations (Ankley et al. 2010).

The unique lifecycle of freshwater mussels includes multiple exposure pathways and periods of exposure that could potentially interrupt the maturation and reduce fitness of an individual mussel (Cope et al. 2008). Currently, glochidia and juveniles are the most studied unionid lifestages in toxicity tests (Ingersoll et al. 2007; Van Hassel and Farris 2007). But generally, the tests are performed only with glochidia that are free from the maternal marsupium and have not yet attached to fish. The standardized tests last 24-48 h; however, the process of glochidia encapsulation only takes a few hours (Rogers-Lowery and Dimock 2003; Rogers-Lowery and Dimock 2006; Watters 2007), while the time period within the marsupia and while encapsulated on the fish lasts weeks to months (Watters 2007; Cope et al. 2008). Jacobson et al. (1997) found no difference from the control viability and attachment of *Villosa iris* glochidia exposed *in marsupia* for 30 d to copper at concentrations below the LC50. When glochidia encapsulated on fish hosts were exposed to an aqueous copper solution, no decrease in metamorphosis success from control groups was found (Jacobson et al. 1997); in part, these findings have led to the prevailing thought that both brooding and encapsulated glochidia are protected from waterborne contaminants (Cope et al. 2008). Until recently, no published work has investigated the effect of a contaminant body burden in fish on the attachment and metamorphosis of glochidia. Dubansky et al. (2011) found that injecting fish with cortisol increased the metamorphosis success of *Utterbackia imbecillis* on bluegill

(*Lepomis macrochirus*). Though cortisol is not an environmental contaminant of concern, this work further isolated the importance of the host immune system to successful attachment and metamorphosis of mussels. Little additional work has investigated these glochidial exposure periods, including the effects of accumulated contaminant transfer on mussel metamorphosis, begging the question if ecotoxicologists are ignoring critical periods of exposure to glochidial maturation and metamorphosis into juveniles.

Juvenile mussels are also commonly used in standard toxicity testing approaches, though normally at durations below 28 d because of high control mortality (Ingersoll et al. 2007). After juveniles excyst from fish hosts, they settle to the stream bed and live the first one to several years burrowed in the sediment. Though they will eventually gain the ability to filter-feed, at first they are dependent on obtaining food from the sediment and pore water using cilia tracks on their foot (Watters 2007). Through this 'pedal feeding' behavior juvenile mussels are even more at risk to sediment borne contaminants than during the adult stage (Cope et al. 2008), yet chronic exposures to contaminated sediments are far less common than aqueous exposures in controlled toxicity tests (Ingersoll et al. 2007). Laboratory toxicity studies involving sediment are becoming more common (Newton et al. 2003; Miao et al. 2010; Wang et al. 2011), and most determine the effects of contaminants on mussel growth which is thought to be a good predictor of general health (Cope et al. 2008). Growth rates throughout the juvenile and adult stages are likely important metrics in mussel ecology because maturity and fecundity are more closely related to size than age (Haag and Staton 2003).

Adult mussels are no longer typically used in acute and chronic toxicity tests for the reasons mentioned above (section 1.2.2), but non-lethal endpoints in laboratory and *in situ* exposures have investigated multiple genetic and physiological markers of stress from contaminant activity (Newton and Cope 2007). Indeed the importance of biomarkers will continue to grow in the field of unionid toxicology, and to realize the predictive potential of these tools it is critical that we find linkages between exposure, biomarkers and deleterious effects to population persistence (Salazar and Salazar 2007; Geist 2011). Along the spectrum of biological organization, organismal behavior encompasses numerous endpoints that lie between the sensitive sub-organismal responses and the consequences realized by populations, communities and ecosystems (Amiard-Triquet 2009). Nevertheless, few studies assess contaminant-induced changes in mussel behavior beyond a valve closure response to contaminant presence (Kádár et al. 2001; Cope et al. 2008). However, recently Bringolf et al. (2010) described increases in the frequency of mussel mantle-lure display and premature release of glochidia in the presence of the antidepressant fluoxetine. Many unionid species use highly specialized mantle-lures to persuade fish hosts into closer range to be inoculated with glochidia (Haag et al. 1995; Haag and Warren 2000; Zanatta et al. 2007; Barnhart et al. 2008). Changes in the timing of lure display and glochidia release could disrupt the interactions between mussels and their host fish, resulting in fewer glochidial infections and encapsulated glochidia (Cope et al. 2008).

Also, Flynn and Spellman (2009) reported reduced rates of movement toward spatial aggregation in *Eliptio complanata* exposed to estradiol or atrazine for 72 h. This is particularly interesting because mussels do aggregate along seasonal cycles, a behavior

that is largely attributed to reproduction (Perles et al. 2003). If mussels were to aggregate less often in response to the presence of a contaminant, then population-wide fertilization rates could suffer.

Unionids are becoming increasingly recognized as important candidates for ecotoxicity testing because of their established sensitivity to some contaminants, their potential as biomonitors due to a sessile lifestyle, and adequate bioaccumulation rates but mostly because the taxa as a whole is critically imperiled and essential to the function of the riverine systems in which they occur. The majority of previous toxicity testing has focused on development of acute and chronic toxicity methods that do not fully incorporate environmentally realistic exposures or ecologically relevant endpoints. It is therefore imperative that future research focus on evaluating the toxicity of contaminants throughout the unionid lifecycle to determine the most sensitive lifestage(s) and better estimate risk to native populations.

1.3. Emerging Contaminants

1.3.1. Perfluoroalkly Acids (PFAAs)

Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) belong to a diverse group of industrial chemicals known as perfluorinated chemicals (PFCs), or more specifically perfluoroalkyl acids (PFAAs). PFAAs are completely fluorinated organic compounds with terminal carboxylate or sulfonate groups. Fluorine-carbon bonds have greater polarity and thus are much stronger than the carbon-hydrogen bonds that are replaced, resulting in greater resistance to microbial and photodegredation, leading to the persistence of the chemical (Lau et al. 2007; Conder et al. 2008). PFAAs are unique as

repellants of both water and oil, which explains their primary use in surface protection of carpets, upholstery, paper, food containers and fabric (Konwick et al. 2008; Zushi et al. 2012). These traits also lead to problematic calculations of partition coefficients (octanol-water, K_{ow}) since the compound is not readily dissolved by water or lipids; resulting in difficulty measuring the PFAA in animal or environmental tissue and potentially unreliable BCF and BAF measurements (Martin et al. 2004; Conder et al. 2008; Valsecchi et al. 2013). Nevertheless, PFAAs with more than seven fluorocarbons (PFOS & PFOA both have 8) exhibit moderate tendencies to bind to blood serum proteins, and can accumulate in hepatic tissue (Conder et al. 2008; Valsecchi et al. 2013), as well as gill and kidney tissue in fish (Martin et al. 2003). Evidence of biomagnification along aquatic trophic systems has been well documented (Martin et al. 2004; Kannan et al. 2005; Lau et al. 2007; Ji et al. 2008; Quinete et al. 2009), and despite difficulties in their quantification, PFAAs have been found in trace or higher concentrations throughout the globe in surface waters, air, soils, sediments and ice caps (Lau et al. 2007; Conder et al. 2008).

The effects of PFAAs on native Unionids have been largely ignored until recently. Fernandez-Sanjuan et al. (2013) found that 10-d exposures of PFOS at concentrations as low as 10 µg/L increased multixenobiotic resistance activity, and both 1-10 µg/L increased respiration in zebra mussels (*Dreissena polymorpha*). Much more data exist on the acute and reproductive effects on other freshwater organisms. Forty-eight hour acute lethality (LC50) of daphnids occurs at 17-35 mg/L (PFOS) and 199-476 mg/L (PFOA). Reduced reproduction occurs at 2.5 mg/L (PFOS) and 25 mg/L (PFOA) in *Daphnia magna*, and 0.3 mg/L (PFOS) and 6.3 mg/L (PFOA) in *Moina macrocopa* (Ji

et al. 2008). Interestingly, Japanese medaka (*Oryzias latipes*) appeared to have a more sensitive reproductive response than ostracods as reductions in gonadosomatic-index (GSI), progeny, and growth of offspring occurred at 0.01 mg/L PFOS (Ji et al. 2008). Sub-chronic exposures (21-28 days) of fathead minnow (*Pimephales promelas*) to 0.3 mg/L PFOS resulted in increased plasma levels of sex steroids testosterone, ketotestosterone and estradiol (Ankley et al. 2005; Oakes et al. 2005), suggesting endocrine disrupting potential caused by PFAAs. Although concentrations used in these tests were several orders of magnitude greater than typical PFAA levels found in the environment (typically < 1 μg/L; Lau et al. 2007), exposures in these laboratory studies are typically short (3-28 days). Persistent exposure to PFAAs may result in chronic decreases in reproduction of aquatic species and the paucity of data on the effects of PFAAs on freshwater bivalves needs to be addressed.

1.3.2. Pharmaceuticals

Human pharmaceuticals have become increasingly important contaminants in the aquatic environment. Pharmaceuticals are released into the environment primarily through wastewater treatment effluent. Continuous release can manifest as a "pseudopersistence" downstream of effluent point sources, effectively causing a chronic exposure to aquatic organisms to one or many chemicals (Daughton 2002; Ramirez et al. 2009). These compounds are designed to be highly bioactive for a therapeutic outcome in their intended target. Thus, biological pathways that have been evolutionarily conserved are most affected from environmental exposure (Brooks et al. 2012). Indeed, there are many questions regarding the fate, transport and effects of pharmaceuticals in

the environment (Brooks et al. 2009; Boxall et al. 2012; Brooks et al. 2012). Increasing concern has prompted discussion of better wastewater treatment techniques (Styrishave et al. 2011) and identified the need for better pharmaceutical design and prescription (Daughton 2002; Daughton and Ruhoy 2013).

Among the most studied pharmaceuticals in the environment is the antidepressant fluoxetine, a selective serotonin reuptake inhibitor (SSRI) used as an antidepressant and anti-anxiety medication under the trade name Prozac© (Eli Lilly and Company). Fluoxetine is highly water-soluble (4 mg/mL , log Kow ~ 1.57-4.05), and has a half-life of 1-4 days in humans (Paterson and Metcalfe 2008). Fluoxetine and its primary metabolite, norfluoxetine, are regularly measured in surface waters at 1-10 ng/L (Conley et al. 2008) and have estimated bioconcentration factors (BCF) in fish between 74 and 117 (Paterson and Metcalfe 2008). Freshwater mussels may concentrate fluoxetine from the water column at greater rates than fish because of their filter feeding strategies. Bringolf et al. (2010) calculated fluoxetine bioaccumulation factors (BAF) in caged mussels (*Elliptio complanata*) after 14-day exposures downstream from a municipal wastewater effluent. Although concentrations of fluoxetine were highest in mussel tissue in the effluent channel (~ 75 ng/g wet wt.), accumulation factors were nearly double at sites 50 and 100 m downstream where water concentrations were much lower (Bringolf et al. 2010).

Fluoxetine can have multiple effects on aquatic invertebrates. Although acute lethal concentrations (*Ceriodaphnia dubia*; LC50s) are typically in the range of 200-500 μg/L (Henry et al. 2004), sublethal effects on reproduction are reported at concentrations an order of magnitude lower. Concentrations of 100 μg/L significantly reduced growth in

Hyalella azteca after 15-day exposures, and 30 μg/L decreased neonate size (but not numbers) in third generation *Daphnia magna* in a 21-day multigenerational experiment (Péry et al. 2008). Interestingly, some freshwater snails (*Potamopyrgus antipodarum*) exposed to fluoxetine for six weeks exhibited increased reproductive output (# offspring/female) at concentrations near 10 μg/L; however, higher concentrations (> 30 μg/L) significantly reduced reproductive output compared with control groups (Péry et al. 2008; Gust et al. 2009). This effect appears to be species-specific as a similar species (*Valvata piscinalis*) was unaffected under the same experimental conditions. The authors speculated that the differences between species may be attributed to differences in snail lipid content which may influence the availability of fluoxetine to the animal. The unaffected *Valvata piscinalis* contains higher body lipid levels which ultimately may protect it against low-level fluoxetine effects (Gust et al. 2009).

The effect of environmental concentrations of fluoxetine on bivalve mollusks is yet unclear. Numerous studies have shown that serotonin is involved in gamete parturition in non-unionid bivalves and in glochidia release in the Unionidae, suggesting that a drug designed to increase serotonin availability (i.e., fluoxetine) may have a similar effect. Male zebra mussels (*Dreissena polymorpha*) significantly increased spawning at ~ 3 μg/L, and females at ~ 31 μg/L fluoxetine (Fong 1998). Glochidia parturition has been induced in the unionid *Alasmidonta cygnea* exposed to ~ 300 μg/L fluoxetine for 24 hours (Cunha and Machado 2001), and the release of non-viable glochidia increased in *Elliptio complanata* exposed at similar concentrations for 48 hours (Bringolf et al. 2010). *In vitro* immunoassays have also been conducted on *E. complanata* to assess how fluoxetine affects phagocytic activity, intracellular esterases, and cell adherence, but the

concentrations producing increases in these processes are not likely to occur in the environment (~ 30 mg/L; Gagne et al. 2006). Concentrations needed to produce deleterious effects in the lab may be dependent on the duration of exposure. Therefore, persistent exposure to fluoxetine at lower doses may elicit a response undetected under previous laboratory exposures. Furthermore, few studies have investigated the effects of fluoxetine on freshwater mussels exposed during brooding, metamorphosis, and early juvenile life-stages.

1.4. Current Research

In the chapters herein, I report novel findings of the effects of two emerging contaminants: PFOS and fluoxetine, on the survival and behavior of freshwater mussels at several life stages. In Chapter 2 my coauthors and I investigate the acute toxicity of PFOA and PFOS to early juvenile and larval freshwater mussels using standard toxicity methods. We also assess the effects of PFOS on glochidia of the fatmucket (*Lampsilis siliquoidea*) through a novel partial-lifecycle assay, which incorporates 30-day exposure *in marsupia*. In Chapter 3, we apply both standard unionid toxicity methods as well as the partial-lifecycle assay to the toxicity of fluoxetine on the wavy rayed lampmussel (*Lampsilis fasciola*). We measured behavioral endpoints on brooding lampmussels throughout the *in marsupia* exposure. We then expanded on these behavioral changes by determining the effects of fluoxetine on movement and feeding behavior in adult lampmussels during a novel 67-day exposure design summarized in Chapter 4. Two smaller experiments are incorporated in Chapters 5 and 6, in which we examined the effects of a PFOS body burden in host fish on the metamorphosis success and survival of

paper pondshell (*Utterbackia imbecillis*; Chapter 5), and the effects of a seven day aqueous PFOS exposure on juvenile fatmucket survival in a new exposure chamber design (Chapter 6).

Though the work in the coming chapters does not include the effects of continuous exposure throughout an entire lifecycle of a unionids species, I have investigated important periods of exposure that may have been missed or disregarded in previous research. By testing environmentally relevant concentrations at environmentally relevant exposure durations, I feel we will better understand the most sensitive life stage(s) to environmental contaminants. Further, by combining data and endpoints from exposures at multiple stages, we will better understand the adverse outcome to populations of unionids.

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CHAPTER 2

PARTIAL LIFE-CYCLE AND ACUTE TOXICITY OF PERFLUOROALKYL ACIDS TO FRESHWATER MUSSELS[1]

[1] Peter D. Hazelton, W. Gregory Cope, Tamara J. Pandolfo, Shad Mosher, Mark J. Strynar, M. Christopher Barnhart, and Robert B. Bringolf. 2012. *Environmental Toxicology & Chemistry* vol. 31(7):1611-1620
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Abstract

Freshwater mussels are among the most sensitive aquatic organisms to many contaminants and have complex life-cycles that include several distinct life stages with unique contaminant exposure pathways. Standard acute (24 to 96-h) and chronic (28-d) toxicity tests with free larva (glochidia) and juvenile mussels are effective at generating data on contaminant effects at two discrete life-stages, but do not incorporate effects on brooded glochidia. We developed a novel partial life-cycle assay that incorporates exposures to brooding adult female mussels and used this method in combination with acute toxicity tests to assess adverse effects of perfluoroctanesulfonic acid (PFOS) and perfluoroctanoic acid, (PFOA) on freshwater mussels. Fatmucket (*Lampsilis siliquoidea*) were exposed to PFOS at two life stages: brooding glochidia (*in marsupia*) for 36 d and free glochidia in water for 24 h. In standard acute tests with glochidia (24- 48 h exposures) and juveniles (48-96 h exposures) of fatmucket and black sandshell (*Ligumia recta*), glochidia were 8 to 25 times more sensitive than juveniles. PFOS significantly reduced duration of glochidia viability and reduced probability of metamorphosis at concentrations 3,000 times lower than the most sensitive acute endpoint (24-h-EC50). The partial life-cycle test is adaptable to a variety of endpoints and research objectives, and is useful for identifying adverse effects at contaminant concentrations below those required for an acute lethal response.

Keywords

Perfluoroalklyl acids, Perfluorinated compounds, Akaike's information criteria, Unionidae

2.1. Introduction

Freshwater mussels (Order Unionoida) are among the most imperiled taxanomic groups worldwide (Williams et al. 1993). In North America, as many as 72% of the native fauna are listed as endangered, threatened, or of special concern, and more than 7% are likely extinct (Williams et al. 1993). Understanding the effects of contaminants on freshwater mussels is critical to conservation efforts and environmental risk assessment as mussels are often among the most sensitive species to aquatic contaminants (Augspurger et al. 2003; Cope et al. 2008; Raimondo et al. 2008). Freshwater mussels have a complex life history that includes internal fertilization of gametes, gestational brooding of larvae (glochidia) within the adult female for one to many months and obligate parasitic metamorphosis on the gills or fin epithelial tissue of host fish, followed by excystment from the host and growth through a juvenile stage to adulthood (Barnhart et al. 2008). Each discrete life-stage is critically important to the recruitment of breeding adults in a population (Barnhart 2006) and represents a unique exposure period to contaminants (Cope et al. 2008).

Internationally approved standard acute and chronic toxicity test guidelines have been developed for larval and juvenile freshwater mussels (ASTM 2006). Acute tests consist of aqueous contaminant exposures to glochidia for 24 and 48 h, and to juveniles for 24, 48, and 96 h and are efficient methods for determining median effective (EC50) and lethal concentrations (LC50; ASTM 2006). Chronic toxicity tests (28 d) of water-only contaminant exposures and aqueous exposures in the presence of sediment, have also been effective at determining lethal and sublethal (e.g., growth effects) contaminant concentrations on juvenile mussels (ASTM 2006; Wang et al. 2007; Wang et al. 2011).

However, because these approaches focus on only two distinct life-stages of freshwater mussels, the relevance to environmental exposures is limited and may differ among toxicants (Cope et al. 2008). Further development of partial life-cycle assays is warranted to determine the most-sensitive life-stages to environmental contaminants, and to improve predictive power of risk assessments.

Perfluoralkyl acids (PFAAs) uniquely repel both water and oil, which explains their primary use in surface protection of carpets, upholstery, paper, food containers, and fabric as well as in fire suppressants (Konwick et al. 2008; Giesy et al. 2010). PFAAs are fully fluorinated organic compounds with terminal carboxylate or sulfonate groups. Fluorine-carbon bonds have greater polarity and thus are much stronger than carbon-hydrogen bonds, resulting in environmental persistence of many chemicals in this class (Giesy et al. 2010). The PFAAs with eight or more fluorocarbons (e.g., perfluoroctanesulfonic acid [PFOS] and perfluoroctanoic acid, [PFOA]) exhibit moderate tendencies to bind to blood serum proteins, can accumulate in hepatic tissue, and demonstrate biomagnification in aquatic food webs (Kannan et al. 2005; Lau et al. 2007; Quinete et al. 2009; Giesy et al. 2010). Concentrations of PFAAs in the aquatic environment are typically in the ng/L range (Lau et al. 2007), but concentrations have been measured near 1 μg/L in areas of long-term pollution (Konwick et al. 2008; Lindstrom et al. 2011), and in excess of 2,200 μg/L in locations of accidental spills of fire-fighting foams (Moody et al. 2001; Oakes et al. 2010). Several studies have reported PFAA bioconcentration in estuarine bivalves (Kannan et al. 2005; Quinete et al. 2009), but the effects of PFAAs on native freshwater mussels have not been published to date.

Persistent exposure to PFAAs may result in decreased reproduction of aquatic species (Lau et al. 2007; Bots et al. 2010); thus, the effect of PFAAs on freshwater bivalves needs to be addressed.

In the present study, we combined use of a novel partial life-cycle approach with standard acute toxicity test methodology to assess the effects of PFAAs on freshwater mussels. In the partial life-cycle study, mussels were exposed to PFOS at one or both of two discrete life-stages: (1) *in marsupia* exposure: glochidia brooding within the adult female, and (2) free glochidia exposure: glochidia in water. This approach facilitated identification of the most sensitive exposure period for larval mussels and presents a unique approach to complement current acute and chronic toxicity tests for freshwater mussels (ASTM 2006).

2.2. Methods & Materials

2.2.1. Test organisms

Fatmucket *Lampsilis siliquoidea* (Barnes) and black sandshell *Ligumia recta* (Lamarck) were used in acute toxicity tests in the present study; however, only fatmucket were used in the partial life cycle tests and juvenile toxicity tests. Brooding female fatmucket were collected from the Silver Fork of Perche Creek in Boone County, Missouri and brooding female black sandshell mussels were collected from the Meramec River in Jefferson County, Missouri. Both rivers support healthy, stable mussel communities. Juvenile test organisms were propagated with previously published (Barnhart 2006) culture methods at Missouri State University and the University of Georgia. Juvenile fatmucket metamorphosed on largemouth bass (*Micropterus*

salmoides) and black sandshell juveniles metamorphosed on walleye (*Sander vitreus*). Glochidia were <24 h old at the start of each acute toxicity test and juveniles ranged in age from 4 to 6 weeks at the time of testing.

2.2.2. Partial life-cycle test

Brooding female fatmucket collected from Perche Creek (Boone County, MO) in March 2009 were shipped overnight to the University of Georgia. Mussels were packaged in individual plastic bags with 1 L of water in a cooler with ice. Upon delivery, water temperature was 9 to 11°C and mussels were unpacked and scrubbed with a non-abrasive sponge to remove any visible periphyton or debris from the periostricum. Mussels were held in a 530-L Living Stream (Frigid Units Inc.) with natural pond water and approximately 5 cm of quartz/silica sand (Playsand, Quickcrete Products Corp). In the Living Stream, pond water was renewed once per week and holding temperatures were maintained at 5 to 10°C to inhibit parturition of glochidia (Wang et al. 2007).

Brooding female mussels (*in marsupia* exposure) were exposed to PFOS (0, 1, 100 µg/L) in water for 36 d followed by extraction of the glochidia and then a 24 h aqueous PFOS exposure (0, 1, 100 µg/L) with the free glochidia (Figure 2.1). We assessed the effects of PFOS on glochidia survival and duration of viability to a subset of the glochidia exposed at each stage. All remaining free glochidia at the end of the 24 h aqueous exposure were then infected on host fish (largemouth bass, *Micropterus salmoides*) to test metamorphosis success to the juvenile stage. We used this approach to

reflect environmentally realistic conditions of exposure for glochidia in the different stages and to determine the stage at which glochidia were more sensitive to PFOS exposure.

Only adult fatmucket with initial glochidia viabilities > 70% were used in the partial-life-cycle study. We used a 19-guage hypodermic needle and dechlorinated city water to flush glochidia from two interlamelar spaces (i.e., watertubes) from the marsupial demibranchs (McMahon and Bogan 2001) of each female. Ten subsamples of 30 to 50 glochidia were assessed for viability using the shell-closure response to NaCl (ASTM 2006). Twelve adult mussels were identified as having adequate viability and four individuals were randomly assigned to each of three marsupial exposure treatment groups. Exposures (36 d) were conducted in 3.8-L glass jars with one adult mussel per jar. Each jar was aerated and all jars were maintained in an environmental chamber. Initial temperatures were similar to the holding temperature (mean = 7.5° C) for the first 7 d of the exposure, and then gradually increased 2° C per day to approximately 15° C and maintained for the remainder of the experiment. All glassware was triple washed with methanol, and preconditioned with PFOS treatment concentrations for 24 h prior to introduction of mussels. All *in marsupia* exposures were conducted in filtered, dechlorinated tap water held for > 24 h in the experimental chamber to reach target temperature prior to water changes. Mean hardness (47.5 ± std. dev. 9.2 mg $CaCO_3$/L), and alkalinity (34.8 ± 4.1 mg $CaCO_3$/L) were measured by titration twice weekly (n = 8) prior to water changes. Water changes (100%) were conducted daily and water quality (pH, temperature, and dissolved oxygen) was measured prior to water change with a Hydrolab Quanta multiprobe (Hach Company) in one replicate of each treatment group.

Replicates used for water quality measurements were changed daily to allow measurements from all four replicates every 4 d. For all treatments, water temperature ranged from 14.6 to 16.1°C, dissolved oxygen ranged from 6.1 to 7.3 mg/L, and pH ranged from 7.6 to 8.5 but did not differ across treatments.

After the 36 d exposure, adult mussels were removed from exposure vessels and approximately 120,000 glochidia were gently flushed (with a syringe) from each mussel. Subsamples of 200 to 250 glochidia from each mussel were used to determine viability immediately following the *in marsupia* exposure using the same procedure as above. Remaining glochidia were used for the 24 h aqueous PFOS exposure (see below). Length and wet weight of each female was recorded and two mussels from each treatment group were euthanized, removed from their shells and whole soft tissues were frozen at -80° C for later chemical analysis.

Glochidia were pooled from females within each *in marsupia* treatment to average individual effects among females. We then divided each group of free glochidia into approximately equal aliquots for the 24h aqueous exposure to PFOS (Figure 2.1). Glochidia from treatment groups of 1 μg/L and 100 μg/L (*in marsupia* exposures) were each divided into four aliquots for treatment at the corresponding PFOS concentration and four aliquots to be treated with only dilution-water during the 24 h exposure. Glochidia from the control groups for the *in marsupia* exposure were divided into twelve aliquots, four of each treated with 0, 1, or 100 μg/L of PFOS, respectively. Therefore, the exposure regimen resulted in four replicates for each of seven treatment groups (Figure 2.1) for use in glochidia viability duration and metamorphosis experiments. Free glochidia PFOS exposures (24 h) were conducted in 150 mL glass beakers with 100 mL

of dechlorinated tap water. All glassware was preconditioned with target PFOS concentrations for 24 h and a water change was performed prior to introduction of glochidia. Exposures were conducted in an environmental chamber at 20°C.

Following the 24 h free glochidia exposure, a subsample of glochidia from each replicate was inoculated on host fish, largemouth bass, to determine if PFOS exposure affected the ability of glochidia to metamorphose to the juvenile stage. Among all treatments, host fish weights ranged from 4.7 to 12.5 g and lengths ranged from 6.4 to 9.2 cm and fish size did not differ among treatments. Inoculations were conducted on individual fish in 1 L of filtered dechlorinated tap water in a 1.5 L glass aquarium for 15 min. Each aquarium was aerated to suspend glochidia and facilitate attachment to host fish. Immediately prior to inoculation, glochidia density was adjusted to approximately 1,000 viable glochidia/L, based on glochidia viability assessed on a subsample of 50 to 100 glochidia using the NaCl shell-closure method (ASTM 2006). After a 15 min inoculation period, the fish were rinsed to remove unattached glochidia and were placed in individual 3-L tanks in a recirculating aquaculture system (Aquatic Habitats, Inc.). The outflow of each tank was fitted with a 150-µm mesh cup to collect sloughed glochidia and metamorphosed juveniles. Cups were monitored every 24 to 48 h for 19 d following inoculation. Metamorphosis success was calculated for each fish as the proportion of successfully metamorphosed juveniles to the total number of juveniles and glochidia sloughed off each fish.

We also tested duration of viability for glochidia from each treatment. Aliquots of approximately 2,000 glochidia from each treatment were placed in 100 mL of dechlorinated tap water in 150 mL glass beakers. All aliquots were kept in an

environmental chamber at 20° C and 50% of the water was replaced every 48 h. Viability, as determined by valve closure response to NaCl, was determined every 48 h for up to 8 d for five subsamples of 30 to 50 glochidia from each aliquot. Viability duration was assessed at 48-h time points rather than every 24 h because this was more logistically feasible given that metamorphosis success was also assessed every other day, thus allowing for alternating efforts on these two assays. Twenty-four hour time points of viability duration would add more precision to the data and are recommended for future studies if resources are available.

2.2.3. Acute toxicity tests

Standard acute toxicity tests of PFOS and PFOA with glochidia and juvenile fatmucket and black sandshell were carried out at North Carolina State University. For tests with glochidia, the organisms were shipped from Missouri State University in coolers via overnight courier. Upon arrival, viability of glochidia was assessed by exposing three sub-samples of 50 to 100 glochidia (each) to a saturated NaCl solution, which initiates shell closure in viable glochidia. We used glochidia for toxicity tests only if initial viability exceeded 90%, in accordance with standard guidelines (ASTM 2006). The average temperature of culture shipping water at the time of receipt was 19.2 °C (range 17.1 to 21.4 °C). Tests were 48-h non-aerated static experiments. At 24 and 48 h, viability (i.e., shell closure) was assessed with the addition of a saturated NaCl solution to a subsample of 50 of the 150 glochidia from each of three replicates per treatment.

Juvenile mussels were also shipped in coolers via overnight courier, and average shipping water temperature was 22 °C (range 18.2-23.5 °C) at the time of receipt. Upon

arrival mussels were acclimated to the test temperature and test water by adjusting their shipping temperature with three 50% water replacements using dilution water held at 20°C. Mussels were given a 48 h acclimation period once the target test temperature of 20°C was reached. Acute toxicity tests consisted of 96 h non-aerated static tests with 90% water renewal at 48 h. Survival (based on foot movement inside or outside of the shell) was assessed visually (7 mussels per treatment replicate and 10 mussels per control replicate) in each of three replicates per treatment at 48 and 96 h with an Olympus SZ61 microscope (ASTM 2006; Bringolf et al. 2007).

Each acute test consisted of three replicates of each of six test chemical concentrations (0.005, 0.05, 0.5, 5, 50, 500 mg/L PFOS or PFOA) and a dilution-water control. A dilution factor of 10 was used because no previously published tests of acute toxicity of PFAAs on freshwater mussels were available at the time of study design. A narrower range of concentrations is recommended for acute toxicity studies when a preliminary range has previously been established (ASTM 2006).

Sodium chloride (NaCl) was used as a reference toxicant for quality assurance purposes; reference toxicant tests have been used in previous studies with glochidia to serve as a measure of relative health and condition of test organisms (Bringolf et al. 2007; Cope et al. 2008). The reference toxicant tests were conducted by preparing treatment concentrations ranging from 0.25 to 8.0 g NaCl/L with 7 concentrations and a dilution factor of 0.5. Concentrations of NaCl were confirmed at the start of the test with a salinity meter (YSI 30, Yellow Springs Instruments) and were within 0.05 g/L of target in all treatments. Glochidia viability was assessed at 24 and 48 h, as described for PFOS and PFOA.

Reconstituted hard water (hardness 160-180 mg/L as CaCO3) was used as dilution water for all toxicity tests (Delinsky et al. 2010) and was prepared by adding reagent-grade salts ($CaSO_4$, $2H_2O$, $MgSO_4$, KCl, and $NaHCO_3$; Fisher Scientific) to deionized water (Delinsky et al. 2010). Standard methods were used for measurement of all water quality parameters as described elsewhere (Bringolf et al. 2007). For all PFAA acute tests, alkalinity ranged from 97 to 110 mg $CaCO_3$/L with a mean of 104.4 mg $CaCO_3$/L, hardness ranged from 132 to 162 mg $CaCO_3$/L with a mean of 149.6 mg $CaCO_3$/L, conductivity ranged from 514 to 643 µs/cm with a mean of 556.5 µs/cm, pH ranged from 8.05 to 8.56 with a mean of 8.46, and dissolved oxygen ranged from 8.16 to 9.46 mg/L with a mean of 8.62 mg/L (n = 12 for alkalinity and hardness, n = 55 for all other parameters).

2.2.4. Test chemicals & quantification

Chemicals (PFOS, >98% purity; PFOA, 96% purity) were purchased from Fisher Scientific or Sigma Aldrich. Certified NaCl (ACS grade, Fisher Chemical) was used in reference toxicant tests. Analyses of PFAA concentrations in water and tissue samples were conducted at the United States Environmental Protection Agency's (U.S. EPA) National Exposure Research Laboratory (NERL) in Research Triangle Park, NC, USA.

During the 36 d *in marsupia* exposure, water samples for PFOS analysis were collected on day 10 and 11 of the exposure. We collected 15 mL from two replicates of each treatment, one immediately prior to renewal and another at 15 min following the water change. Samples were stored in 15-mL conical centrifuge tubes (BD Falcon; Becton, Dickinson & Company) at 4°C. Water samples were shipped overnight to NERL

for solid-phase extraction (SPE) and high-performance-liquid-chromatography/mass-spectrometry (HPLC/MS) analysis following the protocol of Nakayama et al. (2010) for water samples with target concentrations less than 0.1 µg/L PFOS. For target PFOS concentrations over 1.0 µg/L, water samples were directly injected due to sufficiently high analyte concentrations. Two standard curves were used to quantify PFOS water concentrations during the partial-life-cycle experiment: low range (0.5, 0.25, 0.5, 0.75, 1.0, 2.5, 5.0 µg/L) and high range (1, 5, 10, 25, 50, 100, 150 µg/L). Two replicate samples were measured at each standard concentration. Accuracy (recovery) of PFOS in the low range standard curve ranged from 89.5 to 123% (n=7) and for the high range standard curve accuracy was 85.3 to 123% (n=7).

Adult mussel tissue samples were collected immediately following the 36-d *in marsupia* exposure from two replicates in each treatment and stored at -80° C. Samples were shipped to NERL for PFOS analysis. Whole tissue homogenization, SPE, and HPLC/MS procedures followed were based on modifications of the methods previously described by Ye et al. (2008) and Delinsky et al. (Delinsky et al. 2010). Recovery of PFOS in standards used for tissue analysis ranged from 97.2 to 104.5% (n=7).

At the start of each acute toxicity test, water samples were composited from all replicates of each treatment concentration and were shipped overnight to NERL for PFOS or PFOA analysis as described for water samples in the partial lifecycle test. Samples were stored in 15-mL conical centrifuge tubes (BD Falcon; Becton, Dickinson & Company) or 1-L Nalgen HDPE bottles at 4° C until analysis. Standard curves for PFOS

and PFOA ranged from 5 to 500,000 ng/ml (ten-fold dilution series). Recovery of PFOS standards ranged from 95.2 to 102% (mean 98.3%, n=6) and recovery of PFOA standards ranged from 91.2 to 108% (mean 98%, n=6).

2.2.5. Statistical analyses

For the partial life-cycle test, we used a mixed-model logistic regression framework with multi-model inference to assess the effects of exposure type (i.e., *in marsupia* or free glochidia) and PFOS concentration (0, 1, 100 µg/L) on viability duration and metamorphosis success. This approach is different from traditional ANOVA tests in that it allows for mediation of random-effects of nested variables. Additionally, data are not subject to the assumptions of a normal distribution as in ANOVA and allow for more predictive measurements of effect than nonparametric statistics (Bolker et al. 2009). We incorporated Akaike's information criteria (AIC) to assess the relative importance of competing regression models. Model selection through AIC is an established approach that is gaining popularity in analysis of ecological data, and incorporates the principle of parsimony to determine the most efficient explanations in likelihood models (Burnham and Anderson 2002).

All statistical tests were conducted in the R statistical programming platform (R Core Development Team 2013). Count data from viability assessments and host-fish metamorphosis experiments were expressed as a binary response where 0 is a failure and 1 is a success (i.e., viability: 0 = nonviable, 1 = viable; metamorphosis: 0 = untransformed glochidia, 1 = metamorphosed juvenile). This approach resulted in a hierarchically structured dataset, where an individual glochidia or juvenile response is

nested within a group (i.e., aliquot beaker for viability assessment and fish during a host-fish transformation). To account for variation among each group within a treatment, we used a mixed-model logistic regression with the lme4 package (Bates and Sarkar 2007) to model an intercept that varies randomly among groups (i.e., a random effect) unrelated to treatment. The lme4 package allows modeling of more than one random effect (Bolker et al. 2009), and in the viability duration analysis, we used a second random effect parameter to assess changes in slope variation among groups at each day post removal of glochidia from the parent mussel.

We fit generalized linear models (GLM) and mixed models of the response parameter and used AIC (Burnham and Anderson 2002) to compare relative support for the same models with random effects. We used the model (mixed effects or GLM) with the lowest AIC value for further model selection procedures. AIC values were considered directly comparable between GLM and mixed models because the functions used to calculate the maximum likelihood estimates for each model used equivalent algorithms and both model types use the Laplace approximation to determine the log-likelihood and AIC values (personal communication: Dr. Douglas Bates, University of Wisconsin, Madison). With the most accurate model (mixed-effects or GLM), we constructed several competing candidate models to assess the relative importance of each descriptive variable on the response variable and used AIC and relative importance weights (w_i) to determine the most parsimonious model (Table 2.1; Burnham and Anderson 2002). We used the parameter estimates (i.e., logits, or log odds) of fixed-effects from the best fitting model to assess the effect of that parameter on the response. Wald statistics (z) and standard errors were also calculated, and the level of significance

was considered $p < 0.05$. Wald statistics are a standard statistic on the statistical significance of a logit from the null model in a logistic regression and are similar in interpretation to an F-value in an ANOVA (Hair et al. 1998; Bolker et al. 2009). We converted logits to probability estimates using the logit link function (Hair et al. 1998) and calculated 95% confidence intervals around the probability estimate using the Wald standard error of the logit multiplied by 1.96.

For acute toxicity tests, nominal concentrations of PFOS, PFOA, and NaCl were used to calculate the median effective concentrations and 95% confidence intervals (CIs) using the Trimmed Spearman-Karber method with ToxCalc statistical software (version 5.0.231, Tidepool Scientific Software). Estimates of EC50s were considered significantly different within a test when 95% CIs for treatment groups did not overlap.

2.3. Results

2.3.1. Chemical quantification

Measured test concentrations of PFOS and PFOA were within 10% of target in water from acute tests; measured concentrations from the partial life-cycle test were not as close to target (Table 2.2). Calculations for PFOS concentration in the control and low dose were derived from the low range standard curve and the high range standard curve was used to calculate the PFOS concentration for the 100 µg/L target concentration. In the partial life-cycle test, the control treatment contained measurable PFOS concentrations in water; however, whole body tissue concentrations of PFOS in adult

mussels increased in a dose-dependent manner in other treatment groups (Table 2.2). Target PFOS treatments of 1 and 100 µg/L in water are subsequently referred to by the measured concentrations (4.5 and 69.5 µg/L, respectively).

2.3.2. Partial life-cycle exposure to PFOS

There were no mortalities to adult *L. siliquoidea* during the 36-d partial life-cycle test. Immediately following *in marsupia* exposure (0 d post-removal), glochidia viability ranged from 50 to 91% in all treatments and did not significantly differ among treatments (Figure 2.2A); however, viability of PFOS-treated mussels declined precipitously beginning at 1 d post-removal from females. By day 7 post-removal, probability of glochidia viability from females exposed to both PFOS levels was approximately 10%, whereas glochidia from control females had >50% probability of viability at this time point (Figure 2.2A). Compared to the relatively constant rate of decline in viability of the control group, there was rapid reduction in viability in the 4.5 and 69.5 µg/L treatment groups. There was no statistical difference between viability of the 4.5 and 69.5 µg/L PFOS treatment groups, though viability of glochidida from the two treatments decreased substantially after test day 1 post-removal and were significantly lower than the viability in the control on test days 3,5, and 7 (Figure 2.2A).

The mixed-effects model had greater explanatory weight (AIC = 22849) than the GLM (AIC = 25510) for the partial life-cycle assay; therefore, the candidate set of a priori models (Table 2.1) was carried out with terms to account for random-effects. Of the candidate models, the *in marsupia* exposure model held the greatest weight of evidence and explained 78% of the variability in glochidia viability (AIC = 22843, w_i =

0.78). The *in marsupia* X Glochidia Exposure model was the next best model with 17% support (AIC = 22847, $w_i = 0.17$) and all other models had less than 10% support. Therefore, the *in marsupia* exposure model was used to generate parameter estimates (Table 2.3). Logit estimates from the *in marsupia* exposure model were all statistically significant at $p < 0.0001$ with negative relationships (Table 2.3), suggesting that PFOS exposures of 4.5 and 69.5 µg/L, number of days post-exposure, and interactions between exposure treatment and days post-exposure have significant negative effects on the viability of glochidia. For example, interactions between PFOS treatment and time can be explained as a change in the rate of decrease across days with an associated change in treatment, similar to an interaction in a traditional ANOVA (Hair et al. 1998).

Metamorphosed juvenile fatmucket were first collected six days post-inoculation, peaked on day nine, and were not collected after day 17. *In marsupia* PFOS treatment levels of both 4.5 and 69.5 µg/L reduced the probability of metamorphosis; however, only the 100 µg/L treatment was significantly different at the $\alpha = 0.05$ level of significance (Figure 2.2B). The AIC values from mixed-effect models (AIC = 21961) were lower than simple GLM (AIC = 22580) so we accounted for the random variation among fish using the mixed-effect model for the remainder of the candidate models. Of the candidate models, the *in marsupia* exposure model had 83% support as the most explanatory model (AIC = 21955, $w_i = 0.83$) and was used for parameter estimation (Table 2.3). All other candidate models had 13% support or less (Table 2.3).

2.3.3. Acute toxicity tests

All acute tests with glochidia and juveniles met acceptability criteria of >90% survival in control treatments (ASTM 2006; Ingersoll et al. 2007; Wang et al. 2011).

Both PFOS and PFOA caused acute toxicity to fatmucket and black sandshell glochidia, but in both species, PFOS was more toxic to glochidia than PFOA (Table 2.4). Glochidia of both species were more sensitive to PFOS than juveniles; for juvenile tests, we could not calculate an EC50 for PFOA within the range of tested concentrations (EC50 > 500 mg/L) because fewer than 50% of juveniles were affected in even the highest test concentrations. Results of NaCl reference toxicant tests (Table 2.4) were consistent with reference values in the literature for these and other freshwater mussel species (Bringolf et al. 2007; Valenti et al. 2007).

2.4. Discussion

The primary objective of the present study was to develop a novel partial-life-cycle assay for the effects of chemical contaminants on freshwater mussels. The method we describe can be a valuable tool for assessing environmentally relevant exposures to several life-stages of freshwater mussels (e.g., adult, brooding, and free glochidia). Indeed, our study revealed effects of PFOS at concentrations lower than previously described for mussels and other taxa. Application of this approach should increase our knowledge of the contaminant effects on freshwater mussels because it (1) facilitates the identification of the most sensitive life-stage to individual toxicants, (2) incorporates environmentally realistic exposure concentrations, and (3) is adaptable to include further endpoints on adults (e.g., behavioral or physiological measurements) and metamorphosed juveniles (e.g., time to death, physiological parameters, behavior, etc.). Adult exposures also allow for investigation of sublethal contaminant-induced effects including, but not

limited to, changes in reproductive status, physiological parameters, molecular markers, and behavior, although these were not assessed in the present study.

Few studies have compared the effects resulting from contaminant exposures within the brooding female and to free glochidia. In an investigation of the most sensitive unionid life-stage to copper toxicity, Jacobson et al. (1997) exposed brooding female *Villosa iris* to 0, 8, and 17 µg Cu/L for 30 d and compared glochidia viability and attachment rates on largemouth bass. Viability was assessed immediately following removal from copper exposure, and no significant difference was found between treatment and control groups. Because viability was only assessed once, and duration of viability was not measured, no conclusions can be drawn regarding the *in marsupia* toxicity of Cu compared to PFOS in the present study. *In marsupia* exposures and duration of viability will likely be a useful tool to compare freshwater mussel sensitivity among different classes of toxicants which may differ, among other things, in their ability to cross marsupial gill membranes. Jacobson et al. (1997) also compared viability and attachment rates in released (i.e., free) *V. iris* glochidia exposed to 0, 24, 42, 59 µg Cu/L for 24 h. The concentrations tested had no effect on attachment success of *in marsupia* or free glochidia. However, in the Jacobson et al. study (1997), copper concentrations tested were not the same at both exposures (*in marsupia* vs. free glochidia). Thus, the method could not provide an adequate comparison of sensitivity between life-stages. The exposure design in the present study improves on the previous method because it uses the same contaminant concentrations across exposure types and therefore allows direct identification of the most sensitive period of exposure. Use of metamorphosis as an

endpoint may also be more sensitive than glochidia viability or attachment as metamorphosis is a complex process that may be interrupted through a variety of biochemical pathways.

In the present study, freshwater mussels were sensitive to PFAAs in both acute and chronic toxicity tests. Acute toxicity data of PFOS and PFOA presented in the present study suggest that freshwater mussel glochidia are among the most sensitive organisms tested to date with PFAAs (Qi et al. 2011). Qi et al. (2011) reviewed the currently published acute toxicity values for PFOS to aquatic organisms and found that only three organisms (96-h LC50s, shrimp – *Mysidopsis bahia* = 3.6 mg PFOS/L, *Neocardina denticulate* = 10 mg PFOS/L, fish – *Pimephales promelas* = 9.1 mg PFOS/L) had mean LC50s below those of 24- and 48-h glochidia tests reported in the current study. Prior to the present study, only one other study had examined the effects of PFOS on freshwater mussels, but it was not published in peer-reviewed literature (Drottar and Krueger 2000; reviewed by Beach et al. 2006 and Qi et al. 2011). In that study, *Unio complamatus* were exposed to PFOS for 96 h and the LC50 was reported at 59 mg/L, which is lower than the 96 h LC50s for juvenile mussels in the current study (e.g., *Ligumia recta*, Table 2.4). The life-stage of animals used in the *Unio* test was not reported, but length ranged from 45 to 55mm.

We found that *in marsupia* exposure to PFOS for 36 d was significantly more harmful than exposure of free glochidia for both viability duration and metamorphosis. Duration of viability was significantly reduced compared to the control at PFOS concentrations 3,000 times lower (4.5 µg/L) than the most sensitive acute endpoint (glochidia 24-h EC50 = 13.5 mg/L). The *in marsupia* exposure model was the best

explanatory model for both endpoints and free glochidia exposure (24 h) did not significantly affect either endpoint at the concentrations tested. *In marsupia* exposures were not only more diagnostic of adverse effects of PFOS on glochidia survivorship and metamorphosis, but are also environmentally realistic because mussels brood glochidia for several weeks to nearly a year, depending on species (Cope et al. 2008). Conduct of toxicity tests that incorporate *in marsupia* exposures is highly desirable for environmental relevance but exposure of brooding adult mussels is often impossible or impractical because many species are imperiled and large numbers of suitable test organisms are not available. Relationships between acute and chronic (*in marsupia*) glochidia and juvenile exposures should be further examined to determine if chronic toxicity could be predicted from acute test results.

Our dataset for probability of glochidia viability exposed *in marsupia* failed to suggest a linear concentration-response relationship (Figure 2.2A). A threshold response may be present because responses were similar at 4.5 and 69.5 μg/L. We did not test for mechanism(s) of PFOS toxicity in the present study; however, reductions in glochidia viability duration and metamorphosis success may be due to behavioral avoidance of an irritant (i.e., shell closure) by the brooding female rather than mechanistic toxicity of the pollutant. Cope et al. (2008) previously showed that in the presence of Cd, *L. siliquoidea* reduced filtering activity and oxygen consumption. Therefore, the decrease in survival in our study may have been caused by a physiological consequence (e.g., asphyxia of brooding glochidia) of shell closure behavior by the adult female. Nevertheless, the resulting reduction in viability/survival is still a response to the presence of PFOS.

Most existing data on the toxicity of PFAAs to freshwater organisms has focused on PFOS and PFOA (Ankley et al. 2005; Ji et al. 2008; Oakes et al. 2010). In acute tests prior to the present study, the most sensitive taxa tested were daphnids with a PFOS 48-h LC50 of 17 to 35 mg/L and 199 to 476 mg/L for PFOA. Sub-chronic exposures (21-28 d) of fathead minnow (*Pimephales promelas*) to 0.3 mg/L PFOS resulted in increased plasma levels of sexual steroids testosterone, ketotestosterone, and estradiol (Ankley et al. 2005; Oakes et al. 2010), suggesting PFAAs may act as endocrine disruptors. Further, Japanese medaka (*Oryzias latipes*) exposed to 0.01 mg/L PFOS had reductions in gonadosomatic-index (GSI), progeny, and growth of offspring (Ji et al. 2008). Although PFAA concentrations used in previous tests were several orders of magnitude greater than typical levels found in the environment, exposures in laboratory studies are typically short (3-28 d) and may not adequately represent exposure duration in the environment. In a partial life-cycle test of the effects of PFOS on the damselfly *Enallagma cyathigerum,* metamorphosis was significantly reduced at 10 μg/L (Bots et al. 2010), a concentration also associated with significantly increased oocyte death rates in the amphipod *Monoporeia affinis* (Jacobson et al. 2010). Such reductions in reproduction and recruitment at PFAA concentrations at or near environmentally relevant levels could cascade to population level effects.

Species sensitivity distributions have been used to derive a predicted no effect concentration (PNEC) for PFOS (Beach et al. 2006; Qi et al. 2011) and EPA tier II benchmarks for PFOS and PFOA have been established at 0.6 and 1.2 μg/L, respectively. In the present study, reductions in metamorphosis success of glochidia exposed *in marsupia* were seen at PFOS concentrations 50 to 100 times greater than the established

benchmarks. Duration of glochidia viability was reduced at concentrations < 10 times greater than established benchmarks after a 36 d *in marsupia* exposure. However, the ecological relevance of glochidia viability duration is not currently understood because glochidia are typically only exposed to host fish - outside of the marsupium - for a critical window of hours to a few days (Augspurger et al. 2007; Cope et al. 2008). We also attempted a 28 d aqueous exposure of juvenile (90 days post metamorphosis) *L. siliquoidea* to 0, 0.1, 1.0, 10 & 100 µg/L of PFOS (Hazelton et al. University of Georgia, unpublished data) but those results were inconclusive due to an unexplainable low survival rate in control animals (~60 %). To better understand the effects of PFAAs on early life-stages of freshwater mussels, future research objectives should include a 28 d juvenile chronic toxicity test, and recalculation of acute EC50's at dilution factors of less than 10 (ASTM 2006).

We observed some inaccuracy in our target concentrations of PFOS in the 36 d *in marsupia* exposure. First, these samples were taken at day 10 to 11 of the 36 d exposure, and although a 90% water change was conducted daily, some residual PFOS may have been on the glassware or the mussel and thus accumulated within the sample jar throughout the experiment. Second, measureable PFOS concentrations in our control group may have been due to cross-contamination from water quality instruments transferred between treatment groups. We did not analyze tissue from brooding females upon collection from the field; mussels may have had residual PFOS concentrations at the start of our study. Nevertheless, we measured a dose-dependent increase in whole tissue concentrations of PFOS across treatments (Table 2.2). Bioconcentration factors (BCF = [mean tissue PFOS ng/g]/[mean water PFOS ng/ml]) were consistent across

treatments (Control = 4.30, 4.5 µg/L = 3.32, 69.5 µg/L = 3.56), but were 2 to 90 times lower than those reported in marine mussels from polluted ecosystems (BCF = 138-297; Quinete et al. 2009), and resident freshwater mussels from PFOS polluted reaches of the Conasauga River, GA (BCF = 11-300, R. Bringolf, University of Georgia, unpublished data). The low BCF values in the present study suggest that adult *L. siliquoidea* exposed to PFOS for 36 d did not reach equilibrium, even though test concentrations were 4 to 70 times greater than normally found in natural systems (Konwick et al. 2008).

Duration of the free glochidia phase in nature is difficult to estimate experimentally and therefore the appropriate duration of the free glochidia exposure is a major concern for the relevance of acute glochidia tests. Currently the U.S. EPA does not accept acute glochidia toxicity test data for establishing water quality criteria for chemical contaminants (USEPA 2009) because an appropriate duration has not been established. *In marsupia* exposures used in the present study are ecologically relevant because the brooding length of many Unionid species is known, or easier to quantify than duration of the free glochidia stage in situ. Brooding duration and host infection strategies of Unionids are quite diverse (Barnhart et al. 2008; Cope et al. 2008), and differences in sensitivity to *in marsupia* exposures will likely differ among species and contaminant type. Further testing of the partial life-cycle test with different toxicants and life history strategies is necessary to determine the utility of this assay for chemical risk assessment. In addition, incorporation of the partial life-cycle assay into existing toxicity testing procedures will be useful in contaminant risk assessment for freshwater mussel

populations. The assay could be followed by chronic juvenile toxicity tests (Wang et al. 2007; Wang et al. 2011) to further understand the effects of contaminants throughout the early life-stages of freshwater mussels.

The partial life-cycle test used in the present study is adaptable for monitoring a number of endpoints in various mussel life-stages and may be improved by further standardization of techniques (e.g., use of moderately hard water in adult exposures, additional water sample collection for chemical validation; ASTM 2006). The assay described does have some disadvantages compared to the currently accepted standard toxicity tests for freshwater mussels (ASTM 2006). First, this assay is logistically more demanding and may require more financial and temporal resources than standardized methods. However, the assay allows for collection of more data on a wider range of endpoints that may be adapted to a particular mussel species, contaminant, or research objective. Another disadvantage is this assay requires the use of brooding adult mussels. Collection and use of reproductively mature individuals from wild populations for toxicity tests on numerous contaminants may not be the best conservation strategy given the widespread decline in freshwater mussel populations (Williams et al. 1993). Nevertheless, careful application of this assay in combination with contaminant screening and established acute and chronic toxicity test methods (ASTM 2006), along with continued advancement and availability of animals from freshwater mussel propagation (ASTM 2006; Bishop et al. 2007), should allow for the responsible use of adult mussels in toxicity testing.

2.5. Conclusion

The partial life-cycle assay was useful for identifying the most sensitive life-stage of freshwater mussels. This approach is adaptable to new endpoints, and, when combined with current acute and chronic toxicity assays (ASTM 2006), will be useful in further understanding of environmental toxicology and risk assessment to freshwater mussels. Data generated in the partial life-cycle analysis is probabilistic (e.g. probability of survival, metamorphosis) and may be useful in informing in situ responses of freshwater mussels to contaminants. The effects of chronic exposures to low concentrations of PFAAs are not fully understood (Beach et al. 2006) and further research on the effects in aquatic systems is warranted, particularly for estimation of population level changes caused by exposure to PFOS.

2.6. Acknowledgements

This project was funded by the U.S. Fish and Wildlife Service (US FWS) through Research Work Order Number 1434-HQ-08-RU-01568 from the U.S. Geological Survey (USGS). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the US FWS or USGS. We thank Colette Charbonneau, Mike Coffey, Dave Mosby, and Annette Trowbridge of the US FWS for their technical assistance; Andy Roberts of the US FWS for assisting with adult mussel collections in Missouri; and Bob Ratajczak, Jennifer Johnson, Jordan Cohen, Julie Creamer, Kaitlyn Brotman, and Andrea Fritts for logistic and laboratory assistance. We also thank Gregory Grimes and Aquatic Environmental Services, Inc. for supplying fish. We thank Amy Delinsky (U.S. EPA) for assistance in the quantitation of PFOS in mussels and appreciate

friendly reviews and statistical advice from Brenda Rashleigh (U.S. EPA), Colin Shea (University of Georgia), James Peterson (Oregon State University), and Douglas Bates (University of Wisconsin at Madison).

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2.8. Table Captions

Table 2.1: Names and explanations for competing models assessed with AIC model selection for viability duration and metamorphosis success of *Lampsilis siliquoidea*.

Table 2.2: Nominal and measured concentrations of PFOS in water and tissue samples for all exposure assays.

Table 2.3: Best fitting models, model statistics, and parameter estimates (Logit) for logistic regression models of effects of PFOS exposure on *Lampsilis siliquoidea* glochidia viability and metamorphosis.

Table 2.4: Acute toxicity of PFOS and PFOA to larval (glochidia) and juvenile fatmucket (*Lampsilis siliquoidea*) and black sandshell (*Ligumia recta*). Endpoints for EC50s and 95% confidence limits (parentheses) were valve closure for glochidia, and foot movement for juvenile mussels. NaCl was included as a reference toxicant. All EC50s were calculated using nominal concentrations.

Table 2.1. Names and explanations for competing models assessed with AIC model selection for viability duration and metamorphosis success of *Lampsilis siliquoidea.*

Model Name	Explanation	Model
Analysis		
Viability Duration		
In marsupia Exposure	PFOS concentration of 30-d adult exposure (bX) and the days post removal from adult best explain viability.	*p*(viability) = *bX + Day + bX*Day*
Glochidial Exposure	PFOS concentration of 24-hglochidia only exposure (gX) and the days post removal from adult best explain viability.	*p*(viability) = g*X + Day + gX*Day*
In marsupia + Glochidial Exposure	PFOS concentration during *in marsupia* exposure and glochidia only exposure, as well as days post removal affect viability.	*p*(viability) = *bX + gX + Day + bX*Day + gX*Day*
In marsupia X Glochidial Exposure	PFOS concentration during *in marsupia* and glochidia exposures and days post removal affect viability. There is also an interaction between the brood PFOS concentration and glochidial exposure PFOS concentration.	*p*(viability) = *bX + gX + Day + gX*bX + bX*Day + gX*Day*
Time	PFOS concentration did not affect viability, and decreases in probability that an animal is viable are only related to the time since removal from the adult.	*p*(viability) = *Day*
Metamorphosis		
In marsupia Exposure	PFOS concentration of 30-d *in marsupia* exposure is the only parameter of influence on the probability of metamorphosis.	*p*(metamorphosis) = *bX*
Glochidial Exposure	PFOS concentration of 24-h adult exposure is the only parameter of influence on the probability of metamorphosis.	*p*(metamorphosis) = *gX*
In marsupia + Glochidial Exposure	PFOS concentration at *in marsupia* and glochidia exposures decrease probability of metamorphosis.	*p*(metamorphosis) = *bX + gX*
In marsupia XGlochidial Exposure	PFOS concentration at *in marsupia* and glochidia exposures decrease probability of metamorphosis, and there is an interaction between exposures.	*p*(metamorphosis) = *bX + gX + bX*gX*

bX – *in marsupia* exposure PFOS concentration. Modeled as discrete factors: 0, 4.5 and 69.5 µg/L.
gX – glochidia exposure PFOS concentration. Modeled as discrete factors: 0, 4.5 and 69.5 µg/L.
Day – days post removal of glochidia from adult. *Day* = 0 was the day of removal, and initiation time of glochidial treatment.

Table 2.2. Nominal and measured concentrations of PFOS in water and tissue samples for all exposure assays.

Assay	Compound	Nominal Concentration (µg/L)	Measured Water Concentration (µg/L)	Measured Tissue Concentration (ng/g)
Acute toxicity	PFOA	5	5.1	
		50	48.4	
		500	490.0	
		5,000	4,800	
		50,000	51,000	
		500,000	476,000	
	PFOS	5	5.4	
		50	51.4	
		500	456.0	
		5,000	4,680	
		50,000	47,200	
		500,000	490,000	
Partial life-cycle	PFOS	0	2.11[a] (1.94, 2.28)	9.08[b] (8.74, 9.41)
		1	4.52 (3.84, 5.20)	15.00 (14.2, 15.8)
		100	69.5 (65.0, 74.0)	248.00 (208, 287)

[a] Mean PFOS concentration taken on day 10, 1 h after water change and retreatment (n=2). Values in parentheses include lower, upper values.
[b] Tissue concentrations measured in adult mussels after 36-d exposure (n = 2). Values in parentheses include lower/upper measurement. PFOS = perfluoroctanesulfonic acid. PFOA = perfluoroctanoic acid.

Table 2.3. Best fitting models, model statistics, and parameter estimates (Logit) for logistic regression models of effects of PFOS exposure on *Lampsilis siliquoidea* glochidia viability and metamorphosis.

Analysis	Best Model Name		AIC	w_i	k
Probability of Glochidia Viability	*In marsupia* Exposure		22843	0.94	8
Fixed Effects	Logit	Std. Err	*Wald - z*	*p* - value	
Intercept	2.35	0.08	29.38	<0.0001	
In marsupia 4.5µg/L	-0.49	0.11	-4.42	<0.0001	
In marsupia 69.5 µg/L	-0.50	0.11	-4.58	<0.0001	
Days	-0.32	0.02	-14.59	<0.0001	
In marsupia 4.5 µg/L X Days	-0.31	0.03	-9.39	<0.0001	
In marsupia 69.5 µg/L X Days	-0.25	0.03	-7.69	<0.0001	
[a]Random Effects	Variance		Standard Deviation		
[b]Group Intercept	0.016		0.12		
[b]Slope (Days)	0.003		0.05		
Analysis	Best Model Name		AIC	w_i	k
Probability of Metamorphosis	*In marsupia* Exposure		21954.54	0.83	4
Fixed Effects	Logit	Std. Err	*Wald - z*	*p* - value	
Intercept	0.63	0.15	4.27	< 0.0001	
In marsupia 4.5 µg/L	-0.11	0.22	-0.49	0.63	
In marsupia 69.5 µg/L	-0.92	0.23	-3.96	<0.0001	
Random Effects	Variance		Standard Deviation		
Group Intercept	0.21		21947		

[a] Random Effects – modeled variance within a group (exposure beaker) not explained by the predictive variables.
[b] Group Intercept – random intercept associated with variance among groups (exposure tanks) unexplained by the relationship between the explanatory and response variables.
[c] Slope (Days) – random change in slope among groups response to the Day variable not explained by the relationship between the explanatory and response variables. PFOS = perfluoroctanesulfonic acid; AIC = Akaike information criterion.

Table 2.4. Acute toxicity of PFOS and PFOA to larval (glochidia) and juvenile fatmucket (*Lampsilis siliquoidea*) and black sandshell (*Ligumia recta*). Endpoints for EC50s and 95% confidence limits (parentheses) were valve closure for glochidia, and foot movement for juvenile mussels. NaCl was included as a reference toxicant. All EC50s were calculated using nominal concentrations.

Life Stage	Duration (h)	Species	Chemical		
			NaCl (g/L)	PFOS (mg/L)	PFOA (mg/L)
Glochidia	24	Fatmucket	1.36 (0.76-2.43)	16.5 (8.0-33.9)	164.4 (116.0-232.8)
		Black sandshell	1.26 (0.57-2.80)	13.5 (5.7-31.8)	161.0 (135.8-191.0)
	48	Fatmucket	ND[a]	17.7 (7.2-43.5)	162.6 (130.6-202.3)
		Black sandshell	2.02 (0.74-5.56)	17.1 (9.4-31.1)	161.3 (135.0-192.7)
Juveniles	48	Fatmucket	3.57 (2.30-5.55)	158.1	>500[b]
		Black sandshell	3.75 (2.38-5.90)	158.1	>500[b]
	96	Fatmucket	2.63 (1.87-3.69)	158.1	>500[b]
		Black sandshell	2.51 (1.84-3.43)	141.7 (80.4-249.6)	>500[b]

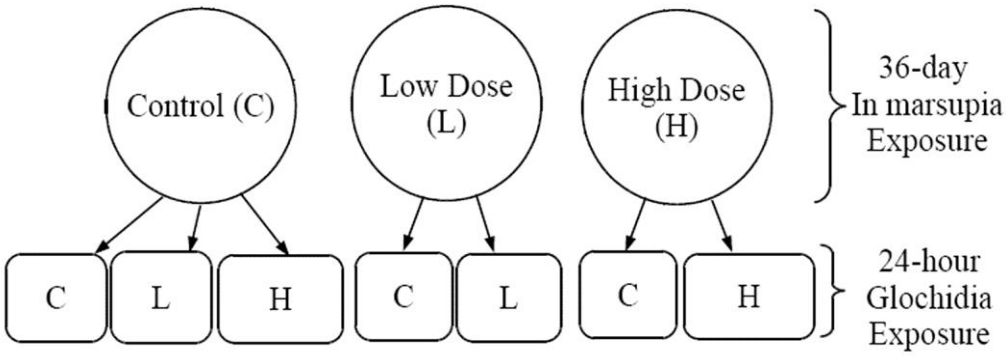
[a] Test not performed

[b] EC50 greater than highest concentration tested

2.9. Figure Captions

Figure 2.1: Exposure regimen of PFOS in a partial life-cycle test with freshwater mussel glochidia. *In marsupia* exposures were conducted as static renewal (100% daily) with four replicates of each treatment, one brooding *Lampsilis siliquoidea* per replicate. Glochidia from all mussels in each *in marsupia*l PFOS treatment group were pooled and four aliquots of the glochidia were used for 24-h glochidia PFOS exposures.

Figure 2.2: Fatmucket (*Lampsilis siliquoidea*) glochidia viability over time (A), and probability of metamorphosis (B) following 36-d PFOS exposure *in marsupia*. Error bars represent 95% confidence intervals. Levels of significance: * $p < 0.05$, *** $p < 0.0005$ when compared to control.



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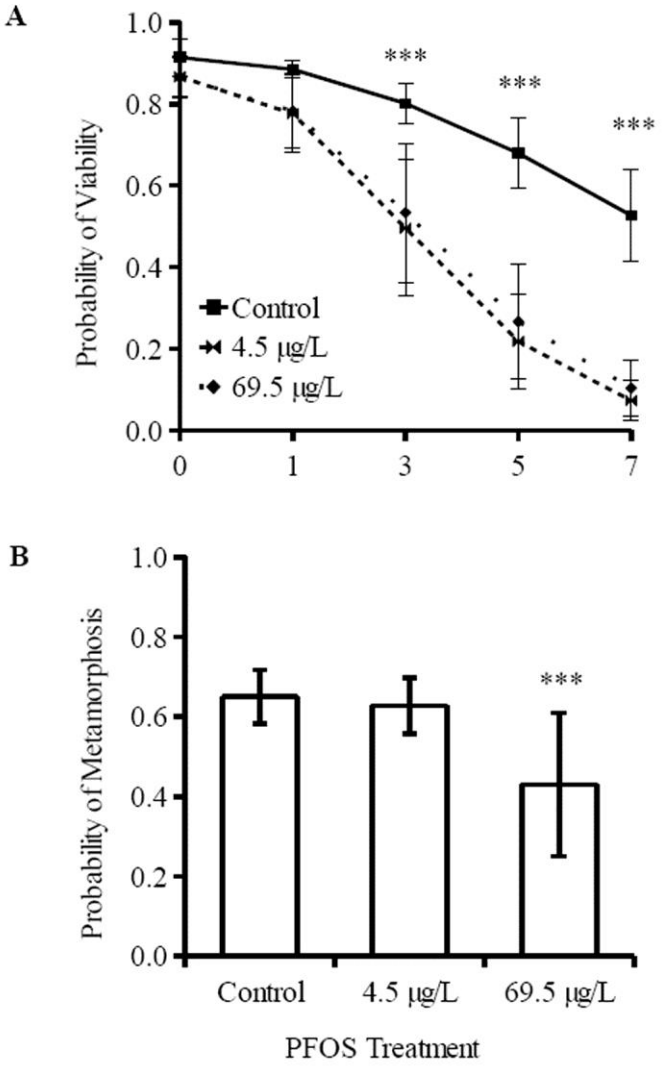

Figure 2.2: Fatmucket (*Lampsilis siliquoidea*) glochidia viability over time (A), and probability of metamorphosis (B) following 36 d PFOS exposure *in marsupia*. Error bars represent 95% confidence intervals. Levels of significance: * $p < 0.05$, *** $p < 0.0005$ when compared to control.

CHAPTER 3

FLUOXETINE ALTERS ADULT FRESHWATER MUSSEL BEHAVIOR AND LARVAL METAMORPHOSIS[2]

[2] Peter D. Hazelton, W. Gregory Cope, Shad Mosher, Tamara J. Pandolfo, Jason B. Belden, M. Christopher Barnhart and Robert B. Bringolf. 2013. *Science of the Total Environment* vol. 445-446:94-100
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Abstract

We used acute and partial-lifecycle tests to examine the effects of the pharmaceutical fluoxetine on freshwater mussels (Unionida). In acute tests lasting 24-48 h, we determined median effective concentrations (EC50s) for fluoxetine with larval (glochidia viability) and juvenile (survival) life-stages of fatmucket (*Lampsilis siliquoidea)* and black sandshell (*Ligumia recta*). In a 28 d behavioral test we exposed brooding adult female wavy-rayed lampmussels (*Lampsilis fasciola*) to 0.37 and 29.3 µg/L fluoxetine to determine effects on adult behavior (foot protrusion, mantle lure display and glochidia parturition). We also assessed the effects of 24-h exposure of 1 and 100 fluoxetine on glochidia viability duration and metamorphosis success for the wavy-rayed lampmussel. Fluoxetine EC50s ranged from 62 µg/L for juveniles (96 h) to 293 µg/L for glochidia (24 h). In adults, statistically significant increases were observed in foot protrusion at 0.37 and 29.3 µg/L fluoxetine and lure display rates at 29.3 µg/L; glochidia parturition was not significantly affected at any test concentration. Twenty-four hour exposure of glochidia to fluoxetine did not affect viability duration, but likelihood of metamorphosis to the juvenile stage significantly increased with 1 and 100 µg/L treatments. Our results demonstrated effects of fluoxetine at concentrations on unionids mussels less than previously and approaching concentrations measured in surface waters.

Keywords

Toxicity, Emerging contaminants, Metamorphosis, Behavior, Pharmaceuticals, PPCP

3.1. Introduction

Apprehension is growing internationally over widespread detection of pharmaceuticals in surface waters. The primary entry of these compounds into the environment is through wastewater discharge into surface waters. Although typically found in relatively low environmental concentrations (e.g., ng/L to μg/L range; Daughton 2001; Ternes et al. 2004; Valenti et al. 2012), continuous release from point-sources results in "pseudo-persistence" in surface waters and effectively creates chronic exposures to aquatic organisms (Daughton and Ternes 1999; Ramirez et al. 2009). Though our knowledge of the effects of pharmaceuticals on many aquatic species continues to grow (Brooks et al. 2003a; Brooks et al. 2003b), we still know relatively little of the effects on freshwater mussels (Order Unionida). However, previous studies have suggested potential endocrine disruption (Gagne et al. 2005), behavior effects and induction of premature larval release (parturition; Cunha and Machado 2001; Bringolf et al. 2010).

The paucity of information on the effects of emerging contaminants on freshwater mussels is of great concern because these species are among the most critically imperiled in North America and Europe (Williams et al. 1993; Strayer et al. 2004) and chemical pollution is often cited as an important potential cause of this decline (Augspurger et al. 2007; Cope et al. 2008; Downing et al. 2010). The unique life-cycle of freshwater mussels includes males' release of sperm into the water column, internal fertilization, brooding of maturing larvae (glochidia) within modified gills of the adult female, and an obligate parasitic stage where glochidia metamorphose to the juvenile stage on a host fish before dropping from the fish to the sediment to become free-living benthic adults. Each

discrete phase throughout ontogeny provides for a unique exposure route to contaminants; adversely affected individuals may have reduced fitness and reduced recruitment to a population (Cope et al. 2008).

The present study focused on the antidepressant pharmaceutical fluoxetine, which is commonly found in municipal or industrial wastewater effluent at concentrations as high as 0.54 µg/L (Brooks et al. 2003). Fluoxetine is a selective serotonin reuptake inhibitor (SSRI) and numerous studies have shown that fluoxetine disrupts reproduction in mollusks (Fong et al. 1996; Fong 1998; Fong and Molnar 2008; Gust et al. 2009; Meechonkit et al. 2010; Lazzara et al. 2012) and stimulates glochidia release in the Unionida (Cunha and Machado 2001; Bringolf et al. 2010). However, to date exposures have only been investigated with adults and there are no published reports of the effects of fluoxetine on early life stages of freshwater mussels. In the present study, we used standard (ASTM 2006b) methods to determine the acute toxicity of fluoxetine to the early life stages of freshwater mussels. We also examined behavioral effects during chronic fluoxetine exposure on adult female freshwater mussels and we conducted a 24 h water only exposure of glochidia to determine effects of fluoxetine on viability duration and metamorphosis success.

3.2. Materials and methods

3.2.1. Test Organisms

Brooding female mussels were collected from the following stable mussel populations: fatmucket (*Lampsilis siliquoidea)* from Silver Fork of Perche Creek, Boone County, MO and black sandshell (*Ligumia recta)* were collected from the Meremec

River, Jefferson County, MO. Wavy-rayed lampmussel (*Lampsilis fasciola*) were collected from the Little Tennessee River, Macon and Swain Counties, NC and transported to the University of Georgia in a 95 L cooler with battery powered aerator. Prior to experimentation, adult brooding females were held in a 530-L Living Stream (Frigid Units Inc., Toledo, OH) with natural pond water and approximately 5 cm of quartz/silica sand (Playsand, QuickCrete Products Corp, Norco, CA). Juvenile mussels were propagated at Missouri State University Barnhart 2006 (Barnhart 2006), and all juveniles and glochidia were the pooled progeny from 3 adult mussels. Fatmucket juveniles were produced from host fish inoculations of largemouth bass (*Micropterus salmoides*) and black sandshell were produced from inoculations of walleye (*Sander vitreus*). Juveniles and glochidia used in acute toxicity tests were shipped via overnight courier to North Carolina State University in moderately hard water in 500mL polyethylene bottles, within an insulated container, without ice. Temperature upon arrival (17.1-21.4 °C) did not deviate markedly from shipment temperature (~15-17 °C). Juveniles ranged in age from 2-6 weeks, but ranged no more than 1 week in age within a given species and toxicant experiment. All glochidia were < 24 hours old at the time of testing.

3.2.2. Chemical concentrations & quantification

All exposures were conducted with fluoxetine hydrochloride (> 98% purity purchased from Fisher Scientific (West Chester, PA, USA) or Sigma Aldrich (St. Louis, MO, USA). Stock solutions were prepared in water (fluoxetine). In acute tests, we used three replicates of each of six test chemical concentrations (5, 25, 50, 250, 500, 1000,

1600 µg/L Fluoxetine) with dilution-water controls. For twenty-eight day adult behavioral tests, target concentrations of 1 and 100 µg/L fluoxetine and a dilution-water control were used. Reconstituted hard water (hardness 160-180 mg/L as $CaCO_3$) was used as dilution water for all acute toxicity tests (ASTM 2006b) and was prepared by adding reagent-grade salts (Fisher Scientific; Fair Lawn, NJ, USA) to deionized water. Certified NaCl (ACS grade, Fisher Chemical, Hampton, NH, USA) was used as a reference toxicant. Test concentrations of fluoxetine were verified using gas chromatography and mass spectrometry in the Environmental Toxicology Laboratory at Oklahoma State University (Stillwater, OK, USA). Water samples were extracted using Oasis hydrophilic-lipophilic-balanced solid phase extraction cartridges (6-mL cartridges for 200-mL samples and 12-mL cartridges for 500-mL samples; Waters, Milford, NH, USA). For all samples, 200 mL of test solution was extracted. Prior to extraction, pH of the samples was reduced to < 2. Following extraction, analytes were eluted from the cartridge using 1:1 ethyl ether: methanol, which was then evaporated to < 1 mL under a stream of nitrogen and brought to a final volume of 1.0 mL with ethyl ether. To measure fluoxetine, 0.2 mL of extract was evaporated to near dryness and then reconstituted in 0.4 mL of ethyl acetate. This extract was analyzed on an Agilent 6850 gas chromatograph coupled with a 5975C mass selective detector with electron ionization, selectively monitoring ion 309 with phenanthrene d10 and perylene d10 included as internal standards. Quality assurance was conducted with matrix spikes of four replicate reagent water samples, spiked with a known concentration of 6 µg/L fluoxetine.

3.2.3. Acute toxicity of fluoxetine to glochidia & juvenile stages

Acute toxicity tests were conducted at the Environmental Toxicology Laboratory at North Carolina State University (Raleigh, NC, USA). Briefly, acute toxicity tests were conducted with glochidia and juvenile stages of fatmucket and black sandshell according to standard guidelines (ASTM 2006a). Glochidia tests consisted of 24-h non-aerated, static experiments of three replicates of 150-200 glochidia per treatment. Viability was assessed at 24 h on a subsample of 50 glochidia from each replicate, determined by the addition of a saturated NaCl solution and measurement of shell closure response (ASTM 2006a). Juvenile tests were 96 h non-aerated, static experiments of three replicates with ten juveniles per treatment. Effects endpoints assessed at 48 and 96 h included the detection of foot movement or the presence of a heartbeat for mussels in each replicate (Pandolfo et al. 2010). Glochidia and juvenile toxicity to sodium chloride (range 0.25-8.0 g NaCl/L) was assessed concurrently with fluoxetine tests for quality assurance purposes (ASTM 2006a), and mussel sensitivity to NaCl has been demonstrated in previous studies to serve as a measure of relative health and condition of test organisms (Bringolf et al. 2007; Valenti et al. 2007; Cope et al. 2008).

3.2.4. Sublethal effects of fluoxetine on adult mussel behavior

We assessed the sublethal effects of fluoxetine to brooding adult wavy-rayed lampmussel. Only adult mussels with initial glochidia viabilities > 80% were used. Each treatment was replicated in triplicate; replicates consisted of one adult wavy-rayed lampmussel in a 3.8 L glass jar containing 3 L of 0, 0.37, or 29.3 μg/L fluoxetine in moderately hard reconstituted water. Thus, there were nine total experimental units

maintained in an environmental chamber at 20°C and 12:12 light:dark photoperiod. Each jar was aerated and had >90% water renewal daily and mussels were fed a mixture of 2.55 x 10^7 cells/mL *Nannochloropsis* (Nanno 3600) and 1.75 x 10^6 cells/mL commercial shellfish diet (Shell Fish Diet 1800: Reed Mariculture Inc., Campbell, CA, USA). Daily water quality measurements were consistent among treatments throughout the partial-lifecycle exposure and were well suited for freshwater mussels. Temperature ranged from 18.4 to 19.1°C, specific conductance varied between 0.297 and 0.325 mS, dissolved oxygen ranged from 6.89 to 8.79 mg/L, and pH ranged from 7.38 to 8.04. Behavioral responses of adult female wavy-rayed lampmussel to fluoxetine were measured daily prior to each water quality measurement and water renewal. We evaluated lure display, foot protrusion beyond the margin of the valves, and parturition of glochidia conglutinates (ie., release of glochidia packets; Bringolf et al. 2010) for each mussel and recorded each endpoint as a binomial response where 0 indicated no response, and 1 indicated a positive response.

3.2.5. Sublethal effects of fluoxetine to glochidia

Following the 28 d exposure, we obtained ~120,000 glochidia from the marsupial gills of two control mussels with viability > 90%. This deviated from the suggested pooled glochidia from 3 females in the ASTM guidelines (ASTM 2006a) because the third female had glochidia viability below 90%. Glochidia viability was assessed from a subsample of 50 -150 glochidia from each female. Glochidia were pooled to average effects of individual females, and divided into 12 aliquots and randomly assigned to an exposure of 0, 1, 100 µg/L fluoxetine, with four replicate aliquots per treatment.

Glochidia exposures (24 h) were conducted in 150 mL glass beakers with 100 mL of un-aerated moderately hard, reconstituted water. All glassware was preconditioned with fluoxetine test concentrations for 24 h prior to experimentation and exposures were conducted in an environmental chamber at 20°C and 12:12 light:dark photoperiod. Every 48 h for eight days we assessed viability (via NaCl exposure) on a subsample of 50 glochidia and performed a 50% water renewal.

Immediately after the glochidia were exposed to 0, 1, and 100 µg/L fluoxetine for 24 h, largemouth bass were inoculated with a subsample of glochidia from each aliquot, resulting in four replicates of each treatment. All inoculations were conducted on individual fish in 0.5 L of filtered and dechlorinated city water, in a 1.5-L aquarium for 15 min with aeration to suspend glochidia (~1000 viable glochidia/L) for host attachment. Fish were rinsed of unattached glochidia following the 15-min inoculation and placed in 3-L tanks (one fish/tank) in a recirculating aquaculture system (Aquatic Habitats, Inc., Apopka, FL, USA). Outflow from each tank was continually filtered through a 150-µm mesh cup to collect sloughed glochidia and metamorphosed juveniles. Contents of the filter cups were assessed every 24-48 h for 19 d after inoculation. We calculated metamorphosis success for each fish as the proportion of live juveniles to the total number of juveniles and glochidia sloughed from each fish.

3.2.6. Statistical analysis

For both glochidia and juvenile acute toxicity tests, estimates of the median effective concentration (EC50) and 95% confidence interval were calculated using the Trimmed Spearman-Karber (TSK) method (ASTM 2006b) with the ToxCalc™ statistical

software (version 5.0.231, Tidepool Scientific Software, McKinleyville, CA, USA). When data were inadequate to use TSK, EC50 values were estimated using the graphical method (ASTM 2006b). EC50 values were considered significantly different when 95% confidence intervals did not overlap (Bringolf et al. 2007).

A mixed-model logistic regression framework was employed to assess the effects of fluoxetine exposure concentration on duration of viability and metamorphosis success in glochidia exposures, and behavioral endpoints in adult exposures. Logistic regression analysis was preferred because responses in our assays were structured as nominal variables, and the probability of the response can be easily estimated as an effect of a numerical or nominal predictor variable. Further, logistic regression is not as sensitive to the assumptions of normality as an ANOVA, particularly when data are structured as a proportion or frequency (Dowdy et al. 2004).

The R statistical programming platform (R Core Development Team 2010) was used for all statistical tests and the statistical methods followed those described in Hazelton et al. (2012). Briefly, data from viability duration assessments, host-fish metamorphosis, and adult behavioral experiments were expressed as a binary response where 0 is a failure and 1 is a success (e.g., viability: 0 = nonviable, 1 = viable; metamorphosis: 0 = unmetamorphosed glochidia, 1 = metamorphosed juvenile; behavior: 0 = no response, 1 = response), resulting in a hierarchically structured dataset, where the response of an individual glochidium or juvenile is nested within a group (i.e., aliquot beaker or fish). This approach is analogous to a nested ANOVA; however it incorporates the benefits of logistic regression. In the case of the adult behaviors, the response was a repeated daily measurement nested within a single adult, thus analogous to a repeated

measures design (Bolker et al. 2009). The lme4 package was used in R (Bates and Sarkar 2007) to model a random effect of each group, should such event be unrelated to the effect of treatment. We modeled the relationship of the response to fluoxetine as a factor (i.e., nominal or categorical) variable rather than a linear value because we used only three treatment levels of fluoxetine (target concentrations = 0, 1, 100 µg/L) for both exposures; the absence of fluoxetine (0 µg/L) was a null relationship (Bates and Sarkar 2007). Logit parameter estimates of fixed-effects from the best fitting models were used to assess the effect of that parameter on the response. Wald statistics (z) and standard errors were also calculated and the level of significance was considered $p < 0.05$ (Hair et al. 1998). Logits were converted to probability estimates with the logit link function (Hair et al. 1998).

3.3. Results

3.3.1. Chemical quantification

Mean recovery of fluoxetine in matrix-spiked samples was 84% with 14% ± SD. Reported concentrations were not corrected for matrix spike recoveries. In acute tests, measured concentrations of fluoxetine ranged from 60-130% of target (measured concentrations: 6.5, 22, 50, 150, 350, 1000 and 1500 µg/L for target concentrations of 5, 25, 50, 250, 500, 1000, 1600 µg/L). Target concentrations were used for calculation of EC50s. Fluoxetine concentrations in test water from the 28 d adult exposures were 29-37% of targets (0.37 and 29.30 µg/L for 1.0 and 100 µg/L target concentrations respectively). We are uncertain of the reason for such a large difference between target and measured fluoxetine concentrations. Test concentrations of fluoxetine used in the 24

h glochidia only exposures were not quantified and are expressed as target concentrations, though the same stock solutions were used as in the adult study. Concentrations used in behavioral endpoints of the 28 d adult exposures are herein expressed as measured concentrations.

3.3.2. Acute toxicity of fluoxetine to glochidia & juvenile stages

Control viability for all glochidia acute toxicity tests exceeded 90%, and control survival for all juvenile acute toxicity tests exceeded 80%. Fluoxetine EC50s were similar between species, life-stages, and testing periods (Table 3.1). EC50s ranged from 62 µg/L for juveniles (96 h) to 293 µg/L for glochidia (24 h). The large confidence intervals may be an artifact of the wide range of concentrations tested because this study was largely a range finding effort given the paucity of information on the toxicity of these compounds to freshwater mussels. In reference toxicant (NaCl) tests, juveniles of both species were less sensitive than glochidia, and our results were similar to reference NaCl toxicant test results reported for fatmucket by Bringolf et al. (2007).

3.3.3. Sublethal effects of fluoxetine on adult mussel behavior

Glochidia viability measured prior to initiation of the 28 d *in marsupia* exposure did not vary among treatment groups (mean % viability ± SD: 91 ± 6 for 0 µg/L, 90 ± 7 for 0.37 µg/L, and 91 ± 7 for 29.3 µg/L), suggesting that all mussels were in the same general reproductive condition at the start of the exposure. One of three adults in the 29.3 µg fluoxetine /L treatment group died on day 19 of the experiment and was not used in the analysis of behavioral endpoints, thus behavioral endpoints were based on 3 mussels

of the control and 0.37 µg/L group, and 2 mussels in the 29.3 µg/L group. There were a total of 25 days during which behavioral observations were taken for each mussel. Foot protrusion counts ranged from 0-20 occurrences, while occurrences of lure display ranged from 7-24 and parturition events ranged from 1-6 occurrences.

Probability of foot protrusion increased in adult mussels exposed to fluoxetine treatments of 0.37 µg/L (logit = 1.49, SE = 1.01, Wald z = 1.47, p = 0.14) and 29.3 µg/L (logit = 4.39, SE = 1.02, Wald z = 4.32, $p < 0.0001$), though this response was only statistically significant in the highest fluoxetine treatment. Control mussels displayed a protruded foot beyond the valve margin in less than 3% (± 2% SD) of observations, whereas individuals exposed to fluoxetine at 0.37 µg/L displayed 11% (± 9%) of the time, and the 29.3 µg/L treatment group displayed a protruded foot in 62% (± 25%) of observations. Parameter estimates from the statistical model were used to calculate probabilities of behavior occurrence (Figure 3.1A).

Probability of lure display (Figure 3.1B) was also positively correlated to fluoxetine treatments of 0.37 µg/L (logit = -0.07, SE = 0.66, Wald z = -0.11, p = 0.91) and 29.3 µg/L (logit = 2.07, SE = 0.83, Wald z = 2.55, p = 0.01), though only statistically significant at the higher concentration. Animals in the 29.3 µg/L concentration displayed their lures in 86 % (± 14%) of the observations, whereas animals in the lower concentration (52% ± 21%) and control (53% ± 20%) were found displaying lures less frequently.

We did not find a statistically significant change in probability of glochidia parturition events in mussels exposed to 0.37 µg/L (logit = -0.27, SE = 0.57, Wald z = -0.46, p = 0.64) or 29.3 µg/L (logit = 0.62, SE = 0.51, Wald z = 1.21, p = 0.23) fluoxetine

compared to control mussels. There was no discernible trend in the parturition rates among control (13 % ± 10%), 0.37 µg/L (11% ± 6%), and 29.3 µg/L (14% ± 3%) treatments.

3.3.4. Sublethal effects of fluoxetine to glochidia

Free glochidia exposure (24-h) to fluoxetine did not affect the duration of viability of glochidia. The logistic regression model had a significant intercept (logit = 4.71, SE = 0.38, Wald $z = 12.55$, $p < 0.001$) and a significant fixed effect of days post exposure (logit = -0.91, SE = 0.04, Wald $z = -22.16$, $p < 0.001$). However, fluoxetine treatment had no significant effects on viability duration at 1 µg/L (logit = 0.49, SE = 0.54, Wald $z = 0.9$, $p = 0.37$), or 100 µg/L (logit = 0.54, SE = 0.54, Wald $z = 1.01$, $p = 0.31$). In all treatment groups, percent viability was greater than 90% at the time of removal (control = 97% ± 0.6% SE, 1 µg/L = 94% ± 2.2% SE, 100 µg/L = 95% ± 3.8% SE). Viability in all treatment groups decreased approximately 10% by day 3, decreased more than 30% by day 5 and decreased approximately 70% by day 7 (Figure 3.2A). Probability of viability was calculated using parameter estimates for days post exposure on glochidia viability.

Metamorphosis success was greater in 1 µg/L (85.1% ± 0.7% SE) and 100 µg/L (82.9% ± 2.3% SE) fluoxetine treatments than the control group (69.7% ± 6.8% SE). Fluoxetine exposure resulted in increased probability of metamorphosis (compared to controls) by a mean of 19.4 % in 1 µg/L fluoxetine, and 16.7% in 100 µg/L fluoxetine (Figure 3.2B). Metamorphosis was 1.57 times more likely than the control in the 1 µg/L treatment and 1.92 times more likely in the 100 µg/L treatment. Parameter estimates for 1 µg/L fluoxetine (logit = 0.87, SE = 0.28, Wald $z = 3.08$, $p = 0.002$), and 100 µg/L

fluoxetine (logit = 0.69, SE = 0.27, Wald $z = 2.53$, $p < 0.01$) were used in calculating metamorphosis probabilities of exposed glochidia compared to the control.

3.4. Discussion

In this study, the effects of fluoxetine were tested on freshwater mussels with a combination of standardized acute toxicity tests and novel approaches to assess sublethal effects. Our results indicate that juvenile and adult mussels are among the most sensitive aquatic organisms tested to date with fluoxetine (Brooks et al. 2003a; Brooks et al. 2003b; Henry et al. 2004; Henry and Black 2008).

In the present study, fluoxetine exposure was associated with a significant increase in metamorphosis success compared to untreated glochidia. A similar trend occurred in a preliminary experiment with fatmucket (P. Hazelton, unpublished data). Serotonin has been reported as a major factor in induction of metamorphosis in gastropod mollusks, e.g., *Ilyanassa obsoleta* (Couper and Leise 1996; Leise et al. 2001; Leise et al. 2004). The similarity of mechanisms regulating metamorphosis in marine gastropod veligers and Unionid glochidia are currently unknown, and we did not find any change in the time to metamorphosis in fluoxetine-treated wavy-rayed lampmussel glochidia compared to controls (P. Hazelton unpublished data), which might be expected if fluoxetine induces metamorphosis through the same serotonergic pathway as found in *I. obsoleta*. A competing hypothesis may be that residual fluoxetine on glochidia may interfere with the host-fish immune response, which is purported to control glochidia attachment and metamorphosis success. Anti-inflammatory effects are well documented for SSRIs (Bianchi and Panerai 1996), and fluoxetine induces increased plasma

corticosterone in rats (Bianchi et al. 1994), likely through a seratonergic activation of the pituitary-adrenocortical axis (Bianchi and Panerai 1996). A similar response in fish treated with fluoxetine should result in increased glochidial metamorphosis success through reduced gill inflammation and sloughed glochidia. For example, bluegill (*Lepomis machrochirus*) exposed to cortisol, a glucocorticoid with anti-inflammatory effects, had increased metamorphosis rates of *Utterbackia imbecillis* compared to controls (Dubansky et al. 2011). Thus, the mechanism by which fluoxetine causes increased metamorphosis in unionids deserves further attention. Further research may lead to a better understanding of the processes that control host suitability and may have important implications for enhancing culture, particularly for rare species. Interestingly, metamorphosis is inhibited in marine mollusks by endogenous synthesis of nitric oxide (Couper and Leise 1996; Leise et al. 2001; Leise et al. 2004), and inhibition is reversed by a nitric oxide synthase inhibitor, nitroindazole (Leise et al. 2004). A comparative study in unionid glochidia including exposures to nitroindazole, the serotonin receptor agonist cyproheptadine (Campos et al. 2012), and greater concentrations of fluoxetine may elucidate the similarities of glochidia development to that of other mollusk larvae. In addition, reduced growth has been observed in the crustaceans *Hyallela azteca* and *Daphnia magna* exposed to fluoxetine (Péry et al. 2008); however, we did not assess the size/growth rate of metamorphosed juveniles exposed to fluoxetine, but measurement of these endpoints may be informative in future studies to determine overall effects of fluoxetine on freshwater mussel populations.

Adult wavy-rayed lampmussel exposed to 29.3 μg/L of fluoxetine were significantly more likely to be observed with a protruded foot, and displayed mantle lure.

We did not measure the extent of foot protrusion, but the condition was marked by a swelling of the foot tissue beyond its normal size and the mussel's inability to retain the foot within the valves and maintain a closed shell. Individuals from the fluoxetine 29.3 µg/L exposure group did not retract the foot during daily water changes (P. Hazelton personal observation). Cunha and Machado (2001) found an increase in foot volume and protrusion in *Anadonta cygnea* exposed to 309,000 µg/L fluoxetine for 24 h, however, in the present study, we observed a significant increase in foot protrusion at concentrations approximately 10,500 times less. Similarly, Bringolf et al. (2010) found that the lure display lowest observed effect concentration (LOEC) for fluoxetine in 96 h tests with *L. fasciola* was 300 µg/L; a concentration 10 times greater than the effective concentration for lure display over 28 d in the present study. Both behaviors may result from disruption of a serotonergic pathway. Ram et al. (1999) found that the mantle tissue forming the incurrent and excurrent siphons in zebra mussels showed cycles of contraction and relaxation induced by the neurotransmitter serotonin. Tissue forming the mantle lure in unionids is located directly anterior to the siphon apertures and is innervated from the same neural ganglia (McMahon and Bogan 2001), supporting a homologous response induced by fluoxetine, a potent selective serotonin reuptake inhibitor. Foot protrusion is caused by a similar mechanism, in which serotonin induced relaxation and dilation of the muscular bands of the ostia (water canals) in the mussel gill results in increased water uptake and swelling (Gardiner et al. 1991). Alterations to these behaviors in the environment may have consequences for survival and reproduction. Foot protrusion due to swelling is atypical for mussels in the wild and likely impedes burrowing, locomotion, and predator avoidance; however, the importance of this behavior has not yet been

investigated and warrants further attention. The lure display behavior is exhibited by freshwater mussels to attract a host fish for infection with glochidia (Barnhart et al. 2008), and changes in lure display could potentially disrupt timing of host-fish attraction. The reproductive consequences of such an occurrence are difficult to quantify across a population, but may reduce recruitment of future mussel generations.

In this study, we observed no increase in glochidia parturition events in mussels exposed to 0.37 or 29.3 µg/L fluoxetine. The role of fluoxetine on parturition of gametes and larvae of bivalves has been previously established (Fong 1998; Cunha and Machado 2001; Fong and Molnar 2008; Bringolf et al. 2010; Lazzara et al. 2012). Lazzara et al. (2012) discovered a signicant decrease in mature oocytes per follicle in female, and reduced spermatazoa density in tubules of male zebra mussels (*Dreissena polymorpha*) at concentrations as low as 0.02 and 0.2 µg/L fluoxetine in a six day test. Though the authors did not quantify expelled gametes, all animals were in spawning stage and the absence of gametes in the lumen of the gonads was attributed to parturition. In unionid mussels there has also been validated concern for the premature release of larvae. Both Bringolf et al. (2010) and Cunha and Machado (2001) reported significant increases in glochidia parturition caused by fluoxetine. But in those studies the LOEC was 300 µg/L during a 96h exposure. We suggest that the LOEC for glochidia parturition is likely between 29.3-300 µg/L because measured fluoxetine concentrations in the present study were approximately 30% of target. Fluoxetine is known to accumulate in mussels during long term exposures (Bringolf et al. 2010) and we are uncertain whether the mussels in

the present study had reached a steady state concentration. The possibility remains that chronic fluoxetine exposures may increase the probability of larval parturition at concentrations more consistent with those found in the environment.

3.5. Conclusions

In summary, we used established acute tests to identify toxic effects of fluoxetine on the early life stages of freshwater mussels and novel methods to examine the sublethal effects on adult females and glochidia. We present the first acute toxicity data of glochidia to fluoxetine, and freshwater mussels were among the most sensitive organisms currently tested. Chronic fluoxetine exposures affected behavior of adult *L. fasciola* (lure display and foot protrusion) at concentrations 3-10 times less than previously published (Bringolf et al. 2010; Cunha and Machado 2001), and much closer to concentrations commonly measured in the environment. However, because of low numbers of replicates in adult behavioral assays (2-3 animals) we recommend further research be conducted prior to establishing a LOEC at values below those found in the current literature. Finally, we found that 24 h sublethal fluoxetine exposures to glochidia resulted in increased metamorphosis success. Further research is needed to better understand the potential role of serotonin in glochidia metamorphosis, and the consequences of toxicants that may disrupt this pathway.

3.6. Acknowledgements

The project described in this publication was funded by the U.S. Fish and Wildlife Service (US FWS) through Research Work Order Number 1434-HQ-08-RU-01568 from the U.S. Geological Survey (USGS). Contents are solely the responsibility of the authors and do not necessarily represent the official views of the US FWS or USGS. We thank Colette Charbonneau, Mike Coffey, Dave Mosby, and Annette Trowbridge of the US FWS for their logistical help. Members of the Bringolf lab group: Andrea Fritts, Jennifer Johnson, Julie Creamer, Jordan Cohen and Bob Ratajczak, all provided technical assistance. We are indebted to Stephen Fraley and his staff at the North Carolina Wildlife Resources Commission, and to Andy Roberts and to the US FWS Staff in Missouri for aid in mussel collection. Previous versions of this manuscript were greatly improved by the comments of two anonymous reviewers.

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3.8. Table Captions

Table 3.1: Acute toxicity of fluoxetine and sodium chloride (NaCl; a reference toxicant) to larval (glochidia) and juvenile fatmucket (*Lampsilis siliquoidea*) and black sandshell (*Ligumia recta*). Target (nominal) chemical concentrations were used to calculate median effective concentrations (EC50's). Endpoints for EC50's and 95% confidence intervals (in parentheses) were valve closure for glochidia, and foot movement or heartbeat for juvenile mussels.

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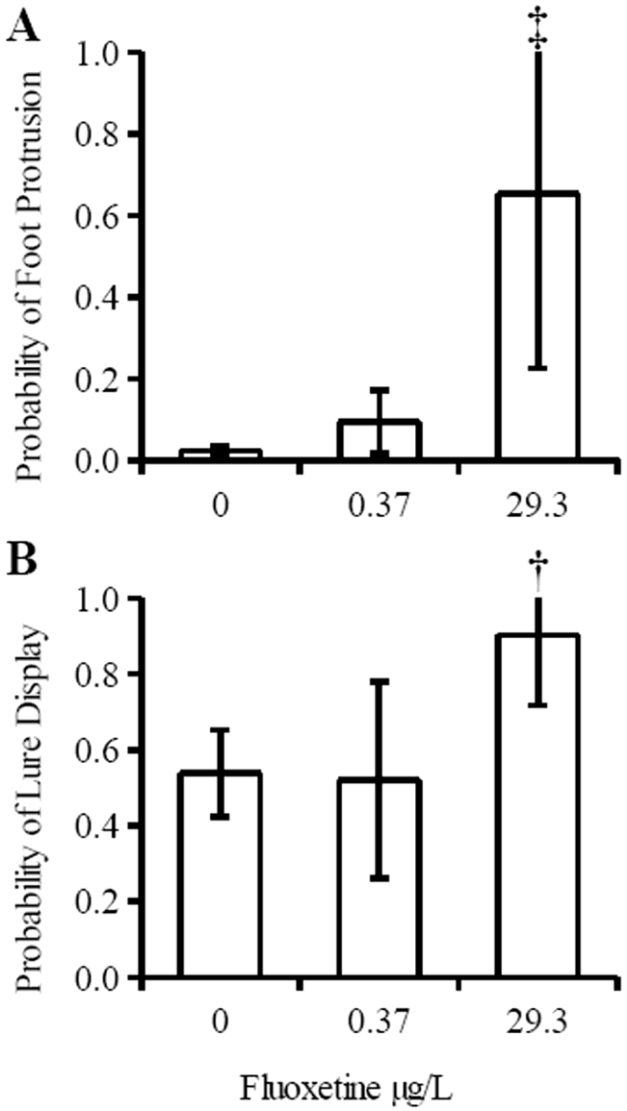
			Chemical	
Life stage	Time Point (hr.)	Species	NaCl (g/L)	Fluoxetine (µg/L)
Glochidia	24	*L. siliquoidea*	1.36 (0.76-2.43)	624.8 (331.4-1177.9)
	24	*L. recta*	1.26 (0.57-2.80)	293.1 (122.6-700.6)
Juvenile	48	*L. siliquoidea*	3.57 (2.30-5.55)	179.0 (90.7-353.3)
	48	*L. recta*	3.75 (2.38-5.90)	265.7 (165.6-426.3)
Juvenile	96	*L. siliquoidea*	2.63 (1.97-3.69)	62.0 (32.6-117.8)
	96	*L. recta*	2.51 (1.84-3.43)	96.9 (35.7-262.9)

3.9. Figure Legends

Figure 3.1. Effects of 28 d fluoxetine exposure on behavior of *Lampsilis fasciola* adult females. Probability of (A) foot protrusion (swelling) beyond mantle and valve margins, and (B) mantle lure display. Error bars represent two standard deviations from the mean and are truncated at probability = 1.0. Level of significance for comparison with control: † $p < 0.05$, ‡ $p < 0.005$.

Figure 3.2. Effects of 24 h fluoxetine exposure on wavy-rayed lampmussel (*Lampsilis fasciola)* glochidia. (A) Glochidia viability duration: there was no difference in viability among fluoxetine and control groups, mean viability across all treatments is shown. Day = 0 corresponds to removal date from adult female and initiation of 24 h exposure to fluoxetine. (B) Probability of glochidia metamorphosis on largemouth bass (*Micropterus salmoides*) following 24 h exposure to fluoxetine. Error bars represent two standard deviations from the mean. Level of significance from control: † $p < 0.05$, ‡ $p < 0.005$.



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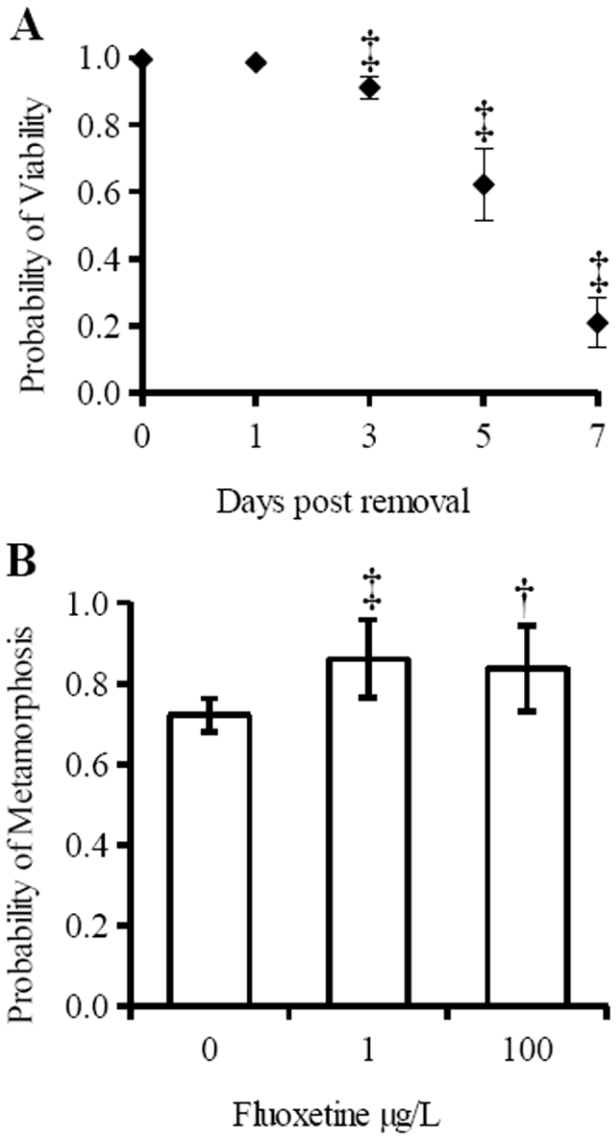

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CHAPTER 4

CHRONIC FLUOXETINE EXPOSURE ALTERS MOVEMENT & BURROWING IN ADULT FRESHWATER MUSSELS[3]

[3] Peter D. Hazelton, Bowen Du, Samuel P. Haddad, Andrea K. Fritts, Bryan W. Brooks and Robert B. Bringolf. To be submitted to *Environmental Pollution*

Abstract

The antidepressant fluoxetine (Prozac) is commonly found in aquatic fauna living near or downstream from point-sources of municipal waste effluent. Continuous release of fluoxetine results in pseudo-persistence in surface waters, creating a chronic exposure for animals downstream. Fluoxetine is known to cause disruptions in reproductive behavior of freshwater mussels (order Unionoida), including stimulating release of gametes, parturition of glochidia (larva), and changes in lure display and foot protrusion. However, the ecological relevance of these effects at environmental concentrations is unknown. We conducted a 67 d exposure of adult *Lampsilis fasciola* to nominal fluoxetine concentrations of 0, 0.5, 5.0, and 50 µg/L in the presence of sediment and assessed impacts on behavior (lateral movement, burrowing and filtering) and metabolism (glycogen storage and respiration). Mussels treated at the 5 and 50 µg/L concentrations were observed with displayed mantle flap lures statistically more frequently than controls. Animals treated with 50 µg/L fluoxetine were statistically more likely to have shorter time-to-movement, greater total movement, and initiate burrowing sooner than control animals. Individuals at the highest fluoxetine treatment were also more likely to be found actively moving, showing foot protrusion beyond shell margins, gaping valves, and displaying abnormal burrowing orientations. Increased activity of mussels exposured to fluoxetine may result in increased susceptibility to predators and may lead to a reduction in energy stores.

Keywords

Unionoida, Diel movement, Prozac, SSRI, Pharmaceutical, PPCP

4.1. Introduction

Evidence continues to grow of the prevalence of pharmaceuticals and personal care products (PPCPs) in the environment (Boxall et al. 2012; Brooks et al. 2012). Particularly in wastewater dominated aquatic ecosystems, continual releases of PPCPs causes a "pseudopersistence" (Daughton and Ternes 1999) leading to chronic exposures and accumulation in aquatic organisms (Ramirez et al. 2009; Bringolf et al. 2010). As pharmaceuticals, many of these compounds are often designed to be biologically active, and there is growing concern of the effects on non-target organisms and ecosystems (Boxall et al. 2012; Brausch et al. 2012), and for potential effects on humans from trace pharmaceutical concentrations in drinking water (Boxall et al. 2012; Daughton and Ruhoy 2013). Such concern has prompted research into the assessment of better wastewater treatment practices (Styrishave et al. 2011; Lajeunesse et al. 2012), and promoted questioning into the need for better drug designs and the potential overuse of pharmaceuticals in modern society (Daughton 2002; Brooks et al. 2012; Valenti et al. 2012; Daughton and Ruhoy 2013).

Among the most studied PIEs is a class of antidepressants known as selective serotonin reuptake inhibitors (SSRIs). These drugs are relatively stable in the environment, resistant to hydrolysis and photolysis in laboratory studies and are found to accumulate in sediments and biota (Bringolf et al. 2010; Mennigen et al. 2011; Brooks et al. 2012), where they are known to affect reproduction, foraging, stress responses, and locomotion of fish and invertebrates (Brooks et al. 2003). SSRI activity in aquatic fauna is due to the conservation of the active serotonin reuptake transporter throughout vertebrates (Mennigen et al. 2011), and documented importance of serotonin receptors in

the neuroendocrine system of invertebrates (Newcomb et al. 2006; Spitzer et al. 2008; Meechonkit et al. 2010). Though concentrations of SSRIs in the environment (e.g. < 1 µg/L) are typically well below acute toxicity levels to native fauna (Kolpin et al. 2002; Ramirez et al. 2009; Brooks et al. 2012), primary metabolites also show bioactivity similar to the parent compound (Fong and Molnar 2008), and cocktails of multiple SSRIs may result in additive or greater than additive effects (Henry and Black 2007; Styrishave et al. 2011). Thus, the number of chemicals and metabolites in the environment capable of inducing a biological response may be larger than predicted or measured thresholds from laboratory studies.

In the present study, we investigated the effects of chronic exposure (67 d) to fluoxetine, a common and potent SSRI, on adult freshwater mussel behavior. Freshwater mussels of the order Unionoida are among the most imperiled taxa worldwide (Haag and Williams 2013), and water quality concerns and pollution are considered an important challenge to their recovery (Downing et al. 2010). Freshwater mussels are often the most sensitive taxa to acute toxicity from aquatic pollutants (Raimondo et al. 2008), including fluoxetine (Hazelton et al. 2013). Serotonin is an important neurotransmitter to unionids (Meechonkit et al. 2010), and serotonergic effects have been reported in mussels exposed to wastewater effluent at threshold concentrations equivalent to those at distances of 4-5 km downstream of a municipal discharge (Gagne et al. 2004). Known effects on freshwater mollusks include release of gametes and parturition of larvae (Fong 1998; Cunha and Machado 2001; Gagne et al. 2004; Bringolf et al. 2010), changes in embryonic or larval development (Gust et al. 2009; Hazelton et al. 2013), increased mantle lure displays (Cunha and Machado 2001; Bringolf et al. 2010; Hazelton et al.

2013), and an apparent loss of control of the muscular foot (Cunha and Machado 2001; Fong and Molnar 2013; Hazelton et al. 2013). However, the importance of these behaviors in an environmental context is not well understood. Therefore, the current study focused on fluoxetine's effects on movement and burrowing behavior in a unionid, the wavy-rayed lampmussel (*Lampsilis fasciola*), in an effort to better understand the adverse outcome pathway (Ankley et al. 2010) of behavioral effects.

4.2. Methods

4.2.1. Animal Care

One year-old female *Lampsilis fasciola* (Length = 42.8 mm ± 1.9 std. dev., Width = 19.0 mm ± 2.8, Height = 28.3 mm ± 1.1) were cultured from wild brood stock at the Alabama Aquatic Biodiversity Center and shipped to the University of Georgia Aquatic Ecology Laboratory by overnight courier on April 18, 2012. Upon arrival, animals were tagged (with cyanoacrylate adhesive) with individually coded Hallprint shellfish tags (Hallprint Inc., Hindmarsh Valley, South Australia). Animals were acclimated over 48 h to filtered (< 25 µm) pond water through two 50% dilutions of shipping water, and eventually maintained in a 530L Living Stream (Frigid Units, Toledo, Ohio) equipped with partial flow-through of filtered pond water. Natural food in the pond water was supplemented bi-weekly with ~ 500 mL solution of commercial shellfish food (stock solution included 6 mL/L *Nannochloropsis* and 14 mL/L Shell Fish Diet; Reed Mariculture, Campbell, CA).

4.2.2. Experimental Procedure

Behavioral experiments were conducted from August 14 to November 10, 2012 using 19 L glass aquaria (39 cm L x 19.6 cm W x 23 cm D). Four liters of Quikrete Premium Play Sand (Quikrete, Atlanta, GA) were spread to an approximately uniform depth (5 cm) in each aquaria. Sand was triple washed with dechlorinated tap water through a 300 µm sieve to reduce turbidity from small particles during water changes. Four replicate aquaria were assigned to each treatment concentration (nominal fluoxetine concentrations: 0, 0.5, 5, 50 µg/L) containing two mussels each for a total of 16 experimental aquaria. Two additional aquaria with no mussels were assigned to each treatment group as control tanks for algal clearance and fluoxetine uptake endpoints.

To facilitate identification between the two individual mussels in an aquarium, each was tagged with a 7 cm long segment of floating fly fishing line colored either yellow or orange. The tag was located on the posterior-dorsal quadrant of the right valve, extending along the anterior-posterior axis. Longer segments of floating fly fishing line have been used successfully in movement studies of freshwater mussels and this method does not appear to harm the animal or affect burrowing activity (Newton et al. 2012). Tests were conducted using standardized-reconstituted moderately hard water (USEPA 2002), and animals were gradually introduced to test dilution water through a series of dilutions. On August 10, animals were introduced to lightly-aerated test aquaria containing reconstituted soft water (32 mg/L CaCO3 hardness, 30 mg/L CaCO3 alkalinity; USEPA 2002) for 48 h, followed by 100 % water renewals of increasing hardness (48 h: 64 mg/L $CaCO_3$ hardness, 32 mg/L $CaCO_3$ alkalinity; 72 h: 74 mg/L $CaCO_3$ hardness, 54 mg/L $CaCO_3$ alkalinity). Final moderately hard dilution water

(USEPA 2002) was reached on August 14 and used for the remainder of the experiment (mean hardness = 86.6 ± 8.4 s.d. mg/L $CaCO_3$, mean alkalinity = 63.21 ± 9.69 s.d. mg/L $CaCO_3$).

Complete water changes were conducted at 72 hour intervals throughout the experiment, during which (1) the mussels were removed from the aquaria; (2) water and organic deposition on the sediment was siphoned and discarded; (3) water was replaced and sediment was graded to a uniform depth; (4) mussels were replaced on top of the sediment on their left valve with their anterior-posterior axis oriented along the narrow width of the tank. Animals tagged with yellow tags were placed at ~ 13 cm along the length of the tank, and orange tagged individuals at ~ 26 cm. Both animals were placed approximately along the midline of the tank width. This served as a starting observation point, from which all behavior and movement could be measured. To further acclimate test animals to the static water renewal procedures, four 72 hour treatment cycles and water changes were conducted prior to introducing fluoxetine exposures on September 4. Water renewal and retreatment continued as above, every 72 h for 67 d for a total of 19 treatment cycles. During a water change on day 18 an incidental mortality occurred to one animal in Tank 11 (0.5 µg/L treatment), this animal was subsequently removed from all experimental analyses.

Upon completion of the experiment, animals were removed from the exposure aquaria, assessed for metabolic activity with flow-through respirometry (see section 4.2.5 below) and then processed for glycogen analysis and chemical quantitation. Foot tissue biopsies (mean weight = 7.3 ± s.d. = 2.3 g) were collected for glycogen assay and hemolymph (~ 100 µL) was extracted and pooled from the sinuses of the anterior and

posterior adductor mussels and stored at -20 °C for later fluoxetine quantitation. Soft tissue was removed from the shells and whole wet weight, shell weight, shell length, height and width were recorded. Whole soft tissue mass was recorded and soft tissue was bisected along the anterior-posterior axis. One half of the soft tissue was frozen at -20 ºC for later fluoxetine quantitation, and the remainder was weighed on (pre-dried & tared) aluminum weighboats then oven dried for 24 h at 87 ºC to determine dry weight and facilitate calculation of % water retention per animal.

4.2.3. Behavioral observations

Observations of mussel behavior were conducted at 0, 1, 3, 6, 12, 24, 48, and 72 h post water renewal and retreatment during each of 16 treatment cycles. During each observation the location of each mussel along the length and width of the aquaria was recorded using a 1-cm mesh grid placed on the top of the aquarium. Movement between observation periods was recorded as the length of an observed track in the sediment from one location to another. In the absence of a track, the linear distance between points was calculated using Euclidean distance. Total movement was calculated as the sum of movements for a mussel during each 72 h treatment cycle. Vertical movement was assessed as the estimated percent of mussel body burrowed at each time point. Horizontal and vertical movement was calculated from a subsample of seven treatment cycles (starting on exposure days: 1, 3, 6, 9, 41, 55, 64) throughout the 67 d exposure. Body orientation was recorded as one of five primary orientations: (1) left – an animal lying on its left valve; (2) right – lying on right valve; (3) horizontal – dorsal surface (umbo) oriented toward top of aquarium, (4) vertical –posterior (siphoning) margin of

shell toward top of aquarium; and (5) inverted – an abnormal behavior where the animal is either vertical with the anterior margin, or horizontal with ventral surface toward the top of the aquarium. Foot protrusion was recorded as a binomial response (i.e. present/absent), where present is when the foot is extended beyond the margins of the valves (Hazelton et al. 2013). Gape was also recorded as a binomial response when valves were opened and right and left mantle tissue beyond the siphon apertures was observed as not touching, thus creating a gape into the mantle cavity. Lure and siphon display were recorded using a modification of the criteria used in Bringolf et al. (2010). Categories included: (0) animal was buried and no display could be observed; (1) valves were closed and thus no display; (2) only siphon apertures were observed beyond valve margins; (3) mantle flap lure was partially displayed beyond valve margins; and (4) the mantle flap lure was extended fully beyond the valve margins.

4.2.4. Algal Clearance

We measured clearance rate of commercial algae to assess potential effects of fluoxetine on freshwater mussel filtering and feeding behavior. Mussels were fed a mixture of *Nannochloropsis* and Shell Fish Diet (Reed Mariculture) during the 24 h observation. Feeding and subsequent algal sampling was delayed for 24 h after a water change to reduce the likelihood of interference of resuspended sediment or old food particles in particle analysis. Fifteen milliliters samples of water were taken from each tank prior to feeding (T = 0), after the algae had reached uniform suspension (T = 1; approximately 1 h after algae introduction), then at 24 and 48 h after feeding. Algal concentrations were quantified using a Beckman Coulter Multisizer III with a 100 µm

aperture tube with filtered (< 0.45µm) 0.9% NaCl as a diluent. Each 15 mL water sample was vortexed, and a 200 µL subsample was diluted to final concentration with 200 mL of diluent. Particle counts and volumes between 2 and 20 µm in diameter were enumerated in 2 mL of final sample solution at electrical current of 1600 µA and a Gain of 2. Particle size calibration constants (Kd = 134.8) were established through machine calibration with Coulter Standard L10 (10 µm) & Coulter Standard L20 (20 µm) latex beads. Samples of diluent were analyzed at each change of diluent fluid to minimize background disturbance in analysis. Algal settling rates were established by concurrent sampling of tanks without mussels (n=8) at 0, 1, 24, 48 h post feeding. Settling tanks were given the same amount of algal suspension as experimental mussel tanks, and were maintained under the same aeration. Algal clearance was calculated as the percentage of algal particles removed from the water column at 24 and 48 h (Equation 1):

$$Clear_{T_x} = \frac{(\Delta T_1 - \Delta T_x)}{(\Delta T_1)} * 100\ \%$$

Where Δ equals the difference in particles after subtracting background particles at $T = 0$ and diluent contamination; T refers to the algal concentration in the experimental tank at time x, and S refers to the algal concentration of the settling tank at time x.

4.2.5. Flow-through Respirometry

We assessed the effects of fluoxetine on respiration using a Q-box AQUARESP flow-through respirometer (Qubit Systems, Kingston, ON, Canada). Following the 67 d fluoxetine exposure, both animals from each tank were placed in dechlorinated tap water in a small respirometer chamber (3.7 ID, 15 cm long) and run for a total of 45 min using repeated cycles of 300 sec circulation during which dissolved O_2 depletion was measured,

and a 60 sec flushing cycle of freshly oxygenated water between circulations. Volume of oxygen consumed was measured using Equation 2:

$$VO_2 = \frac{y_{DO} * (Vol_C - Vol_M)}{DW_M * 3600}$$

Where VO_2 is the volume of oxygen consumed, y_{DO} is the slope of the dissolved oxygen concentration during a circulation cycle, Vol_C is the volume of the respiration chamber, and Vol_M is displacement volume of the mussels, and DW_M is the dry weight of mussel soft tissue. Four representative dissolved oxygen depletion slopes were used to calculate a mean VO_2 for each replicate.

4.2.6. Tissue Glycogen

To further assess metabolic effects of fluoxetine exposure on mussels, we measured total glycogen in foot tissue biopsies (~ 15-30 g) from all mussels following the 67 d fluoxetine exposure. We followed the methods of Naimo et al. (1998). Glycogen standards were produced by dissolving 25 mg powdered oyster glycogen (CAS N 9005-79-2: Sigma-Aldrich Corp., St. Louis, MO) in 5 mL deionized water to a final stock solution concentration of 5000 mg/L, then diluted to 2000, 1000, 500, 250 and 125 mg/L through serial dilution. Internal standards from homogenized foot tissue of *Lampsilis cardium* collected from Pool 8 of the Mississippi River in October 2011 were used for quality assurance and were digested and extracted in the same procedure as test samples. All standards, internal standards and samples were transferred to a 96-well plate and absorbance was measured at 490 nm using a SpectraMax M2 spectrophotometer and SpectraMax Pro software (Molecular Devices, Sunnyvale, CA). Standards and internal standards spiked with each standard concentration were analyzed in triplicate while one

replicate was analyzed of each experimental sample. A standard curve of total glycogen (mg) was used to quantify each sample, and then normalized to the mass of tissue sampled (mg/g). Average glycogen concentrations for both mussels in an experimental aquarium were calculated and used for statistical analyses.

4.2.7. Test Chemicals and Quantitation

Fluoxetine exposures were conducted with fluoxetine hydrochloride (>98% purity) from Fisher Scientific (West Chester, PA, USA). Stock solutions were prepared as needed throughout the experiment. Initial stock for 50 µg/L treatments was prepared by dissolving 15.5 mg fluoxetine hydrochloride in 20 mL deionized water for a final stock concentration of 775 mg/L fluoxetine. Serial dilution of 1:10 (stock: DI water) was used to prepare stock solutions for 5 µg/L and 0.5 µg/L treatments for final stock concentrations of 77.5 mg/L and 7.75 mg/L fluoxetine, respectively. Aquaria were treated with 1 mL of assigned stock concentration in 15.5 L of aquarium dilution water, resulting in target experimental concentrations of 0.5, 5, 50 µg/L. Control aquaria were not treated with stock dilution water because no vehicle was needed and the volume of stock was minimal compared to the total volume of the aquaria (1 mL/ 15.5 L < 0.01 % total volume).

Water samples (10-15 mL) were collected from each experimental aquarium for fluoxetine quantitation at 0, 24, 48, 72 h post treatment during the first and last treatment cycle (exposure days 1-3, 64-67), and at 0 h post treatment during the second and fourth treatment cycle (exposure day 3 and 9). At 0, 24, 48 h time points during the first treatment cycle (exposure day 1-3), we collected duplicate samples from aquaria without

mussels to assess the binding of fluoxetine to algal suspension. One sample was unfiltered and the second was vacuum filtered through a 0.45 µm nitrocellulose membrane filter (Whatman, GE Healthcare Life Sciences, Piscataway, NJ). All water and tissue samples were frozen at -20 °C and then shipped on dry ice via overnight courier to Baylor University for fluoxetine analysis. The average tissue fluoxetine concentration per tank was used to calculate the mean tissue concentration per treatment. Mean aqueous and tissue fluoxetine concentrations were used to calculate bioaccumulation factors (BAF: Newman 1995; Thorsen et al. 2007).

Instrumentation and extraction for analyses of fluoxetine in tissue followed previously reported protocols (Ramirez et al. 2007; Du et al. 2012). Pharmaceuticals were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) on a Varian model 410 autosampler, ProStar model 212 binary pumping system, and model 1200L triple quadrupole mass analyzer. An isocratic mobile phase condition that resulted in elution at 5 minutes was identified for fluoxetine. Salts and other highly polar sample constituents were diverted from the mass spectrometer during the first two minutes of each run. Chromatography was performed with a 15 cm × 2.1 mm Extend-C18 column (5µm, 80 Å; Agilent Technologies, Palo Alto, CA, USA) connected to a 12.5 mm x 2.1 mm Extend-C18 guard cartridge (5 µm, 80 Å; Agilent Technologies). The ionization mode and monitored transitions for fluoxetine and internal standard fluoxetine-d_6, which had not been reported previously by our group, were as follows: ESI+ fluoxetine 310>148 and fluoxetine-d_6 316>154. Method detection limits (MDL) were determined by extracting and analyzing reference samples (uncontaminated mussel tissue) fortified with fluoxetine at a concentration ≤ 10 times the previously reported MDL for fish tissue

(Du et al. 2012). MDL of fluoxetine for whole mussel tissue was determined to be 2.37 ng/g. Six standards, ranging in concentration from below fluoxetine's MDL to 500 ng/mL, were used to construct linear calibration curve ($r^2 \geq 0.998$). Instrument calibration was monitored over time via analysis of continuing calibration verification (CCV) samples with an acceptability criterion of ± 20%.

Water samples were processed with a method adapted from a previously reported protocol (Lajeunesse et al. 2012). Two replicates of each nominal concentration were combined to form an aliquot for samples with concentrations of 0 (control) ng/mL, 0.5 ng/mL, and 5 ng/mL. Additionally, 50 µL of fluoxetine-d_6, 5 µL of 85% phosphoric acid, and 250 µL of methanol were added to the sample. The aliquot was then gravity fed through a Strata SCX (Phenomenex, Torrance, CA, USA) solid phase extraction cartridge. Cartridges were preconditioned with 5 mL of methanol. The sample was then eluted with 5 mL of methanol and blown down to dryness under a gentle stream of nitrogen. The dried sample was reconstituted with a liquid chromatography mobile phases. A 500 µL aliquot was taken for samples with a nominal concentration of 50 ng/mL and combined with 450 µL of liquid chromatography mobile phases as well as 50 µL of fluoxetine-d_6 in an autosampler vial.

4.2.8. Statistical Analyses

Total movement, percent burrowed, percent algal clearance, and tissue glycogen were analyzed using general linear mixed models through the nlme package (Pinheiro et al. 2013) in R statistical platform, version 3.0 (2013). Fixed effects were assessed at each fluoxetine treatment as a nominal variable. A random intercept was incorporated for the

effect of aquaria on movement, burrowing and tissue glycogen of individual mussels, thus data from all mussels could be analyzed without jeopardy of pseudoreplication (Bolker et al. 2009). Random intercepts for treatment cycle were also incorporated in models for percent burrowing and percent algal clearance. Models for percent algal clearance also incorporated a random effect for the slope of the starting algal concentration (T = 0). The predictive model for percent burrowing also included a fixed effect term for the observation hour, and an interaction term for each treatment level at each hour; observations 0 h post treatment were omitted because animals were always replaced into the aquaria on top of the sediment in a uniform starting position. Normality and uniformity of variance were assessed for each model through graphical inspection of the model residuals. If models deviated markedly from a normal probability distribution, data was transformed to better approximate a uniform distribution. Main effects were considered statistically significant at $\alpha = 0.05$ threshold.

A proportional hazard approach was used to assess the effects of fluoxetine on time to first movement. These methods are powerful semi-parametric approaches to assess the risk that an intervention (i.e., fluoxetine exposure) will cause relative to the underlying risk of a control group (Newman 1995). Specifically, we used a marginal Cox proportional hazard model where multiple responses for each individual were incorporated through stratifications (i.e., treatment cycles), and variability among individuals was accounted for by clustering the data for each animal (Therneau and Grambsch 2000). Such models are often used in epidemiological studies to analyze repeated time to event data, controlling for non-independence among observations for each subject (Therneau and Grambsch 2000). Three hundred twenty-two observations

were used to calculate time to first movement, incorporating all observation sets without missing measurements. Treatment groups that were statistically different from the control were visually represented using a Kaplan-Meier survival curve to estimate the probability that an animal will maintain its initial position at each time point.

The effect of fluoxetine on percent frequency of siphoning and lure display behaviors was analyzed using multivariate analysis of variance (MANOVA) with a Wilks-λ test statistic; the same approach used in Bringolf et al. (2010). To avoid multicolinearity in our analysis, we only tested hypotheses of significant differences in display of mantle margins, and lure display. We omitted observations at 0 h post treatment because all animals were typically closed for the first 10-15 minutes after handling during water renewal and retreatment. The model was assessed graphically for multivariate normality and uniformity of the data, and significant ($\alpha = 0.05$) responses were followed by general linear models to determine the effect of each treatment concentration on each response behavior. We assessed the effects of fluoxetine on mean frequency (count) of less common behaviors (gape, foot protrusion, actively moving, inverted position) using non-parametric Kruskal-Wallis tests because these behaviors were rare and the data were determined to deviate from a normal distribution according to either Bartlett test or Shapiro-Wilks test (Newman 1995). All 0 h post treatment observations were omitted, and a threshold of statistical significance was set at $\alpha = 0.10$ for these tests.

4.3. Results

4.3.1. Chemical Quantitation

Measured aqueous fluoxetine concentrations varied across treatment cycles, and trace concentrations of fluoxetine were routinely detected in our control samples (Table 4.1). Mean fluoxetine concentrations across treatment cycles were 0.209 µg/L (± 0.166 std. dev n = 2 at each of 4 treatment cycles) for control samples; 0.545 (± 0.638) for 0.5 µg/L treatments; 2.520 (± 1.638) for 5.0 µg/L treatments; and 22.325 (± 12.937) for 50 µg/L treatments. Tissue concentrations of fluoxetine ranged from 59-490 ng/g in control animals; 150-390 ng/g in 0.5 µg/L; 300-890 ng/g in 5.0 µg/L; and 12,000 – 40,000 ng/g in 50 µg/L treatments (Table 4.1). In tanks without mussels (settling tanks), we saw marked decreases in aqueous fluoxetine concentrations after the removal of algae through 0.45 µm filtration (Table 4.1). In tanks containing mussels, we saw significant depletion of fluoxetine from time 0 to 72 h post treatment (Figure 4.1). Depletion estimates for the best fit log-normal curves of the 5 and 50 µg/L treatments had significantly different slopes and interaction terms for time and nominal treatment concentration (fluoxetine (µg/L) = -8.48 ln(h +1) + 22.40; $p = 0.003$, adjusted $R^2 = 0.93$), suggesting greater rates of depletion in groups with higher fluoxetine concentrations.

4.3.2. Behavioral Observations

Mean total movement per tank across 72 h treatment cycles ranged from 0 to 127 cm (mean = 10.62 cm ± 11.79 std. dev.). We saw a dose dependent increase in total movement with increases in fluoxetine concentration, where mean total movement in the 50 µg/L treatment group was more than double that of the control group, and statistically

significant at $p < 0.001$ (Figure 4.2A). Log +1 transformed data was used in mixed model regression to assess the fixed effects of fluoxetine concentration on total movement. Random effect variation included standard deviations of treatment cycle (0.60) and tank within treatment cycle (0.31). Estimates for fixed effects intercept parameters were 1.99 ± 0.26 std. dev. (102 d.f., t-value = 7.64, $p < 0.0001$), and slopes for 0.5 µg/L (-0.03 ± 0.19 std. dev., 102 d.f., t-value = -0.17, $p = 0.87$), 5 µg/L (0.22 ± 0.19 std. dev., 102 d.f., t-value = 1.17, $p = 0.25$), and 50 µg/L (0.70 ± 0.19 std. dev., 102 d.f., t-value = 3.79, $p = 0.0003$).

Cox and Kaplan-Meier proportional hazard models showed that there were no observations of movement prior to 3 h post treatment, at which time only 4 movements were recorded (p(movement) = 0.02 ± 0.02 std. dev.), all of which were from the 50 µg/L treatment group. The probability of movement at 6 and 12 h was 0.08 (± 0.02) and 0.23 (± 0.03), respectively. Greatest change in movement occurred between 12 and 24 h (0.63 ± 0.03), and nearly all mussels had moved by 48 h (0.87 ± 0.02) and 72 h (0.96 ± 0.05). The Cox proportional hazard marginal model found statistically significant differences in risk of movement (in proportion to the risk of movement in the control group) in both the 0.5 (Cox risk coefficient = 0.096, robust std. error = 0.049, Wald $z = 2.00$, $p = 0.046$) and 50 µg/L treatment groups (Cox risk = 0.263, robust std. error = 0.088, Wald $z = 2.98$, $p = 0.0028$). Odds ratios were calculated as exponents of Cox risk coefficients (Therneau and Grambsch 2000), thus animals treated at 0.5 µg/L were 1.1 times as likely (i.e., 10% more likely) to move, and animals treated at 50 µg/L were 1.3 times as likely (i.e., 30% more likely) to move than control animals. Animals in the 5 µg/L treatment were not significantly different from the control group (Cox risk = 0.023, robust std. error = 0.059,

Wald z = 0.38, p = 0.70). Kaplan-Meier survival curves for control and 50 µg/L groups revealed the greatest change in movement probability occurred within the first 10-h (Figure 4.2B). Probability of remaining in starting position differed significantly (based on non-overlapping 95% confidence intervals) between control animals and the 50 µg/L treatment at 3 h and 6 h post treatment. However, by 12 h post treatment probability of maintaining position had been reduced to 0.83 (± 0.05 std. error) in control groups and 0.67 (± 0.06) in 50 µg/L animals. At 24 h, the probability of holding position in control animals was 0.27 (± 0.06), and 0.11 (± 0.04) in the 50 µg/L treatment. By 48 h post treatment, all animals had moved from their starting position.

Vertical movement (i.e.. burrowing) mirrored trends in horizontal movement, and little burrowing activity took place in the first 6 h post treatment, with the exception of the 50 µg/L treatment group. Within the first hour, mean % burrowed ranged from 0 across control animals to greater than 9 % (± 1.98 % std. dev.) in the 50 µg/L treatment. By 6 h post treatment, all lower treatments and control groups were less than 4 % burrowed, but the 50 µg/L group had progressed to 19 % (± 5.55 %) burrowed. Residuals from linear regression models only deviated slightly from a normal distribution, thus no data transformation was used. Effects of randomly varying intercepts for treatment cycle (std. dev. = 6.76) and tanks within treatment cycle (std. dev. = 0.003) were accounted for using a mixed model regression with fixed effects terms for fluoxetine treatment, hour post treatment and interactions of treatment and time (model intercept = 12.61 ± 2.23 std. error, d.f. = 2824, t value = 5.65, p < 0.0001). We found significant relationships for the 50 µg/L treatment (14.16 ± 2.12 std. error, d.f. = 252, t value = 6.68, p < 0.0001), hour (1.39 ± 0.04 std. error, d.f. = 2824, t value = 34.52, p <

0.0001), and a significant interaction term for the 50 µg/L group and hour (-0.22 ± 0.06 std. error, d.f. = 2824, t value = -3.83, $p < 0.0001$). A negative coefficient for the interaction term indicates that the differences in % burrowing between the 50 µg/L test group and the control group are greater at earlier hours, and this difference becomes less significant as time increases. At hours 1, 3, and 6 post exposure the 95% confidence intervals around % burrowing parameter estimates do not overlap between 50 µg/L and control groups.

Mean frequencies of siphon and lure displays were correlated with fluoxetine treatment. We saw dose dependent decreases in frequency of closed and siphon-only behaviors, and dose dependent increases in mantle margin displays and lure displays (Figure 4.3B). Residuals of all responses exhibited normal distributions with the exception of the buried behavior; however, we did not include this behavior in the MANOVA analysis, nor in post-hoc general linear models to assess multiple comparisons. The overall MANOVA model was significant (d.f. = 3, Wilks' $\lambda = 0.25$, $\sim F = 3.67$, $p = 0.011$). Post-hoc general linear models revealed that mantle margin displays were significantly more likely in all fluoxetine treatments when compared to the control group: 0.5 µg/L = 0.067 ± 0.028 std. error, t- value = 2.398, $p = 0.03$; 5 µg/L = 0.079 ± 0.028 std. error, t- value = 2.817, $p = 0.02$; 50 µg/L = 0.103 ± 0.028 std. error, t-value = 3.654, $p = 0.003$; model intercept = 0.093 ± 0.02 std. error, t- value = 4.683, $p = 0.001$. Lure display behaviors were statistically more common in 5 µg/L (0.169 ± 0.061 std. error, t- value = 2.78, $p = 0.02$) and 50 µg/L (0.247 ± 0.061 std. error, t- value = 4.06, $p = 0.002$), with a model intercept = 0.032 ± 0.04 std. error, t- value = 0.745, $p = 0.47$.

Other behaviors (i.e., gape, foot protrusion, active movement and inverted position) were found in less than 3% of all observations (Table 2.2). Animals were found with an open gape in 2.3% of all observations, and greater than half of those were in the 50 µg/L group. Occurrences for individuals ranged from 0 to 11 observations, and the mean occurrences across all animals was 2.68 (± 2.75). The overall Kruskal-Wallis model was not significant at $p = 0.10$; however, there was a significant one-way comparison between the 50 µg/L group and control (observational difference = 8.25 > critical difference = 7.16). Foot protrusion ranged from 0 observations to 10 occurrences throughout the 67-d experiment, and a grand mean of 0.68 (± 1.83) observations per animal. Foot protrusion and active movement both had statistically significant Kruskal-Wallis models and one-way comparisons between 50 µg/L animals and the control. Animals were observed in active movement a mean of 0.77 (± 0.84) times per animal, and the greatest number of occurrences per individual was 3 times. Incidentally, nearly all observations of active movement in control, 0.5, and 5 µg/L groups occurred at the 12-h observation time point. Indeed only one observation in the 0.5 µg/L group occurred 1 h post treatment, and animals in the 50 µg/L group were seen actively moving at each time point except 24 h post treatment. Only 3 animals in the 50 µg/L treatment exhibited the inverted behavior, two of which were from the same tank. The mean number of occurrences per animal was 0.42 (± 1.46), and seven observations were from the same animal. Kruskal-Wallis model statistics were significant for occurrences of inverted position; however, a one-way pairwise comparison was not possible given 0 occurrences in the control group.

4.3.3. Algal Clearance

Mean and standard deviation of % settling were calculated using the eight settling tanks (i.e. aquaria without mussels), and the same calculation used for % clearance in other tanks. Initial cell densities ranged from 297,052 to 1,117,960 (mean = 698,810 ± 239,525 std. dev.) cells/mL in settling tanks and 166,127 to 912,043 (328,687 ± 107,177) cells/mL in experimental tanks containing mussels. Mean % settling was 20.9 % (± 9.5 %, n = 49) at 24 h and 42.7 % (± 17 %, n = 49) at 48 h post feeding. We saw no biologically meaningful or statistically significant difference in clearance rates among treatments at 24 or 48 h. Mean clearance across all groups was 73.1 % (± 12.2 %, n = 154) at 24 h and 86.1 % (± 9.0 %, n = 154) at 48 hr. Within group means and model estimates of clearance all varied within 5% of the grand mean.

4.3.4. Flow-through Respirometry

Only two replicate samples for each treatment were available for respirometry analysis. Mean and standard deviation of the control group (1346.25 ± 785.24 mg/g wet weight); 0.5 µg/L group (1105 ± 393.15); 5 µg/L group (2246 ± 147.19); and 50 µg/L group (1993.75 ± 931.26) were not significantly different according to a Kruskal-Wallis test ($\chi^2 = 3.17$, df = 3, $p = 0.37$).

4.3.5. Tissue Glycogen

Recovery of glycogen standards ranged from 78% (± 17% std. dev.) at 125 ng glycogen, to 105% (± 5%) at 500 ng glycogen with an overall mean recover of 96% (± 13%). Coefficient of variation of internal standards ranged from 1.5 to 6.7. Overall, the

mean calculated glycogen concentration was 34 (± 7.8) ng/g dry weight, and means varied little among treatments. The mixed model predicted a standard deviation of 2.97 accounting for the randomly varying intercept of each tank. Control animals had lower glycogen concentrations than animals in all other treatments (model intercept = 31.1 ± 2.8 std. dev., d.f. = 15, t = 10.95, p = 0). Both 5 and 50 µg/L treatment groups had slightly higher glycogen concentrations (5 µg/L group = 34.4 ± 6.8 ng/g, d.f. = 12, t = 0.84, p = 0.41; 50 µg/L group = 31.5 ± 6.8 ng/g, d.f. = 12, t = 0.11, p = 0.0.91), while the lowest fluoxetine treatment group had the highest glycogen concentration (0.5 µg/L group = 39.9 ± 7.0 ng/g, d.f. = 12, t = 2.16, p = 0.052).

4.4. Discussion

Our results indicate that prolonged exposure to fluoxetine can considerably alter the behavior of freshwater mussels. Animals treated with 50 µg/L fluoxetine traveled significantly greater distances than control animals. And though not statistically significant, mussels treated at 5 µg/L moved 50% further than control animals during 72 h exposures (Figure 4.2A). An increase in movement by mussels caused by fluoxetine was not expected. Earlier work of two of the authors (Bringolf et al. 2010; Hazelton et al. 2013), and others (Cunha and Machado 2001) have reported a significant swelling and protrusion of the foot in animals treated with fluoxetine. This has been attributed to potential increase in water uptake at the gills, and loss of control of the hydrostatic skeleton, which would presumably reduce the motility of affected mussels (Cope et al. 2008; Hazelton et al. 2013). Conversely, movement may increase and become more erratic than in untreated animals, evidenced by changes in position behavior and time to

movement. Incidences of foot protrusion in the present study were also more common in fluoxetine-treated animals, but rates of this behavior were less than previously reported (Cunha and Machado 2001; Hazelton et al. 2013). Reduced rates of foot protrusion are likely an artifact of the experimental design and the presence of sediment, because the foot was not visible by the observer when the mussel was mostly burrowed. Nevertheless, changes in total movement are likely important factors that may have underlying physiological effects (e.g. increased energy expenditure & reduced fitness). Changes in movement rates may also alter the roles that mussels play in maintaining sediment stability and structure, providing habitat for other macroinvertebrates and in sediment nutrient dynamics and oxygenation through bioturbation (Vaughn and Hakenkamp 2001).

We saw decreases in the time to first movement (Fig 4.2B) and the time to burrowing (Fig 4.3A), and these responses were closely correlated with an increased likelihood of movement during daylight observation periods. Control animals rarely moved during the first 12 h of a 72-h treatment cycle, and were never seen actively moving during daylight observation periods; however, mussels in the 50 µg/L treatment were observed actively moving at each daytime observation. Diel movement patterns in freshwater mussels have not been well studied and may be species specific. Amyot & Downing (1997) saw little difference between diurnal and nocturnal movement in Great Lakes populations of *Eliptio complanata*. But females of a more closely related species to *L. fasciola*, *Ligumia nasuta*, have been observed to move more frequently at night associated with lure display behaviors (Corey et al. 2006). It is possible that mussels in higher treatments had higher, and earlier movement patterns as an escape response to the

presence of the contaminant. To our knowledge, no previous research has looked at movement rates in mussels in the response to a toxicant, but mussels do increase movement and burrowing rates in response to increased temperature and other environmental variables associated with drought (Gough et al. 2012; Newton et al. 2012).

Our data also corroborate a previously described (Bringolf et al. 2010; Hazelton et al. 2013) increase in lure display behavior in fluoxetine treated animals (Fig 4.3B), which combined with changes in movement patterns, could alter interactions between affected mussels and host fish. Furthermore, increased daytime movement may increase the susceptibility of mussels to predators such as mammals or fish, which can have a considerable effect on a local mussel population. Muskrats (*Ondatra zibethicus*), have been shown to hamper conservation efforts and reduce populations of several species (Neves and Odom 1989; Diggins and Stewart 2000; Owen et al. 2011). We also saw increased likelihood of other abnormal behaviors (e.g. foot protrusion, gape & inverted positioning) caused by fluoxetine treatment, all of which could potentially be signs of stress or lead to increased predation.

Our chemical data contained some discrepancies, and we did see signs of fluoxetine contamination throughout water and tissue samples in our control group, which was likely a result of shared equipment use between treatments or spalshover from adjacent aquaria during water changes. Attempts were made to keep contamination to a minimum. Nevertheless, these levels were typically less than the lowest fluoxetine treatment (0.5 µg/L). High variability in fluoxetine tissue concentrations in the control treatment group indicates that fluoxetine contamination may have been driven by cross contamination of one or two of the tanks. Though effort was made to minimize or

eliminate reuse of equipment between tanks, incidental contamination must have occurred through equipment transfer or incidental splashing during water changes. We also saw approximately 80% decrease in the aqueous concentration of fluoxetine after algal removal through vacuum filtration. This suggests that fluoxetine may be binding to the algae and suspended organics, and ingestion may be increasing fluoxetine exposure disproportionately from lower concentrations, leading to tissue concentrations 50-140 times greater than those found in lower dosage groups. Kinetics investigating exposure to fluoxetine and similar contaminants *in situ* may need further investigation as uptake at the gill from dissolved pharmaceuticals may not be the only route of exposure, but that ingestion of bound contaminants is a likely source of contamination in freshwater bivalves.

The binding of fluoxetine to algae may be responsible for disproportionately elevated internal doses in mussels from the highest treatment concentration, yet interestingly we did not see any differences in algal clearance rates across our different treatments. Fluoxetine has been associated with a disruption of feeding behavior in frogs (Conners et al. 2009), striped bass (Gaworecki and Klaine 2008) and goldfish (Mennigen et al. 2010). The methods we used did not measure direct consumption of algae because we were unable to determine the relative production of feces and pseudofeces, but we also saw no significant change in glycogen stores among our treatments, which suggest that there was no differential change in feeding behavior. The lack of differences in algal clearance rates and glycogen stores in our study may be explained by our use of relatively high concentrations of algae; all animals may have been fed in excess of meeting metabolic needs and energy storage, masking any change in feeding behavior or feeding

physiology. Algal clearance rates may be useful as an endpoint in further ecotoxicity studies though, and should focus on short term clearance rates (i.e. less than 24 h) to determine if there is any change in foraging caused by toxicant presence.

Respirometry is another promising endpoint for consideration in unionid ecotoxicity testing. We saw an increase in oxygen consumption in our two highest treatments, though we only conducted two replicates of each treatment and the difference was not statistically significant. Other investigators have demonstrated that increases in respiration may be indicative of changes in metabolic activity (Levine and Cheney 2000) in response to a toxicant Conversely, respiration rates may decrease as a sign of toxicant avoidance if an animal responds to an irritant by closing its valves and reducing its respiration (Cope et al. 2008).

In conclusion, we tested the effects of the fluoxetine, a potent SSRI, on the behavior and physiology of adult *Lampsilis fasciola* over a 67-d experiment with retreatment at 72-h intervals. Previous research has shown changes in lure display and foot extension behavior associated with fluoxetine treatments as low as 39 µg/L (Cunha and Machado 2001; Bringolf et al. 2010; Hazelton et al. 2013). Foot extension (protrusion) was previously thought to be a likely impediment to movement in mussels; however, in the present study we saw statistically significant increases in movement, decreased time to movement, and increased likelihood of diurnal movement in mussels treated with fluoxetine at less than 50 µg/L. Mussels in higher fluoxetine concentrations also had increased rates of lure display, and higher rates of abnormal behaviors such as gaping, foot protrusion, and inverted position. Though we saw no changes in algal clearance rates, fluoxetine appeared to be binding to algae, thus ingestion is likely an

important exposure pathway for SSRI's and similar pharmaceuticals released in wastewater effluent. Changes in mussel movment *in situ* is likely to increase susceptibility to predation, and may also alter sediment nutrient cycling and oxygenation through changes in bioturbation provided by mussels.

4.5. Acknowledgements

Support for this project was provided by the University of Georgia Warnell School of Forestry & Natural Resources, the University of Georgia Interdisciplinary Toxicology Program and the Baylor University Institute for Ecology, Earth & Environmental Sciences. We are indebted to the laboratory assistance of Kristen Kellock and Robert E. Ratajczak at the University of Georgia and Colin Shea (USGS TN Cooperative F&W Research Unit), Alison Price-Stodola (Il NHS), and Andrew Gascho-Landis & Jason Wisniewski (GA DNR) for feedback and literature recommendations. Earlier drafts were considerably improved from the remarks of Brenda Rashleigh (US EPA), Marsha Black (UGA) and Robert Gogal (UGA).

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4.7 Table Captions

Table 4.1: Measured water and tissue fluoxetine concentrations (± std. dev.) and bioaccumulation factors (BAF) across treatment cycles during a 67-d exposure to *Lampsilis fasciola.* Percent reduction in fluoxetine from algal removal was calculated in tanks without mussels.

Table 4.2: Summary of occurrences (mean ± std. dev.) and Kruskal-Wallis test statistics for effects of fluoxetine treatment on abnormal behaviors of *Lampsilis fasciola* exposed for 67 days. Mean frequencies per tank were used in Kruskal-Wallis $\chi 2$ analysis to determine difference among treatment groups.

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Target	Measured Concentration at T = 0 [a]				% Removal from Algal Filtration [b]	ng/g Tissue Concentration [c]	BAF [d]
	Cycle 1	Cycle 2	Cycle 4	Cycle 19			
0 µg/L	0.10 (na)	0.24 (0.01)	0.43 (0.13)	0.065 (0.04)	na	196.5 (160)	941
0.5 µg/L	0.66 (0.33)	0.12 (0.02)	1.40 (0.14)	0.001 (0.001)	na	277.5 (62)	509
5 µg/L	3.5 (0.85)	2.05 (0.21)	4.10 (0.28)	0.43 (0.03)	81% (13%)	577.5 (122)	229
50 µg/L	25.00 (14.00)	16.50 (0.71)	39.00 (1.41)	8.80 (0.71)	78% (2%)	27250 (5172)	1221

[a] Fluoxetine concentration in water at beginning of 72 h treatment cycles 1, 2, 4 & 19 in µg/L averaged across tanks including mussels. n = 2 per treatment.

[b] Averages are % change of one measurement sample across time points 0, 24, & 48 h.

[c] Mean values were calculated for mussels within each tank prior to calculating treatment means.

[d] BAF calculated as mean tissue concentration divided by the mean 0 h water concentration.

Table 4.2: Summary of occurrences (mean ± std. dev.) and Kruskal-Wallis test statistics for effects of fluoxetine treatment on abnormal behaviors of *Lampsilis fasciola* exposed for 67 d. Mean frequencies per tank were used in Kruskal-Wallis $\chi 2$ analysis to determine difference among treatment groups.

Fluoxetine Treatment	Gape	Foot Protrusion	Actively Moving	Inverted Position
Control	7 (0.88 ± 1.11)	1 (0.125 ± 0.25)	2 (0.25 ± 0.29)	0
0.5 µg/L	15 (2 ± 0.91)	1 (0.125 ± 0.25)	6 (0.75 ± 0.65)	0
5 µg/L	18 (2.25 ± 1.85)	4 (0.50 ± 1.00)	4 (0.5 ± 0.41)	0
50 µg/L	44 (5.38 ± 3.4)†	16 (1.88 ± 2.43)†	12 (1.50 ± 0.71)†	12 (1.63 ± 2.63)
Total (% all obs)	84 (2.3 %)	22 (0.59%)	24 (0.64%)	12 (0.32%)
Kruskal-Wallis χ^2 , df, p	$\chi^2 = 6.11$, df = 3, $p = 0.106$	$\chi^2 = 6.68$, df = 3, $p = 0.083$	$\chi^2 = 7.55$, df = 3, $p = 0.056$	$\chi^2 = 6.4$, df = 3, $p = 0.094$

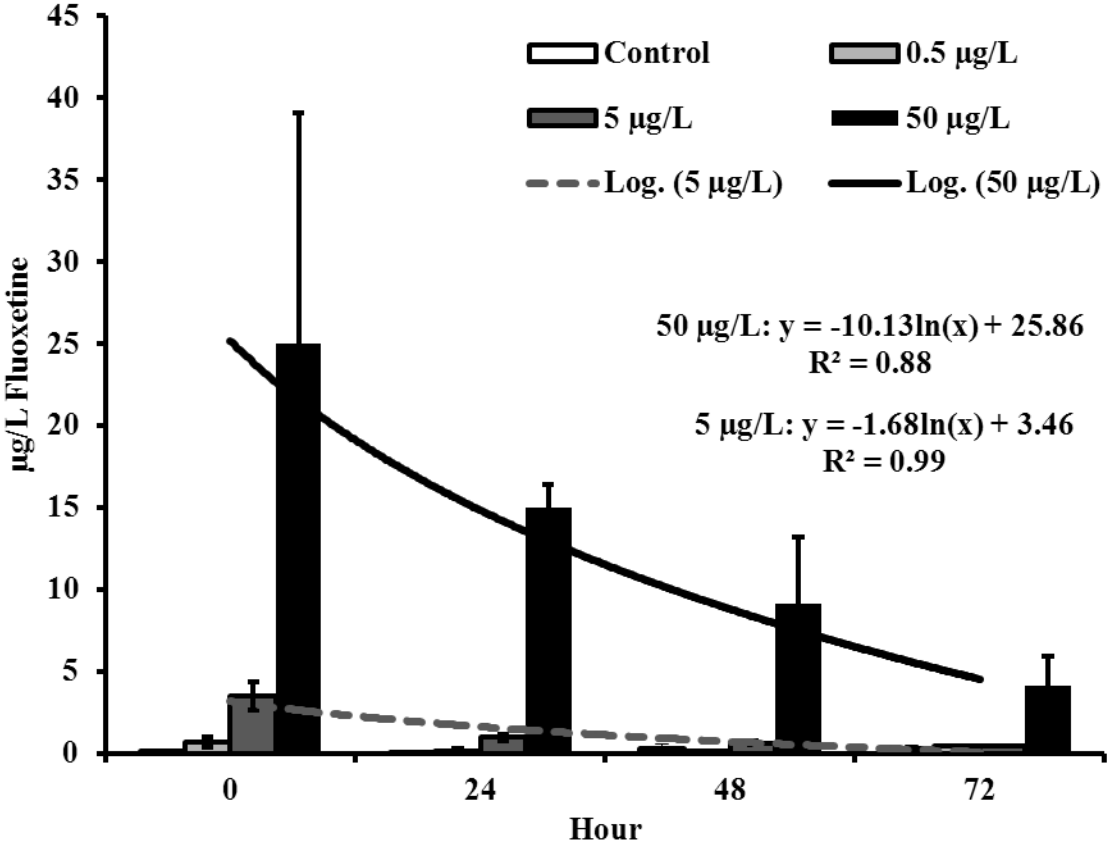
† denotes statistically significant difference from control group at $\alpha = 0.1$ in post hoc multiple comparison tests.

4.8 Figure Captions

Figure 4.1: Fluoxetine depletion throughout 72 h static-renewal treatment cycle of a 67 d exposure to *Lampsilis fasciola*. Data represents mean (± std. dev., n = 2 replicates at each treatment) of fluoxetine measurements of tanks with mussels present during treatment cycle 1 (exposure days 1-3). Depletion rates calculated using lognormal regression model.

Figure 4.2: Effects of fluoxetine treatment on *Lampsilis fasciola* movement. Error bars represent one standard deviation from the mean. (A) Raw scores of mean total movement of mussels per 72 h fluoxetine treatment cycle. ‡ denotes statistical significance from control at $p < 0.001$, based on log+1 transformed data (B) Kaplan-Meier survival curve of time to movement, expressed as probability of animal to retain starting position at tested time points. † denotes statistical significance from control at $p < 0.05$.

Figure 4.3: Effects of fluoxetine treatment on *Lampsilis fasciola* burrowing, siphoning and lure display throughout a 67 d exposure. (A) Modeled mean percent mussel body burrowed at hours post retreatment. (B) Mean frequency of observations of siphoning and lure display behavior. Error bars represent 95% confidence intervals, † and ‡ denote statistical significance from control at $p < 0.05$ and $p < 0.001$, respectively.



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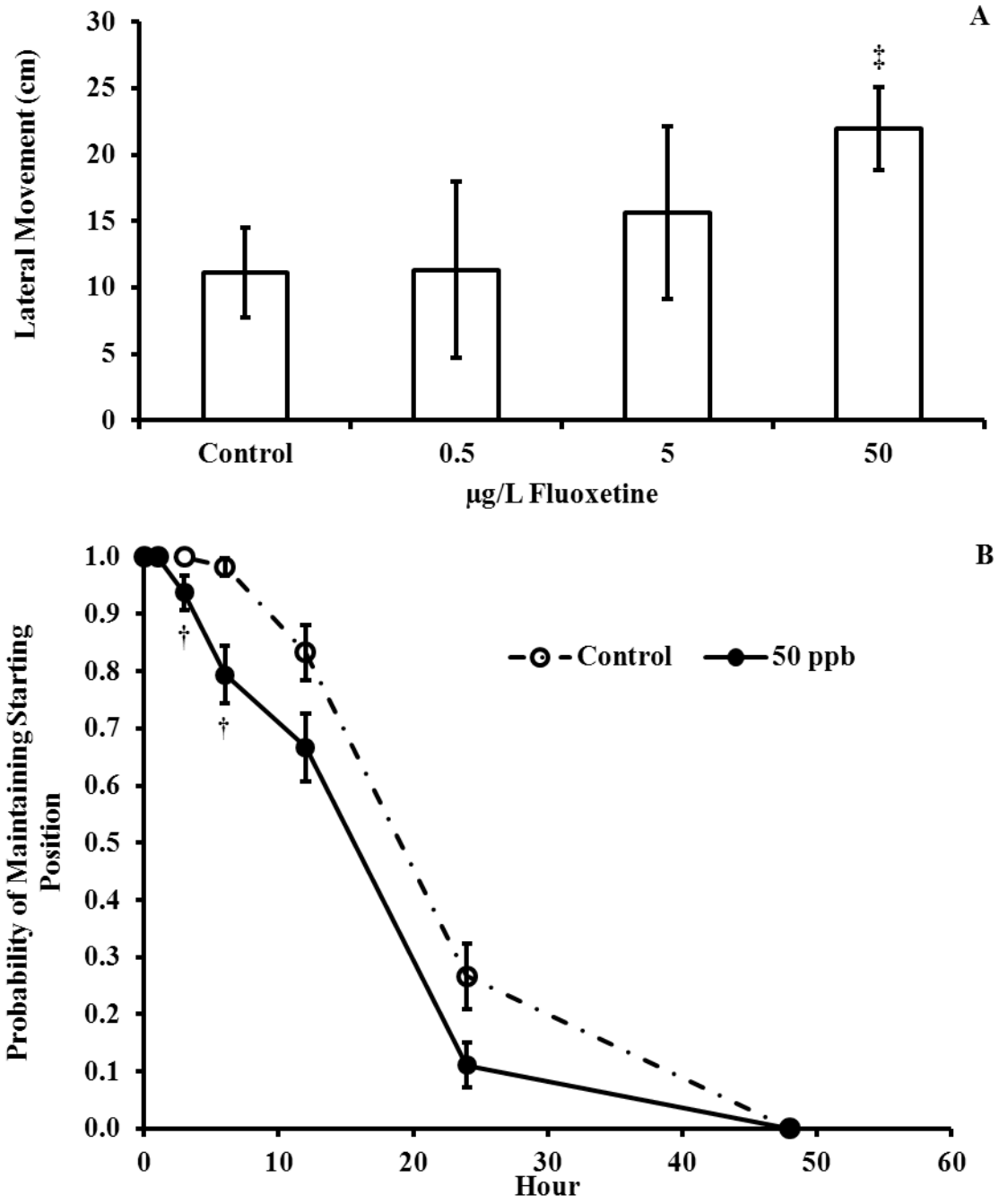

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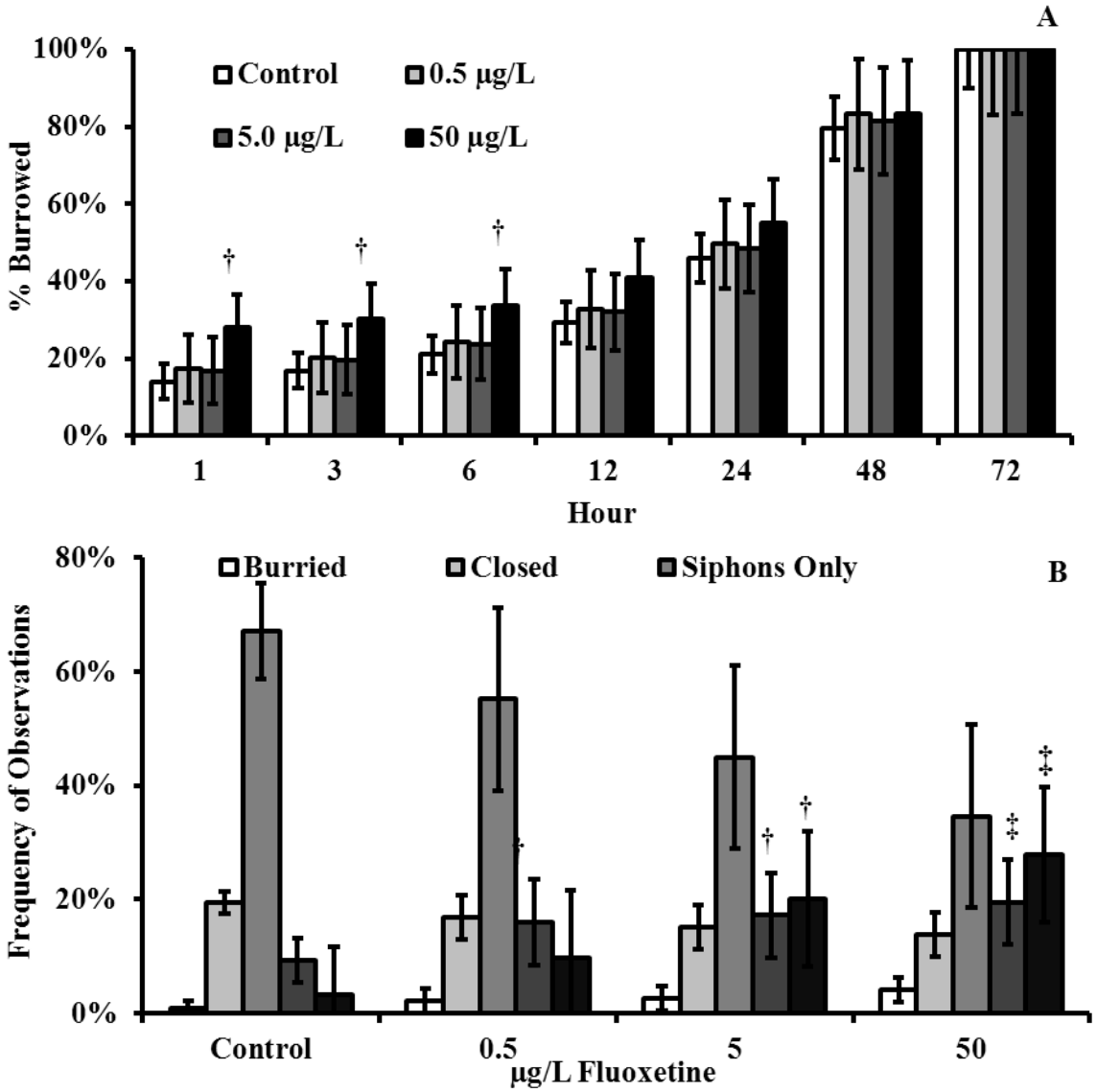

Figure 4.3: Effects of fluoxetine treatment on *Lampsilis fasciola* burrowing, siphoning and lure display throughout a 67 d exposure. (A) Modeled mean percent mussel body burrowed at hours post retreatment. (B) Mean frequency of observations of siphoning and lure display behavior. Error bars represent 95% confidence intervals, † and ‡ denote statistical significance from control at $p < 0.05$ and $p < 0.001$ respectively.

CHAPTER 5

PFOS ACCUMULATION IN FISH AND THE MUSSEL-HOST RELATIONSHIP[4]

[4] Peter D. Hazelton and Robert B. Bringolf. To be submitted to *Walkerana*, the journal of the Freshwater Mollusk Conservation Society

Abstract:

Freshwater mussels in the order Unionoida are among the most sensitive organisms to aquatic pollutants, and this is often cited as a reason for the decline in abundance and diversity of these taxa in North America. Perhaps the most critical stage in the lifecycle is an obligatory parasitic stage when the larvae (glochidia), while encapsulated on a fish host, metamorphoses to a free living juvenile. The effects of accumulated contaminants in host tissue on the metamorphosis and survival of glochidia and early transformed juveniles has not been investigated. In the present study we built a body burden of perflorooctane sulfonate in largemouth bass (*Micropterus salmoides*) over a 105 day exposure to 0, 10, and 100 µg/L, and then challenged these fish with paper pondshell (*Utterbackia imbecillis*) glochidia inoculations at two densities (1000 and 4000 glochidia/L). We monitored metamorphosis success and survival of juveniles as well as the robustness of juveniles assessed through a 48-h NaCl acute toxicity challenge. Fish showed several signs of PFOS effects including significant decreases in liver-somatic index and a decreasing trend in body condition associated with increased PFOS. We saw no significant trends in percentages of attachment, metamorphosis or mortality of juveniles transforming on fish from different glochidia densities or PFOS concentrations. However, EC50s from 48 h NaCl challenge were significantly lower in juveniles raised on bass from 10 µg/L PFOS (EC50 = 2.92 ± 95% CI = 2.7 - 3.15 mg/L NaCl) and 100 µg/L PFOS (EC50 = 2.72 ± 2.48 – 2.98 mg/L NaCl) than control animals (EC50 = 3.89 ± 3.43 – 4.41 mg/L NaCl), suggesting that juveniles produced from PFOS exposed fish were less robust than those produced on fish with no PFOS exposure. Our data suggest that juvenile mussel health may be influenced by host fish condition.

Keywords: PFAA, Immunotoxicology, Host-parasite relationship, Cortisol

5.1. Introduction

Each stage in the lifecycle of a freshwater mussel represents a unique exposure to environmental contaminants (Cope et al. 2008). While others have shown that glochidia are somewhat sheltered to some contaminants while they are encapsulated on fish (Jacobson et al. 1997), there remains question of whether mussels are exposed to accumulated contaminants released from their host. Fritts et al. (2013) have shown that glochidia obtain some nutrients from their hosts, thus direct transfer of toxicants to encapsulated glochidia may be possible. Furthermore, the relationship between fish host and its unionid parasite is mediated through the immune system, and a contaminant that acts upon this system may alter the relationship of host-parasite by mediating the immune response. For example, Dubansky et al (2011) found that *Utterbackia imbecillis* had higher metamorphosis success rates on bluegill (*Lepomis machrochirus*) injected with cortisol. Presumably anti-inflammatory properties of the cortisol caused reduced localized swelling around a glochidium, allowing more organized cell migration and encapsulation.

Perfluorooctane sulfonate (PFOS) is an industrial compound in a class of chemicals known as perfluoroalkyl acids (PFAAs), and is commonly used as a stain guard and for non-stick surfaces because of its unique qualities of repelling both water and oil (Giesy and Kannan 2002; Giesy et al. 2010). Growing concern over the persistence of PFAAs and PFOS in particular has led to a voluntary phase out of its use by manufacturer 3M in 2002 (Renner 2008; USEPA 2012). PFOS has shown immunotoxic effects in fish (Mortensen et al. 2011). This compound also causes reduced metamorphosis success to glochidia exposed *in marsupia* at higher concentrations

(Hazelton et al. 2012). Additionally, PFOS is known to bind to the gills and plasma proteins in fish, and therefore may be available to encapsulated glochidia (Martin et al. 2003). In the present study, I tested whether an accumulated body burden of PFOS in largemouth bass (*Micropterus salmoides*) would affect metamorphosis and early survival of the paper pondshell (*Utterbackia imbecillis*) at two inoculation densities.

5.2. Methods

Fourteen largemouth bass were exposed to 0, 10, 100 or 1000 µg/L PFOS in a circular 67-L high-density polyethylene (HDPE) recirculating tank for 105 d with 90% static renewal every 72-96 h (i.e. twice weekly) and dechlorinated tap water. Each tank was fitted with aeration and an aquarium pump emptying to a 5-L HDPE headtank suspended above the main tank. The head tank was filled with biological filter media and a screen for solid wastes. Exposure containers were pretreated with target PFOS concentrations for 4 treatment cycles (14 d) prior to fish introduction in an attempt to saturate all tank surfaces. Fish were fed a maintenance diet of 2% body weight of fish pellets throughout the exposure. Only one exposure system was used for each treatment and variances among fish within each system are used for statistical analyses; therefore, our design does not pass assumptions of independence typically required for statistical analysis.

Paper pondshell were collected via snorkeling or scuba diving from Lake Chapman, Clarke County, GA on 8/24/11 and held in a living stream at the University of Georgia Whitehall Fisheries Laboratory. Animals were fed *ad libitum* with a mixed diet of *Nannochloropsis sp.* and mixed algal & diatom culture (Nanno1800 and ShellFish

Diet, Reed Mariculture, Campbell, CA), in addition to flow-through pond water that had been filtered below 25 µm. Viability of female paper pondshells was assessed using the salt test (ASTM 2006), and glochidia from three mussels were pooled for use in inoculations (initial viability = 82, 91 and 93 %).

Upon completion of the uptake exposure, fish were weighed and measured (standard length) and LeCren's condition (Kn) factor was calculated as a measure of general condition (Froese 2006). Five fish from each treatment were assigned at random to either a 1000 or 4000 glochidia/L inoculation density. Each fish was inoculated for 15 minutes to the assigned glochidia density in 1 L of highly aerated water, with occasional mixing using a 500-ml bulb syringe. Unattached glochidia were then rinsed from the fish with a garden sprayer and each fish was placed in a separate 3-L aquaria of an Aquatic Habitats (AHAB, Aquatic Ecosystems, Lake Apopka, FL) system with a 150-µm screen at the outflow to catch sloughed glochidia and transformed juveniles. The recirculating AHAB was equipped with a carbon filtration to minimize transfer of PFOS between fish. The number of sloughed glochidia and juveniles from each fish were enumerated every 23-48 h. Attachment was calculated as the sum of glochidia and transformed juveniles divided by the inoculation density. Metamorphosis success was calculated as the number of transformed juveniles divided by attachment, and mortality was estimated as the number of dead juveniles divided by the total number of transformed juveniles. Mortality was assessed through absence of foot movement, heartbeat, or shell movement within 5 min period of observation (Pandolfo et al. 2010).

After a 14-d metamorphosis period, fish were removed from the AHAB and euthanized in ice. Standard length and weights were measured, blood was collected by slicing the caudal vein and collecting with a hematocrit tube, and the fish was dissected for sex determination and liver removal. Livers were weighed and liver-somatic index (% of bodyweight; Adams et al. 1989) was calculated using the final weight of the fish.

Mean metamorphosis data (% metamorphosis, % attachment, mortality, and total juveniles) were analyzed through visual comparison of treatment means, and if an obvious trend was present, were followed by means comparisons through a general linear model. Likewise, mean fish metrics were also analyzed through general linear models at $\alpha = 0.05$ level of statistical significance.

Two weeks following the cessation of the metamorphosis trial we used a 48-h NaCl toxicity challenge to assess the relative health of juvenile paper pondshell produced from fish in each of the PFOS treatments. Juveniles from each PFOS-fish concentration were exposed in triplicate to 0, 1, 2, 4 and 8 mg/L NaCl in unaerated moderately hard water at 20 °C in 120 mL beakers. Each beaker contained 11-14 juveniles (43 out of 55 beakers had 12 juveniles, and beakers with other numbers were randomly distributed). At 48-h after exposure initiation, effects endpoints were assessed as the presence of heartbeat, foot movement, or valve movement within a 5 min period of observation (ASTM 2006). The NaCl concentration at which 50 % of individuals were affected (EC50 ± 95% Confidence Intervals) was assessed for each PFOS-fish concentration separately using the Trimmed Spearman-Karber method (Hamilton et al. 1977; Hamilton 1978).

5.3. Results & Discussion

Through the 105-d PFOS exposure, mortalities included 3 fish in the control group and 2 fish each in the 10 and 100 µg/L PFOS treatments. All but one fish died in the 1000 µg/L PFOS treatment, though nearly all of these fish were found entangled in the escape net or outside of the tank, thus mortality was largely due to attempted escapement from PFOS treatment rather than lethal toxicity. Fish exposed to 10 µg/L had a mean condition factor that was 4% (± 2.5% std. dev.) lower than control fish (t value = -1.66, p = 0.11), and fish exposed to 100 µg/L had a mean condition factor approximately 5% (mean = 4.9% ± 2.5% std. dev.) lower than control fish (t value = -1.98, p = 0.06; Figure 5.1.A). Fish exposed to PFOS had significantly lower LSI than those in the control group (10 µg/L = 30 ± 0.1% lower, t value = -3.02, p = 0.006; 100 µg/L = 34 ± 0.1% lower, t value = -3.72, p = 0.001; Figure 5.1.B).

Metamorphosis success of paper pondshell ranged from 47 to 83% (mean = 71% ± 4% std. dev., n = 5 fish per treatment), and there were no trends of obvious effects from fish PFOS concentration or glochidia density. Mortality of metamorphosed juveniles (3 ± 2%) and percent attachment (15 ± 3%) also followed no consistent trend, though number of juveniles per fish was significantly greater in the 4000 glochidia/L treatment by a mean of 294 (± 47 std. err. across PFOS treatments) juveniles (Figure 5.2).

Juveniles from all three fish PFOS exposures had 100% survival in control groups during 48 h NaCl challenge tests. Both PFOS treatments and control juveniles showed dose dependent increases in mortality to NaCl, though we saw an all-or-nothing response in the 10 µg/L PFOS group where 100% mortality was experienced at 4 and 8 mg/L NaCl, thus 95% confidence intervals were not able to be calculated. To allow model

convergence and be able to calculate confidence intervals, one mortality was added in the 2 mg/L treatment, and one mortality removed from the 4 mg/L treatment. This did not markedly change the EC50 estimate (2.93 mg/L before and 2.92 mg/L after adjustment). However, EC50 estimates decreased in a dose dependent manner from the control PFOS group, and 95% Confidence Intervals between control and 100 µg/L PFOS treatments did not overlap (Figure 5.3).

After a 105-d exposure to aqueous PFOS concentrations, we saw general signs of stress in largemouth bass, consistent with previous studies on PFOS exposure in fish. First, we saw decreases in relative condition factors in fish (Oakes et al. 2005), and a decrease in liver-somatic index (Oakes et al. 2005). Though not quantified, we also noted general listlessness, reduced feeding, and increased presence of external lesions in fish treated with 100 µg/L and 1000 µg/L consistent with other studies (Ankley et al. 2005). Reduced feeding may have led to increased liver glycogen metabolism and a reduced LSI in PFOS treated fish (Hagenaars et al. 2008).

Although we did not analyze cortisol levels in fish, others have seen changes in cortisol levels in fish treated with PFOS. For example, Mortensen et al. (2011) reported a significant decrease in plasma cortisol levels in Atlantic salmon force-fed PFOS compared to the control on day 2 of an 8-d experiment. But at 5 d post treatment, fish in the PFOS group had significantly higher cortisol concentrations than control groups. The authors suggested that the initial reduction in cortisol was likely because PFOS induces an increase in CYP3A mRNA, and CYP3A is known to catalyze steroid metabolism (Mortensen et al. 2011). Increased cortisol likely plays a role in increasing metamorphosis attachment and metamorphosis success of mussels by reducing localized

inflammation at infection site. On bluegill hosts, paper pondshell have higher attachment and metamorphosis rates when infection intensities are increased from 1000 glochidia/L to 2000, and 4000 glochidia/L associated with significant increases in endogenous cortisol (Dubansky et al. 2011). When fish are injected with cortisol, metamorphosis improves from the control even at lower inoculation densities (Dubansky et al. 2011). It may be possible that changes in endogenous cortisol levels could affect a host's ability to shed mussel glochidia during the first 24-48 hours of attachment, leading to changes in mortality rates. Our results are inconclusive in this area as we saw no increase or decrease in metamorphosis, but also did not measure cortisol concentrations in our fish. Furthermore, other studies have shown increases in parasite infestation due to PFOS exposure (Jacobson et al. 2010), and this remains an important research topic to follow up on.

The role of nutrient transfer from fish hosts to encapsulated mussels was until recently not well understood. Traditional thought was that the parasitic benefit to the glochidia was largely one of dispersal (Barnhart et al. 2008). Recently this has been disproven through a comparison of stable isotope ratios of host fish gill tissue, maternal mussel marsupia tissue, glochidia and transformed juveniles (Fritts et al. 2013). While glochidia had similar isotope ratios to maternal marsupia tissue, metamorphosed juveniles showed a characteristic shift toward signatures more similar to host fish, suggesting that nutrients from fish gill tissue are sequestered by the encapsulated mussel. It is still unclear though, whether only a small amount of gill tissue within the closed glochidial shell is digested, or whether there is active uptake of nutrients from the surrounding tissue throughout the encapsulation. Jacobson et al. (1997) found that

encapsulated glochidia were largely protected from aqueous copper exposures ranging up to 200 µg/L. Thus the layer of gill or epithelial tissue that encapsulates the metamorphosing glochidium is likely a barrier to some water soluble toxicants, but we are still unsure whether mussels are protected from contaminants within the fish. In the present study, we saw no changes in attachment, metamorphosis success, or early mortality. Because of known nutrient transfer from fish hosts to mussels, investigations of the transfer of accumulated contaminants to juvenile mussels are still warranted.

In a 48 h acute toxicity challenge to NaCl, we saw lower EC50s on 2 week old paper pondshells that were metamorphosed on fish exposed to PFOS concentrations of 10 and 100 µg/L, suggesting a potential decrease in mussel health. It is currently unknown whether the health of juvenile mussels in this study was affected from transfer of accumulated PFOS in host fish, or whether the poor host condition results in reduced energy transfer to encapsulated glochidia, thus reducing initial survival of metamorphosed juveniles. Fish treated with PFOS had lower mean condition factors and statistically significantly lower LSIs than control animals, the cause of which could be from PFOS toxicity or stress related reductions in feeding. Little work has been conducted on host fish health effects on early stage unionid survival, but the conservation implications of this relationship are extensive. To better our understanding of the role of contaminants on mussel populations, general stress and condition of host fish needs to be a consideration of future research.

5.4. Acknowledgements

Funding for this project was secured through a student research grant from the University of Georgia Interdisciplinary Toxicology Program. Thank you to Dr. Robert Gogal, who gave initial consultation on immunotoxicity experimental design. We would like to thank Robert Ratajczak, Andrea Fritts, Joseph Styga, Derek Colbert, and Amos Tuck for their assistance in mussel collection and laboratory aid.

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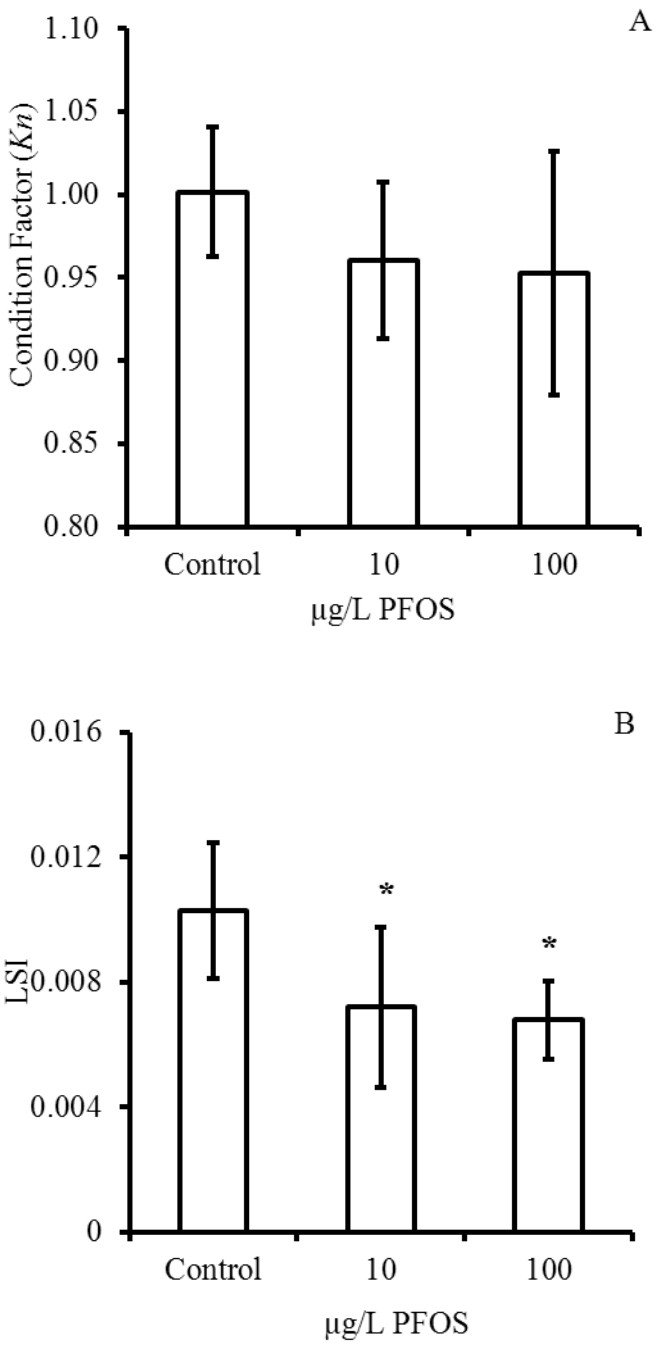
5.6. Figures & Tables:

Figure 5.1: Aqueous exposure of PFOS in largemouth bass (*Micropterus salmoides*) over 105 d. Effects of PFOS exposure on LeCren's condition factor (A) and liver-somatic index (B). Error bars represent ± one standard deviation, and ‡ denotes statistical significance from control at $p = 0.05$.

Figure 5.2: Effects of accumulated PFOS in fish, and glochidia density on number of juveniles produced per fish. PFOS concentrations are target aqueous concentrations during 105 d fish exposure. Error bars represent ± one standard deviation.

Figure 5.3: Forty-eight hour NaCl challenge EC50s for 2 week old *Utterbackia imbecillis* metamorphosed on fish exposed to varying PFOS concentrations. EC50 (mg/L NaCl) based on movement or heartbeat presence during 5 min observation, calculated using trimmed Spearman-Karber method. Error bars represent 95% confidence intervals.



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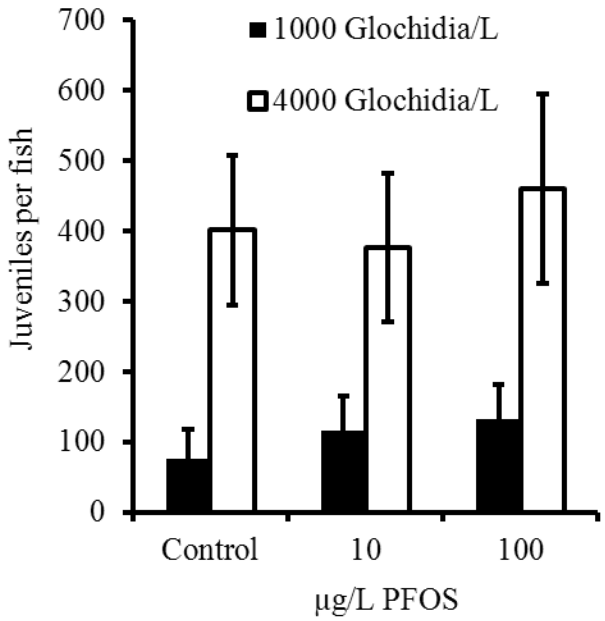

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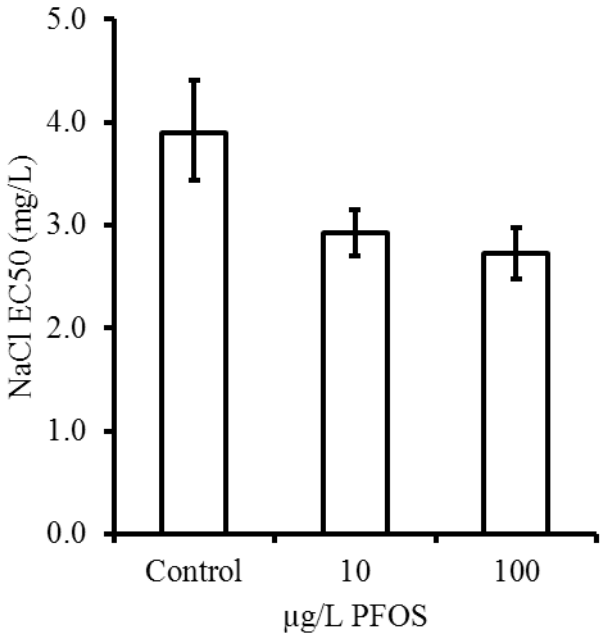

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CHAPTER 6

TOXICITY OF PFOS TO JUVENILE FATMUCKET IN A 7-D AQUEOUS EXPOSURE[5]

[5] Peter D. Hazelton, W. Gregory Cope, Mark J. Strynar, and Robert B. Bringolf. To be submitted to *Bulletin of Environmental Contamination & Toxicology*

Abstract

The aim of the present study was to assess the effect of aqueous perfluorooctane sulfonate (PFOS) on the survival of juvenile freshwater mussels. We exposed 11-week-old *Lampsilis siliquoidea* (Fatmucket) to three concentrations (1, 10 and 100 µg/L) of PFOS for 7 d in a novel exposure apparatus, and compared survival to an untreated control with logistic regression. We also assessed the probability of survival through all early life stages based on a deterministic model that combines our data with previously published data from a maternal exposure. Mean survival in control animals was 65%, and all PFOS treated animals had significantly lower survival probabilities. Mussels exposed both in the maternal marsupial gills and as juveniles to 1 µg/L were on average 50% as likely to survive, and mussels exposed to 100 µg/L were only 15% as likely to survive as control animals.

Keywords PFC, Population modeling, Odds ratio, AIC, Unionidae, Emerging contaminant

6.1. Introduction

Perfluorooctane sulfonate (PFOS) and other perfluoroalkyl acids (PFAAs) are contaminants of emerging concern because of their persistence in the environment (Kannan et al. 2001; Giesy and Kannan 2002; Kannan et al. 2005; Valsecchi et al. 2013). Though concentrations of PFAAs in the aquatic environment are typically in the ng/L range (Lau et al. 2007), concentrations above 1 µg/L have been measured in areas of long-term pollution (Lau et al. 2007; Konwick et al. 2008). Environmental concentrations are typically below acute toxicity thresholds for most aquatic organisms (Beach et al. 2006; Qi et al. 2011). But evidence is growing of potential chronic effects to the environment and human health, particularly regarding PFOS, the most persistent of the PFAAs (Du et al. 2009; Jacobson et al. 2010; Li 2010; Hazelton et al. 2012; Zhang et al. 2012; Chen et al. 2013; Fair et al. 2013). As a result, international concern and regulation of PFOS have increased in the past decade (Zushi et al. 2012).

Freshwater mussels of the order Unionoida are among the most threatened taxa worldwide (Haag and Williams 2013), and environmental contamination is often cited as a major obstacle to the recovery of freshwater mussels (Downing et al. 2010). In standard toxicity tests, freshwater mussel juveniles and larvae (glochidia) are among the most sensitive species to acute exposure to PFAAs (Hazelton et al. 2012). Concentrations of PFOS near environmental relevance (e.g. < 5 µg/L) significantly reduced the duration of larval viability after a ~ 30 exposure within the brooding female (Hazelton et al. 2012). Effects of long-term (> 96 h) exposures to juvenile fresh water mussels are largely unknown, though physiological changes to zebra mussels (*Dresseina polymorpha*) have been reported during 10-d exposures (Fernandez-Sanjuan et al. 2013).

The goal of the current study was to assess the effects of PFOS on juvenile freshwater mussels during a 7-d exposure to environmentally relevant concentrations with a novel exposure apparatus, and to assess the cumulative risk to freshwater mussels exposed as larvae and early juveniles.

6.2. Methods

We conducted a 7-d experiment to examine the effect of PFOS concentrations (0, 1.0, 10, 100 µg/L) on survival of 11-wk old juvenile (length = 350-500 µm) *L. siliquoidea*. Juveniles were cultured at Missouri State University and shipped to the University of Georgia for testing. Each PFOS concentration was replicated five times for a total of 20 experimental units. Each experimental unit consisted of a 3.8-L glass aquarium with 2.0 L of moderately hard reconstituted water (ASTM 2006). In each aquarium, 15 juvenile mussels were contained within a chamber that consisted of two 5.1 cm diameter poly vinyl chloride (PVC) filter cups (modified from Barnhart 2006) with 300 µm nylon mesh (Nitex) fitted to a 6.35 cm long (5.1 cm diameter) PVC coupling with two 1.37 cm holes drilled in the bottom to facilitate water flow through the chamber (Figure 6.1). A 1.27 cm diameter by 25.4 cm long PVC pipe extended from the bottom of the jar to above the mouth of the jar with a T-junction and 5.08 cm horizontal pipe (spout) at the halfway point. Aeration was maintained within the vertical PVC pipe to lift water above the water line and out of the spout onto the top screen of the chamber (Figure 6.1). Care was taken to ensure that air was not trapped within the chamber and that all airlifts were circulating water at an approximately equal rate. At 48-h intervals, we completed a 90% water renewal and measured water quality (pH, temperature, DO & specific conductivity) with a Hydrolab Quanta multiprobe. After 7 d, we removed the

chambers from the apparatus and assessed juvenile survival. Juveniles were viewed under a stereoscopic dissection microscope at 6.3 – 50X magnification for up to 5 min. We considered foot movement or the presence of a heartbeat as evidence of survival (ASTM 2006).

Water samples from two replicates of each treatment group were collected and analyzed for PFOS. Samples were collected in 1-L high-density-polyethylene (HDPE) bottles at the start of the juvenile exposures and shipped on ice via overnight courier to the United States Environmental Protection Agency, National Exposure Research Laboratory in Raleigh, NC for quantitative analysis. PFOS quantitation methods are described elsewhere (Hazelton et al. 2012).

Statistical analyses for juvenile survival followed the same approach as that listed previously for glochidia viability and metamorphosis analyses (Chapter 2 & Hazelton et al. 2012). Briefly, to assess the effects of PFOS on juveniles we used a logistic regression framework in a generalized linear model (GLM; Bolker et al. 2009) where survival of each juvenile after 7 days was normalized to a binary response where 0 = death, and 1 = survival. Parameter estimation in and survival probability calculation was carried out as previously described in Hazelton et al. (2012).

We multiplied the probability of survival at each life stage (i.e., viability, metamorphosis, and juvenile survival) to determine the probability that an individual mussel would survive a PFOS exposure through all three early life stages. Probability of viability and metamorphosis after *in marsupia* exposure are based on estimates from Hazelton et al. (2012). We carried the 95% confidence intervals across each stage, such that upper and lower limits represent best and worst case scenarios for all exposures. The

final survival probability was used to calculate an odds ratio of survival of mussels exposed to PFOS compared to the mean probability of survival of an untreated mussel. Odds ratios were calculated with the equation:

$$OR(y) = \frac{p(y) / 1 - p(y)}{p(null) / 1 - p(null)}$$

where the odds ratio (OR) is a function of the probability of survival at a given PFOS treatment, $p(y)$, and the probability of survival for an untreated mussel, $p(null)$ (Hair et al. 1998). The resulting proportion is how many "times as likely" to survive a treated mussel is compared to an untreated mussel.

6.3. Results & Discussion

PFOS was quantified in two replicates of each treatment, and we did see some evidence of PFOS contamination in our control group (Table 6.1). One control sample was found with 0.28 µg/L, while nothing was detected in the second replicate, suggesting accidental cross-contamination in at least one replicate of the controls despite the care taken to minimize the chances of cross contamination with water quality equipment, air stones, etc. Percent of target concentration ranged from 79 -99 % in the 1 µg/L treatments, 130-150% in 10 µg/L, and 197-202 % in the 100 µg/L group.

Juvenile *L. siliquoidea* survival ranged from 6% to 65% (mean = 37%, ± 17% st.dev.) across all treatments during the 7-d test. Survival in the control treatment was well below 80% (mean = 57%, ± 10% st.dev.), and therefore did not meet the requirements for a standard mussel toxicity test (ASTM 2006). Nevertheless, all three PFOS exposure concentrations had significant negative effects on survival (Figure 6.2).

Survival in PFOS treatments of 1 µg/L and 100 µg/L was significantly less than controls at $p < 0.0001$, whereas the 10 µg/L treatment was significantly less at $p = 0.05$ (Table 6.2). The mean modeled probability of survival for an unexposed (control) juvenile in our assay was 0.56 (± 0.11, 95% CI). PFOS treated juveniles had a probability of survival of 0.28 (± 0.19), 0.41 (± 0.23) and 0.21 (± 0.15) for 1, 10 and 100 µg/L respectively (Figure 6.2). Probability of survival was greater in 10 µg/L than in 1 µg/L PFOS concentrations, which was likely caused by two replicates in the lower concentration having survival of 20% or less. This concentration also had the greatest range in percent survival 13% - 60%.

By multiplying probability of survivorship at the larval life stages (Hazelton et al. 2012) by the probability of juvenile survival in the current study, we were able to calculate the odds of survival of a mussel treated with 1, or 100 µg/L PFOS surviving through all three early lifestages (glochidia viability, metamorphosis & juvenile survival) in comparison to the mean odds of survival of an untreated mussel (Figure 6.3). Mussels exposed to a hypothetical PFOS concentration between 1 and 5 µg/L were 0.46 times as likely to survive as an untreated mussel (95% confidence limits = 0.13 – 1.12). And mussels treated with a PFOS concentration between 65 and 100 µg/L PFOS were 0.23 times as likely to survive as an untreated mussel (95% confidence limits = 0.05 – 0.73).

Because our data did not meet the established control survival criteria for a standard freshwater mussel toxicity testing, these data should not be used to establish standardized environmental effects thresholds (i.e. LOEC, PNEC…) for PFOS. But we do feel that these data represent a relevant proportional risk of mortality resulting from toxicant exposure. High mortality rates are common in the culture of early juvenile

freshwater mussels (Barnhart 2006; Bishop et al. 2007; Ingersoll et al. 2007). However, since the completion of the current study several methods have been developed which aid in the survival of early juveniles in toxicity test control groups. In particular, chronic freshwater mussel toxicity tests are currently conducted in the presence of sediment (Wang et al. 2011). Additionally, older juveniles (e.g. > 2 months) have better control survival than younger animals (Wang et al. 2010) and are easier to find in the sediment following a trial (Hazelton, pers. obs.) but appear to be less sensitive to at least some contaminants than the younger juveniles (Wang et al. 2010). We also used a newly designed exposure system, which may not have been optimized for early juveniles.

We are not certain of the mechanism of mortality in our study, but other work suggests that bivalves may be stressed by relatively low PFAA concentrations. Fernandez-Sanjuan et al. (2010) found that zebra mussels (*Dresseina polymorpha*) exposed to PFOS had increased multixenobiotic resistance rates at 10 µg/L after 10 d compared to initial exposure, and respiration rates at 1 – 10 µg/L were significantly higher than control animals throughout a 10-d exposure (Fernandez-Sanjuan et al. 2013). When our juvenile toxicity test results were combined with data from a previous study (Hazelton et al. 2012), we found that PFOS exposure throughout the brooded glochidia and early juvenile stages had a significant decrease on the probability of survival, relative to unexposed mussels. Even though the change in odds of survival through all early stages is not statistically significant at a 1 µg/L PFOS exposure, these data are strongly suggestive of a decrease in mussel recruitment caused by contaminant exposure. More sophisticated population ecology models could (and should) be used to assess the uncertainty around population persistence of freshwater mussels exposed to

environmental contaminants (Forbes et al. 2011). Nevertheless, in the present study we have shown that concentrations of PFOS at established predicted no effect concentrations (1 µg/L: Beach et al. 2006; Qi et al. 2011) reduced survival of early juvenile mussels relative to control animals. Interestingly, similar or greater PFOS concentrations have been reported in areas of long-term release (Konwick et al. 2008), and at sites of accidental releases (Moody et al. 2001; Oakes et al. 2010). Further research efforts are needed in the direction of *in situ* effects of PFAAs, the role of sediment in juvenile mussel toxicity testing, and the relevance of early life cycle exposures to population persistence.

6.4. Acknowledgements

The project described in this manuscript was funded by the U.S. Fish and Wildlife Service (US FWS) through Research Work Order Number 1434-HQ-08-RU-01568 from the U.S. Geological Survey (USGS). Further assistance was provided by the Interdisciplinary Toxicology Program at the University of Georgia. Juvenile mussels were provided by Dr. Christopher Barnhart and Megan Bradley (currently at VA DGIF) at Missouri State University. We are indebted for the laboratory assistance of Robert Ratajczak, Derek Colbert, Julie Creamer, Kristin Kellock, Andrea Fritts and Jennifer Johnson.

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6.6. Table Captions

Table 6.1: Target and measured PFOS concentrations in water during 7-d juvenile *Lampsilis siliquoidea* exposure. Mean and 95% confidence intervals are presented for n = 2 samples.

Table 6.2: Generalized linear model parameter estimates for 7-d PFOS exposure effects on juvenile *Lampsilis siliquoidea* survival probability.

Table 6.1: Target and measured PFOS concentrations in water during 7-d juvenile *Lampsilis siliquoidea* exposure. Mean and 95% confidence intervals are presented for n = 2 samples.

Target PFOS Concentration	Measured PFOS Concentration	95% Confidence Limits	
		Lower	Upper
Control (0 µg/L)	0.09	0.00	0.28
1 µg/L	0.89	0.61	1.17
10 µg/L	14.14	11.42	16.89
100 µg/L	199.40	192.38	206.41

Table 6.2: Logistic generalized linear model parameter estimates for 7-d PFOS exposure effects on juvenile *Lampsilis siliquoidea* survival probability.

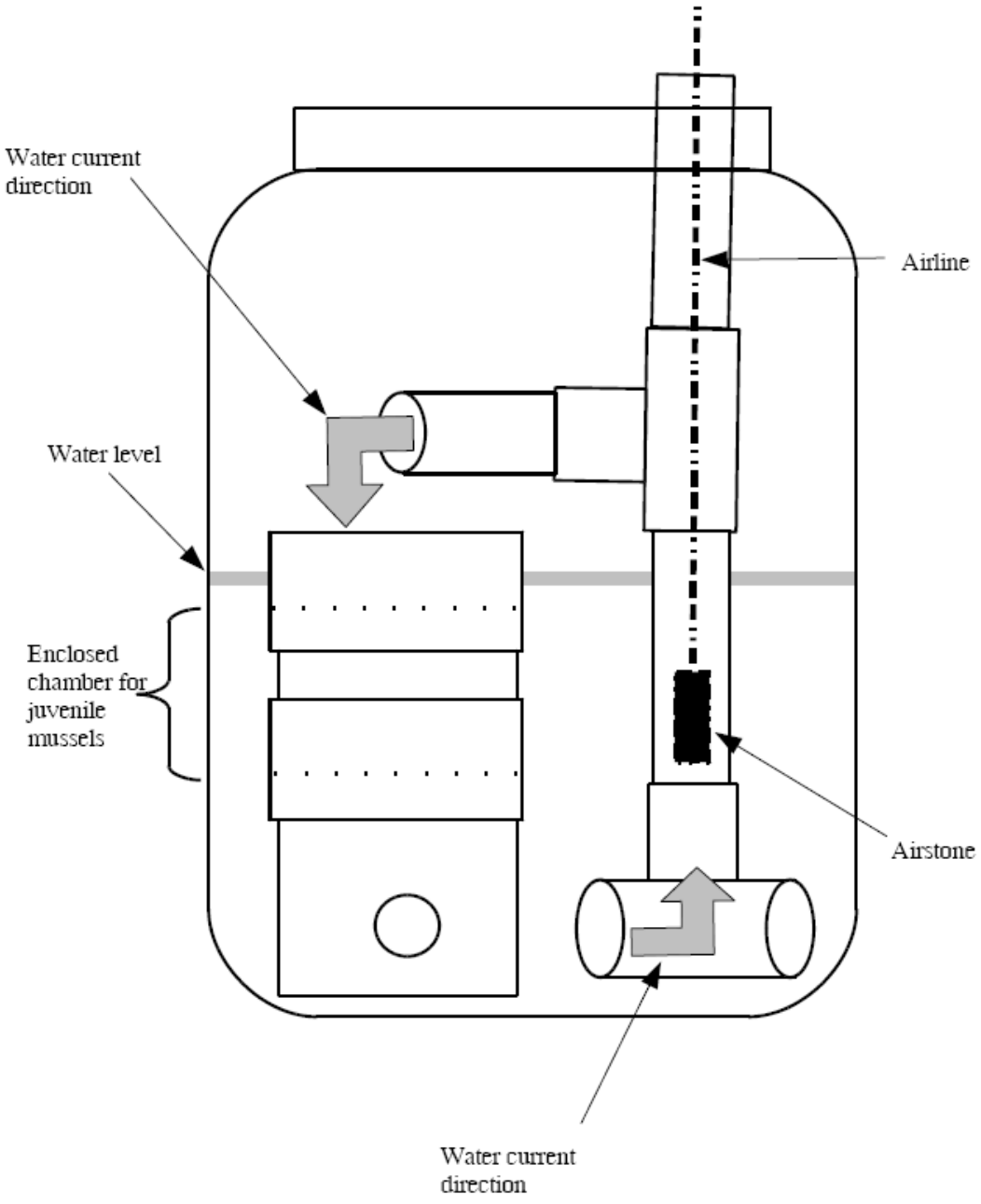
	Logit	Std. Err	*Wald - z*	*p* - value
Intercept	0.26	0.22	1.19	0.23
1 µg/L	-1.19	0.33	-3.67	< 0.0001
10 µg/L	-0.62	0.31	-1.99	0.047
100 µg/L	-1.57	0.34	-4.58	< 0.0001

6.7. Figure Captions

Figure 6.1: Exposure system used for 7-d PFOS exposure to juvenile *Lampsilis siliquoidea*. The system is enclosed within 3.8-L glass jar and the chamber is modified from Barnhart (2006) with 300 µm mesh upper and lower screens in 2 L dilution or treatment water. Two 1.7-cm diameter holes in the base of the chamber allow for water circulation through the enclosed chamber. Water circulation is driven by an adjustable airstone in a 4.8-cm diameter PVC tower, which delivers water to the top screen of the mussel chamber.

Figure 6.2: Percent survival of juvenile *Lampsilis siliquoidea* during 7-d aqueous PFOS exposure. Error bars represent one standard deviation from the mean (n = 4 replicates per treatment). Levels of statistical significance (***** $p < 0.05$, ****** $p < 0.001$ compared to control) are based on survival probability model estimates.

Figure 6.3: Odds ratios and 95% confidence intervals of *Lampsilis siliquoidea* survival of hypothetical PFOS exposure through all early lifestages. Viability and metamorphosis probabilities from Hazelton et al. 2012. Data assumes constant exposure through all lifestages and is quantified based on mean probability of survival of an unexposed mussel, thus the survival odds ratio is the times-as-likely to survive as the mean survival of a control animal. Hypothetical PFOS concentrations are approximate because of differences in concentrations between this study and Hazelton et al. 2012.



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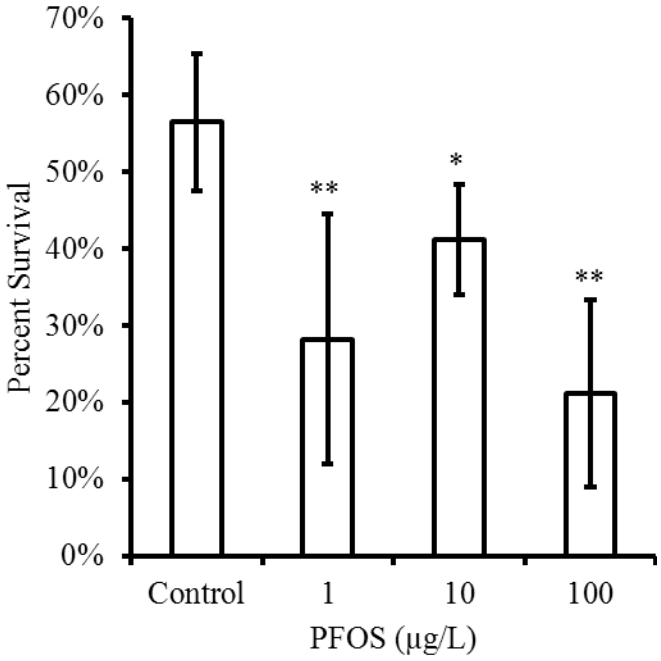

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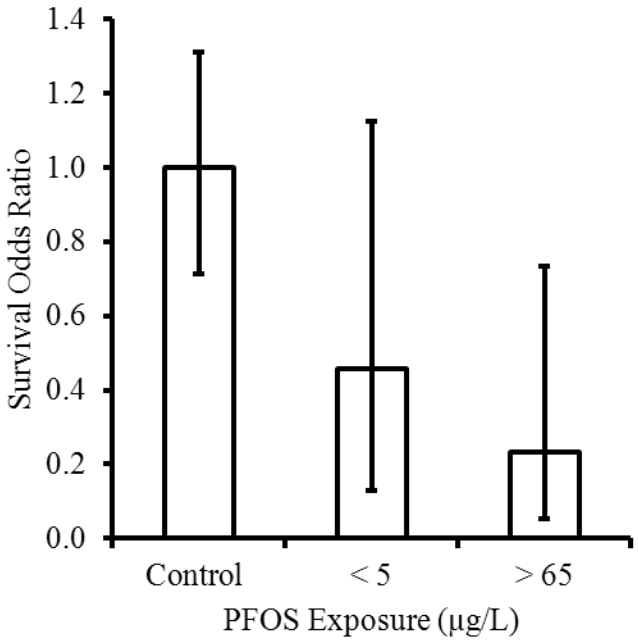

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CHAPTER 7

SUMMARY: ECOLOGICAL AND TOXICOLOGICAL LESSONS LEARNED, AND FUTURE NEEDS IN UNIONID ECOTOXICOLOGY RESEARCH

7.1. Introduction

Methods development in ecotoxicology of freshwater mussels is important on two fronts: First, freshwater mussels have many characteristics that make them ideal candidates for biomonitoring and toxicity testing (Van Hassel and Farris 2007 summarized in Chapter 1.2.1). They appear to be one of the most sensitive organisms to aquatic contaminants, and data generated during the early lifestages of freshwater mussels with standardized acute and chronic toxicity are critical for establishing water quality criteria under current risk assessment policies and procedures (Augspurger et al. 2003, ASTM 2006, Ingersoll et al. 2007). Perhaps more importantly, successful conservation of freshwater mussels is dependent on a better understanding of the role of aquatic contaminants as a cause of their decline (Cope et al. 2008, Downing et al. 2010, Haag and Williams 2013). Such understanding is only possible through investigation of the effects of environmentally realistic contaminant concentrations at all levels of biological organization.

In the preceding chapters (and associated appendices), I have reported observations on the effects of emerging contaminants on freshwater mussels tested at different stages in their complex lifecycle. Indeed, the contaminants investigated are currently associated with important environmental quality debate, and are among the most investigated emerging contaminants in their respective chemical classes. The new toxicity information is therefore intrinsically valuable; however, I contend that the greatest contribution of this effort lies in the presentation of novel methods that can be further used to assess the action of other contaminants on freshwater mussels. Furthermore, these methods and similar approaches should be used to understand the role of contaminants in freshwater mussel diversity loss. Through this process, we will gain further knowledge of the ecology and biology of unionids, and isolate adverse outcome pathways that will be vital to establishment of effect-based risk assessment methods.

7.2. Sensitivity of Unionids to emerging contaminants

Larval and juvenile unionids are often the most sensitive organisms to freshwater contaminants in acute toxicity tests (Augspurger et al. 2003, Ingersoll et al. 2007, Keller, Lydy et al. 2007). Particularly, mussels are more sensitive to metals and ammonia than most other commonly tested species (Augspurger et al. 2003, March et al. 2007), and this has led to proposed adjustments in water quality criteria for ammonia (USEPA 2009). Mussels are also relatively less sensitive than other species to some organic contaminants and pesticides (Milam et al. 2005, Bringolf et al. 2007a-c). It is important to note that contaminant toxicity is often species specific within the unionids (Milam et al. 2005,

Bringolf et al. 2007b), and that there are also contaminant specific differences in sensitivity of each lifestage (Bringolf et al. 2007b).

In chapters 2 and 3, my coauthors and I found that early lifestages of two unionid species were among the most sensitive organisms tested to acute toxicity from PFOS and PFOA (Chapter 2 and Hazelton et al. 2012), and fluoxetine (Chapter 3 and Hazelton et al. 2013). Perhaps more poignant is that the more sensitive stage differed between contaminants. For instance, glochidia LC50s were a fraction of juvenile LC50s for PFOS (Figure 7.1) and PFOA (Figure 7.2). However, juveniles were 50-75% more sensitive to fluoxetine in acute tests (Figure 7.3). Interestingly, when we assessed the effects of fluoxetine with our partial lifecycle assay we did not see detrimental effects on glochidia (Chapter 3 and Hazelton et al. 2013). In fact, we observed increases in metamorphosis success in glochidia treated for 24 h with 1 and 100 µg/L fluoxetine. However, adult behavior appears to be significantly altered at these lower concentrations (Chapter 3 and Hazelton et al. 2013; Chapter 4). Presumably juveniles are more similar in physiology and behavior to adult mussels than glochidia (McMahon and Bogan 2001, Watters 2007), and it is worth questioning whether the more sensitive lifestage in standard acute tests is likely the more sensitive stage during lifecycle exposures. Certainly, this is reasoning for more compounds to be tested using a lifecycle or partial lifecycle framework and to compare stage specific sensitivities to acute toxicity data. If trends in lifecycle data mirror sensitivities in acute toxicity data, then acute tests could be better used as a screening method for toxicants with biological and ecological effects at lower doses.

7.3. Most sensitive lifestage(s)

The partial-lifecycle test and a holistic lifecycle approach to unionid toxicity testing is the only way in which we will better understand what lifestage(s) is most sensitive. Glochidia of most mussel species are only exposed freely in surface water for hours to days (Barnhart et al. 2008, Cope et al. 2008), and may only remain viable outside of the marsupia for a number of days (Cope et al. 2008, Fritts et al. *in review*). Thus, *in marsupia* exposure, and exposure while encapsulated on fish hosts are the only opportunities to estimate chronic toxicity in mussel glochidia. The partial-lifecycle test my coauthors and I describe in Chapter 2 (Hazelton et al. 2012) incorporates a prolonged exposure of glochidia to environmental contaminants while still *in marsupia*. Through direct comparison of *in marsupia* vs. free glochidia exposures, we saw that the 30-d exposure *in marsupia* had a greater effect on the viability, viability duration, and metamorphosis of ghlochidia, whereas no effect was seen on glochidia exposed in 24-h water only exposures.

The effects of contaminants at each lifestage are likely contaminant specific. Though we saw that PFOS is more toxic to ghlochidia exposed *in marsupia* (Hazelton et al. 2012), Jacobson, Neves et al. (1997) found that water-only exposure to copper was the most sensitive exposure route to larval mussels for that contaminant. This highlights the need for further use of the partial-lifecycle assay to determine the most sensitive lifestage(s) to different contaminants at concentrations near environmental relevance. Continued use of standardized acute toxicity testing could focus on screening the relative toxicity of contaminants to early stages, and determining predictive power of these tests for effects at later stages. Data generated could then be used to prioritize further use of

the partial-lifecycle assay and other chronic exposure tests. Further investigation should be conducted on mussels with different lifecycles too (i.e., brooding duration, infection strategy, host fish specificity). Brooding duration *in marsupia* as well as the host infection strategy of a given species or group of unionids are likely important factors that may drive differences in species sensitivities to contaminants (Barnhart et al. 2008, Cope et al. 2008, Fritts et al. *in review*). As is the case with standard acute toxicity testing (ASTM 2006), tests should be conducted with more than one species and tribe of mussel, to better estimate diversity of potential effects.

7.4. Non-toxicological lessons learned

In a review of The 1997 National Strategy for the Conservation of Native Mussels, Haag and Williams (2013) state that continued understanding of the general biology and ecology of unionids is critical to the conservation of these species. While continued standard acute and chronic toxicity testing may give us some insight into the relative toxicity of particular contaminants, these studies are not designed to give us more information about the general biology of a species. Use of the partial-lifecycle assay and incorporation of behavioral ecotoxicology into the assessment of contaminant risk on unionids can provide valuable insight into mussel physiology and ecology.

In Chapter 3 (Hazelton et al. 2012), we observed some previously unexpected results when we determined that fluoxetine exposure to glochidia increased metamorphosis when compared to the control. We felt this was attributable to one of two pathways: first, fluoxetine and other selective serotonin reuptake inhibitors have anti-inflammatory properties (Bianchi and Panerai 1996), such that residue on glochidia could

reduce an inflammatory response in fish tissue at the site of infection and result in fewer sloughed glochidia. This was similar to what has been observed in mussels transforming on fish injected with anti-inflammatory cortisol (Dubansky et al. 2011). Or secondly, serotonin is known to play an important role in the induction of settling and metamorphosis of some marine mollusks (Leise et al. 2001, Leise et al. 2004), thus it is interesting to question whether serotonin is a driving factor in unionid metamorphosis, a pathway that has not yet been elucidated. Thus, by using toxicity testing approaches that investigate a mode of action rather than levels of overt toxicity, we can gather further insight into unionid biology and generate further hypotheses for testing.

In Chapter 4, testing the effects of fluoxetine on adult movement gave us further insight into the behavior of the wavy rayed lampmussel (*Lampsilis fasciola*). First, we saw significant increases in movement rates in mussels treated with fluoxetine when compared to controls. This was counter intuitive to our assessment of behavioral effects in Chapter 3 (Hazelton et al. 2013), where we assumed that foot swelling and protrusion (as a result of fluoxetine exposure) would reduce burrowing ability in mussels. We saw enhanced movement rates in fluoxetine treated animals and a change in timing of movements. Animals treated with fluoxetine moved more during daylight hours, whereas animals in the control group were unlikely to move during daylight. Only a few researchers have investigated the diel movement patterns of mussels, with mixed implications. Amyot and Downing (1997) found no differences in the diurnal or nocturnal movement in *Elliptio complanata*, but Corey et al. (2006) saw significant differences where displaying female *Ligumia nasuta* were more likely to move at night. *Ligumia nasuta* and *Lampsilis fasciola* (our study) are both members of the Lampsilini

tribe and thus closer related than *E. complanata*, Pleurobemini (Graf and Cummings 2007), thus we can assume that *L. fasciola* are typically more likely to follow movement patterns similar to *Ligumia nasuta* despite any pharmaceutical effect. Had we not tested the effects of fluoxetine on movement and burrowing behaviors we would not have found information contrary to our earlier assumptions, nor would we have contributed interesting information regarding the movement habits of this species to the dearth of research on the topic of diel migration behavior in unionids.

7.5. Failures and future approaches

All research that addresses new hypotheses comes with potential failures, and this dissertation is certainly not without failed attempts. However, it is important to determine if the data fail to refute a null hypothesis, or whether this failure is simply an artifact of experimental design. Critical assessment of methodologies is essential to developing alternative, and perhaps improved, approaches. In Chapter 4, we saw no difference in the algal filtration rates of *L. fasciola* treated with fluoxetine compared to control animals. Mussels in all tanks cleared approximately 73% of the algae at 24 h and 86% at 48 h. Furthermore, there were no differences in tissue glycogen levels among control and treatment groups after the 67-d experiment, suggesting no change in nutritional state among mussels. Curiously, fluoxetine has shown anorexigenic effects in both fish (Gaworecki and Klaine 2008, Mennigen et al. 2010) and some invertebrates (e.g. *Chironomus tentans* and *Hyalella azteca*; Brooks et al. 2003), but did not alter feeding or growth in the polychaete *Capitella telata* (Méndez et al.). It is quite possible given the increase in activity in *Lampsilis fasciola*, that feeding is not altered by

fluoxetine at the concentrations tested in Chapter 4. But in our experiment animals may have been fed a greater amount of algae than is typically available under natural conditions, and the 24-h feeding period may have been too long to assess algal clearance rates. More preferred approaches to filtration rates as an endpoint of health in unionids are currently under development where juvenile feeding rates are assessed within 6 h of initial feeding time (Pletta and Barnhart 2013). These new methods are likely more precise to those we used in Chapter 4, and should be considered when testing sublethal effects of contaminants on mussels.

We assessed the effects of a PFOS body burden in host fish on the attachment, metamorphosis and robustness of previously unexposed paper pondshell (*Utterbackia imbecillis*; ApChapter 5). In this experiment, my goal was to assess two alternative hypotheses: (1) PFOS toxicity reduced metamorphosis rates in glochidia exposed for 30 d *in marsupia* (Chapter 2 and Hazelton et al. 2012), and should decrease metamorphosis success in encapsulated glochidia that are exposed to PFOS through the tissue of the host fish; and (2) PFOS is a known immunotoxicant in fish (Mortensen et al. 2011) that could inhibit an immune response in the host fish by increasing endogenous cortisol levels, thus allowing higher rates of metamorphosis (Dubansky et al. 2011). Metamorphosis rates did not differ between our control groups and fish with a PFOS body burden, thus it is unlikely that an immunotoxic mechanism is present that would interfere with metamorphosis. However, juveniles that metamorphosed on PFOS laden fish were significantly more sensitive to a 48-h NaCl acute toxicity challenge, suggesting juvenile mussel health may be affected by accumulated contaminants in the host. We do not know if the PFOS body burden in our hosts affected the immune system because we did

not test fish cortisol levels. Mortensen et al. (2011) reported that the endogenous cortisol levels in salmon fed a PFOS contaminated diet fluctuated at different times in the experiment, peaking at day 5 and falling below those of the control by day 8. Cortisol may only be important at mediating attachment of glochidia within the first 24 h of an infection as this is when encapsulation occurs (Rogers-Lowery and Dimock 2006), but the fish in our study had already been exposed to aqueous PFOS for 105 days at this time. The ecological relevance of the acute NaCl robustness challenge is not clear, and it is likely that contaminants that affect the immune system of fish may alter the relationship between host and unionid parasites. It is therefore exceptionally important that further work examine the relationship between pollutants and the host fish – parasite interaction.

In Chapter 6, we reported the effects of a 7-d aqueous PFOS exposure on juvenile *Lampsilis siliquoidea*. Probability of survival was significantly lower than the control in all PFOS concentrations, including an environmentally relevant 1 µg/L PFOS treatment (Figure 6.2.). However, mean control survival (56%, ± 9% std. dev.) was below the 80% control survival benchmark for chronic juvenile toxicity tests, and 90% control survival for acute juvenile tests (ASTM 2006). Control survival is an important quality assurance measure in standard toxicity testing approaches (Newman 1995), but juvenile mussels are known to undergo high mortality rates, particularly within the first 2 months post metamorphosis (Barnhart 2006, Ingersoll et al. 2007), making them particularly difficult organisms to work with. While some other studies have achieved 90% or greater survival in 30-d juvenile toxicity tests (Bringolf et al. 2007a-c, Wang et al. 2007, Wang et al. 2008, Wang et al. 2010, Wang et al. 2011), these studies were often performed with older and larger individuals and optimization of juvenile mussel culture is badly needed to

maintain survival throughout the juvenile stage. Nevertheless, data generated from studies with low control survival are still valuable for identifying proportional risk associated with contaminant exposure.

7.6. Conclusions

In the preceding chapters my coauthors and I have detailed novel approaches for estimating the effects of pollutants on freshwater mussels throughout their lifecycle. The partial-lifecycle assay introduced in Chapter 2 (Hazelton et al. 2012), is effective at isolating whether glochidia are more sensitive to a given contaminant within or outside of the marsupial gills at ecologically relevant durations of exposure. However, this is not the only lifecycle analysis that should be considered in unionid ecotoxicology. Freshwater mussels are also exposed to contaminants while encapsulated on the host fish, and through sediment and water exposure routes as juveniles and adults (Cope et al. 2008). To better estimate the effect of contaminants on unionid populations, it is important to consider all routes of exposure throughout the lifecycle and to examine ecologically relevant endpoints beyond acute lethality.

Behavioral and physiological data should be investigated to better understand the modes of action of individual contaminants, and to further identify how these modes of action may manifest an adverse outcome to a population (Ankley et al. 2010). The partial-lifecycle assay provides opportunities for data collection on contaminant uptake and kinetics, biomarkers, behavioral and physiological responses on adult mussels exposed for 30-d. Incorporation of these data will result in new discoveries in unionid biology and ecology as well as ecotoxicology. The goal of test methods development should be to determine the effects of contaminant exposure at environmentally relevant

concentrations and exposure durations, and where possible combine mesocosm and *in situ* exposures with lifecycle endpoints (i.e., reproduction, attachment rates, metamorphosis, juvenile survival). Acute toxicity methods should still be used as a screening process, and further research should be conducted comparing the relative sensitivity of early lifestages in acute tests to data from chronic or partial-lifecycle assays.

Use of wild-caught adult unionids in toxicity testing should be avoided, and efforts made to increase culture of unionids for ecotoxicology and conservation purposes. In the current revisions to the National Strategy for the Conservation of Freshwater Mollusks, the Freshwater Mollusk Conservation Society has identified that increasing production efforts and abundance of unionids in the environment should be a goal of conservation agencies and stakeholders (FMCS 2013). To accomplish this goal, greater effort is needed in understanding limitations and overcoming barriers to unionid culture throughout the lifecycle. Specifically, achieving higher juvenile survival rates will help establish captive populations that can be drawn from for toxicity testing as well as population augmentation. As dietary and environmental needs of early juveniles become better understood, these should be applied directly to improve control survival rates in juvenile toxicity tests.

Current ecological risk assessment methodology is largely based on acute and chronic lethality measurements using unrealistic chemical concentrations and exposure durations (Chapman 2002, Dale et al. 2008, Connon et al. 2012). The scientific community has recently placed greater emphasis on the need to use effects-based approaches to risk assessment that incorporate realistic exposure conditions (Chapman 2002), more sublethal endpoints (Connon et al. 2012) and determination of pathways that

link exposure, biological mechanisms of toxicity, and organismal health endpoints to adverse outcome scenarios for a population (Ankley et al. 2010). Population modeling is an exceptional tool at extrapolating the effects of organismal data to population level consequences (Forbes et al. 2008, Forbes et al. 2011), but is underused in ecological risk decision making. Interestingly, modeling of population demographics also has not often been applied to understanding freshwater mussel ecology despite its potential utility (Berg et al. 2008, Strayer 2008). Because the unionid lifecycle is composed of several discrete lifestages, understanding the survival from one stage to the next and how contaminants can affect this progression will be useful at estimating population level changes and risk to unionid populations. The partial-lifecycle assay, combined with other lifestage specific toxicity approaches described in this dissertation is directly applicable to use with population modeling efforts, and should be considered for future unionid ecotoxicology study and risk assessment decision making.

7.8. References

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7.9. Figure Captions

Figure 7.1. Median lethal concentrations (LC50; bars) and test durations (closed circles) for acute PFOS toxicity tests to common aquatic test species. Error bars represent 95% confidence intervals where reported. Light gray bars are unionid glochidia data, dark gray bars are unionid juvenile data, black bar is unionid juvenile data from another author. Sources of data: Planaria – *Dugesia japonica* (Li 2009); Cladocera - *Daphnia magna* (USEPA 1999, Ji, Kim et al. 2008, Li 2009), *Moina macrocopa* (Ji, Kim et al. 2008); Malacostraca crustaceans – *Mysidopsis bahia* (Qi, Wang et al. 2011), *Neocardina denticulate* (Li 2009); Unionoida mollusks – *Lampsilis siliquoidea* & *Ligumia recta* (Chapter 2 and Hazelton, Cope et al. 2012), *Unio complanatus* (Qi, Wang et al. 2011); Gastropoda mollusks – *Physa acuta* (Li 2009); Fish – *Lepomis macrochirus* (USEPA 1999), *Onchorynchus mykiss* (Li 2009), *Pimephales promelas* (Qi, Wang et al. 2011).

Figure 7.2. Median lethal concentrations (LC50; bars) and test durations (closed circles) for acute PFOA toxicity tests to common aquatic test species. Error bars represent 95% confidence intervals where reported. Light gray bars are unionid glochidia data, dark gray bars are unionid juvenile data. Sources of data: Green Algae – *Chlorella vulgaris* (Latala, Nedzi et al. 2009); Planaria – *Dugesia japonica* (Li 2009); Cladocera - *Daphnia magna* (Ji, Kim et al. 2008, Li 2009), *Moina macrocopa* (Ji, Kim et al. 2008);

Malacostraca crustaceans – *Neocardina denticulate* (Li 2009); Unionoida mollusks – *Lampsilis siliquoidea* & *Ligumia recta* (Chapter 2 and Hazelton, Cope et al. 2012); Gastropoda mollusks – *Physa acuta* (Li 2009).

Figure 7.3. Median lethal concentrations (LC50; bars) and test durations (closed circles) for acute fluoxetine toxicity tests to common aquatic test species. Error bars represent 95% confidence intervals where reported. Light gray bars are unionid glochidia data, dark gray bars are unionid juvenile data. Sources of data: Green Algae – *Ankistrodesmus falcatus* (El-Bassat, Touliabah et al. 2011), *Chlorella vulgaris* (El-Bassat, Touliabah et al. 2011); Protozoa – *Paramecium caudataum* (El-Bassat, Touliabah et al. 2011), *Sirostomum amibuum* (Nalecz-Jawecki, Kaza et al. 2008); Rotifera – *Brachionus calyciflorus* (Nalecz-Jawecki, Kaza et al. 2008, El-Bassat, Touliabah et al. 2011); Cladocera – *Ceriodaphnia dubia* (Brooks, Foran et al. 2003, Henry, Kwon et al. 2004), *Daphnia magna* (Brooks, Foran et al. 2003, Hansen, Frost et al. 2008), *Daphnia longispira* (El-Bassat, Touliabah et al. 2011); Anostraca – *Thanmnocephalus platyurus* (Nalecz-Jawecki 2007); Unionoida mollusks – *Lampsilis fasciola* & *Ligumia recta* (Chapter 3 and Hazelton, Cope et al. 2013); Fish – *Gambusia affinis* (Henry and Black 2008), *Oryzias latipes* (Nakamura, Yamamoto et al. 2008), *Pimephales promelas* (Brooks, Foran et al. 2003).

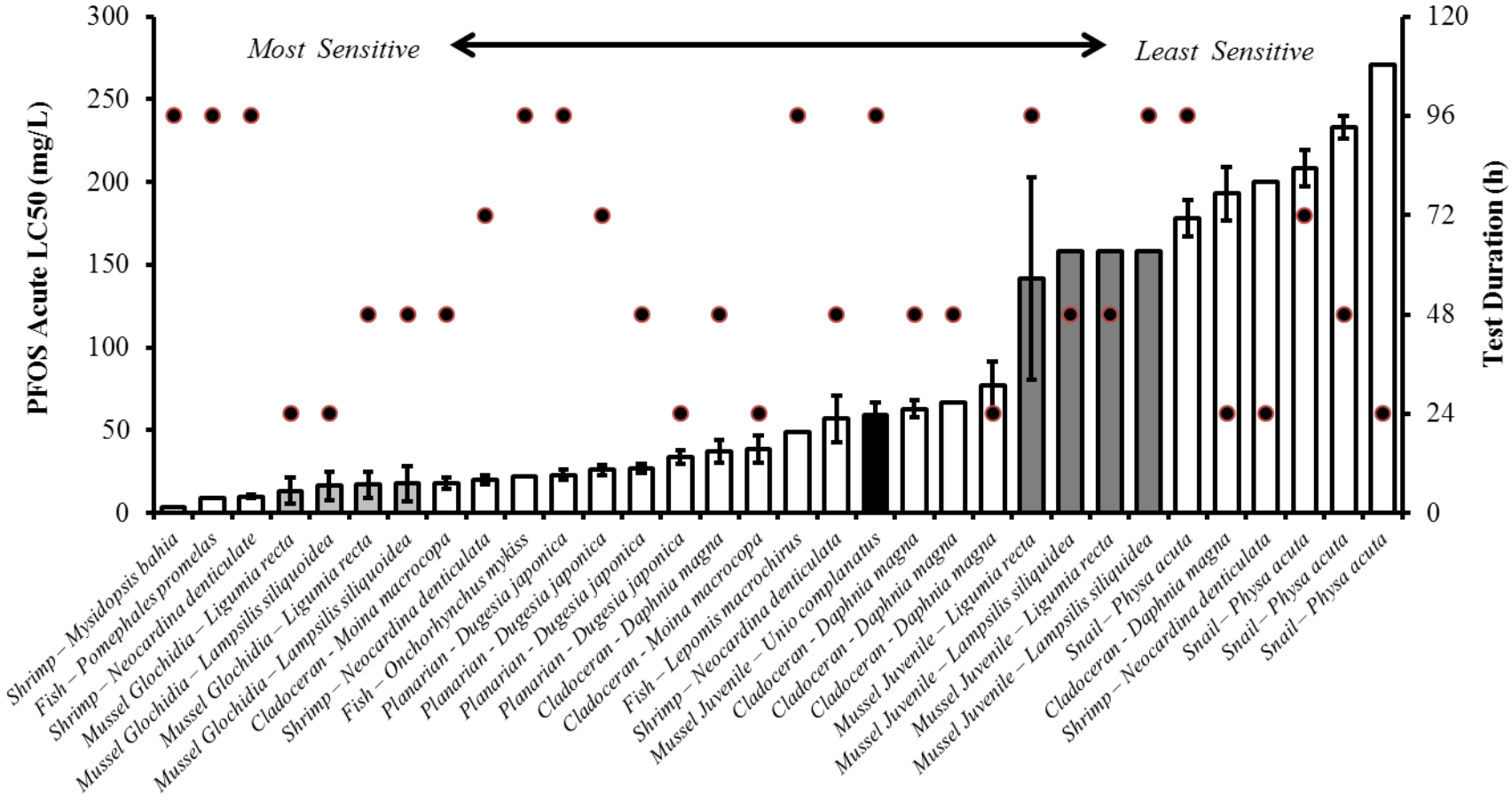

Figure 7.1. Median lethal concentrations (LC50; bars) and test durations (closed circles) for acute PFOS toxicity tests to common aquatic test species. Error bars represent 95% confidence intervals where reported. Light gray bars are unionid glochidia data, dark gray bars are unionid juvenile data, black bar is unionid juvenile data from another author. Sources of data: Planaria – *Dugesia japonica* (Li 2009); Cladocera - *Daphnia magna* (USEPA 1999, Ji, Kim et al. 2008, Li 2009), *Moina macrocopa* (Ji, Kim et al. 2008); Malacostraca – *Mysidopsis bahia* (Qi, Wang et al. 2011), *Neocardina denticulate* (Li 2009); Unionoida mollusks – *Lampsilis siliquoidea* & *Ligumia recta* (Chapter 2 and Hazelton, Cope et al. 2012), *Unio complanatus* (Qi, Wang et al. 2011); Gastropoda mollusks – *Physa acuta* (Li 2009); Fish – *Lepomis macrochirus* (USEPA 1999), *Onchorynchus mykiss* (Li 2009), *Pimephales promelas* (Qi, Wang et al. 2011).

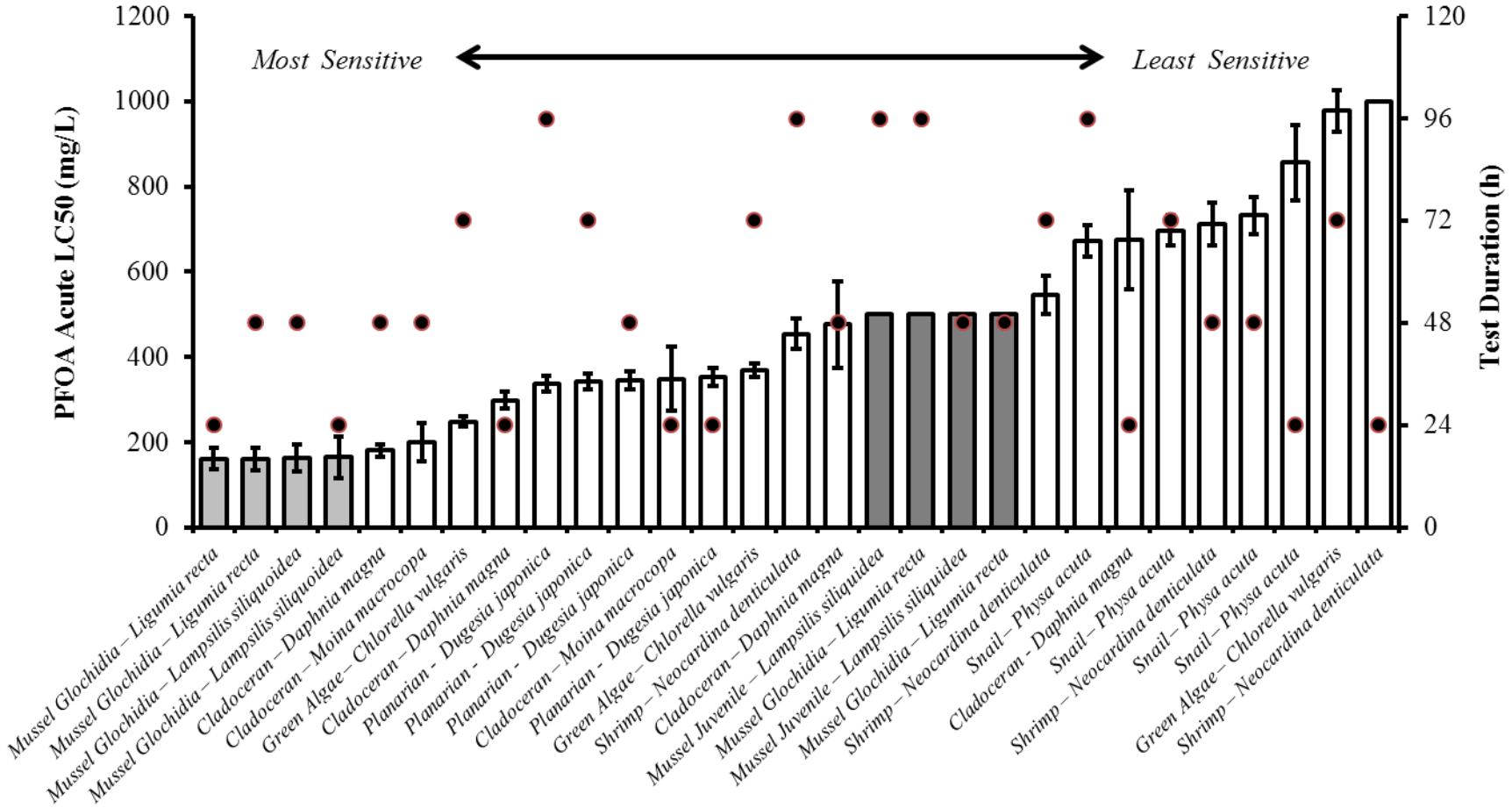

Figure 7.2. Median lethal concentrations (LC50; bars) and test durations (closed circles) for acute PFOA toxicity tests to common aquatic test species. Error bars represent 95% confidence intervals where reported. Light gray bars are unionid glochidia data, dark gray bars are unionid juvenile data. Sources of data: Green Algae – *Chlorella vulgaris* (Latala, Nedzi et al. 2009); Planaria – *Dugesia japonica* (Li 2009); Cladocera - *Daphnia magna* (Ji, Kim et al. 2008, Li 2009), *Moina macrocopa* (Ji, Kim et al. 2008); Malacostraca – *Neocardina denticulate* (Li 2009); Unionoida mollusks – *Lampsilis siliquoidea* & *Ligumia recta* (Chapter 2 and Hazelton, Cope et al. 2012); Gastropoda mollusks – *Physa acuta* (Li 2009).

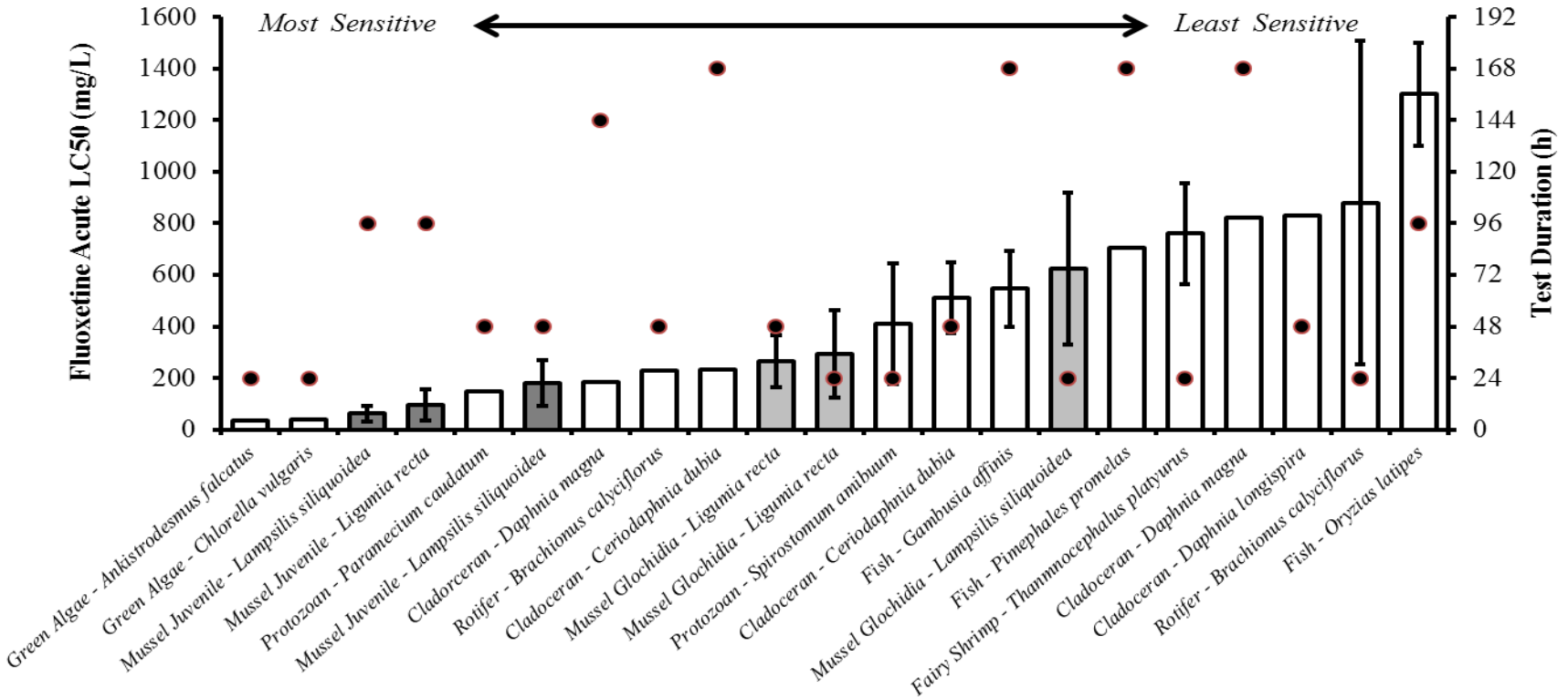

Figure 7.3. Median lethal concentrations (LC50; bars) and test durations (closed circles) for acute fluoxetine toxicity tests to common aquatic test species. Error bars represent 95% confidence intervals where reported. Light gray bars are unionid glochidia data, dark gray bars are unionid juvenile data. Sources of data: Green Algae – *Ankistrodesmus falcatus* (El-Bassat, Touliabah et al. 2011), *Chlorella vulgaris* (El-Bassat, Touliabah et al. 2011); Protozoa – *Paramecium caudataum* (El-Bassat, Touliabah et al. 2011), *Sirostomum amibuum* (Nalecz-Jawecki, Kaza et al. 2008); Rotifera – *Brachionus calyciflorus* (Nalecz-Jawecki, Kaza et al. 2008, El-Bassat, Touliabah et al. 2011); Cladocera – *Ceriodaphnia dubia* (Brooks, Foran et al. 2003, Henry, Kwon et al. 2004), *Daphnia magna* (Brooks, Foran et al. 2003, Hansen, Frost et al. 2008), *Daphnia longispira* (El-Bassat, Touliabah et al. 2011); Anostraca – *Thanmnocephalus platyurus* (Nalecz-Jawecki 2007); Unionoida mollusks – *Lampsilis fasciola* & *Ligumia recta* (Chapter 3 and Hazelton, Cope et al. 2013); Fish – *Gambusia affinis* (Henry and Black 2008), *Oryzias latipes* (Nakamura, Yamamoto et al. 2008), *Pimephales promelas* (Brooks, Foran et al. 2003).