Report as of FY2007 for 2006NC61B: "Endocrine and Reproductive Effects of the Pharmaceutical Fluoxetine on Native Freshwater Mussels: Proximity to Measured Environmental Concentrations"

Publications

- Other Publications:
  - Bringolf, R. B., R. M. Heltsley, C. Eads, T. J. Newton, S. Fraley, D. Shea, and W. G. Cope. 2007. Effects of fluoxetine on freshwater mussel reproduction: relation to environmental occurrence. Annual Meeting of the Water Resources Research Institute of North Carolina, Raleigh, NC, March 27-28, 2007
  - Bringolf, R. B., R. M. Heltsley, C. Eads, T. J. Newton, S. Fraley, D. Shea, and W. G. Cope. 2007. Environmental occurrence of fluoxetine and its effects on freshwater mussel reproduction. 5th Biennial Symposium of the Freshwater Mollusk Conservation Society, Little Rock, AR, March 12-15, 2007.
  - Heltsley, R. M., W. G. Cope, R. B. Bringolf, C. B. Eads, and D. Shea. 2006. Environmental concentrations of prozac induce spawning in freshwater mussels. 27th Annual Meeting of the Society of Environmental Toxicology and Chemistry, Montreal, Canada, November 5-9, 2006.
  - Heltsley, R. M., W. G. Cope, R. B. Bringolf, C. B. Eads, and D. Shea. 2006. Prozac elicits spawning in native freshwater mussels. 232nd Annual Meeting of the American Chemical Society, San Francisco, CA, September 10-14, 2006.

Report Follows

Title

Endocrine And Reproductive Effects Of The Pharmaceutical Fluoxetine On Native Freshwater Mussels: Proximity To Measured Environmental Concentrations

Project Summary

Recent research by Johnson et al. (2005), Kolpin et al. (2002; 2004) and others have measured concentrations of pharmaceuticals and personal care products (PPCPs) in surface waters that have the potential to adversely impact human and ecological health. The ubiquitous detection of these compounds in the environment has revealed an emerging class of contaminants that has largely been unrecognized or ignored in the past (Sanderson et al. 2004). Although some of these compounds are not as persistent as the traditionally studied priority pollutants (e.g., polychlorinated biphenyls and organochlorine pesticides), the continuous release of these PPCPs and other polar compounds into our rivers and streams presents similar exposure conditions as that of a persistent organic pollutant (Johnson et al. 2005). Studies have indicated that many of these compounds enter the environment completely un-metabolized or as a mixture of metabolites (Daughton and Ternes 1999). Therefore, compounds that were manufactured with the intent of being bioactive enter surface waters and may be responsible for not only acute toxicity but also chronic abnormalities and endocrine disruption in aquatic organisms (Colborn et al. 1993; Desbrow et al. 1998; Routledge et al. 1998).

Native freshwater mussels (family Unionidae) may unfortunately be among the groups of aquatic organisms adversely affected by persistent, low-level exposure to PCPPs and other endocrine disrupting compounds (EDCs) in our surface waters. Unionid mussels are filter and deposit feeding, long-lived (30-100 yr) benthic organisms that live burrowed in sediments of streams and rivers. They are one of the most rapidly declining faunal groups in North America. About 67% of the nearly 300 freshwater mussel species found in North America are considered vulnerable to extinction or already extinct (Bogan 1993; Williams et al. 1993). The decline of mussel populations in North America has occurred steadily since the mid 1800s and has been attributed to pollution, construction of dams and impoundments, sedimentation, navigation, and habitat degradation (Fuller 1974; Bogan 1993; Neves 1997; Brim Box and Mossa 1999; Vaughn and Taylor 1999). The surface waters of North Carolina have historically supported 56 species of unionid mussels (Bogan 2002). Today, 82% of these species are listed as endangered, threatened, or of special concern by the U.S. Fish and Wildlife Service and the State of North Carolina (Code of Federal Regulations 1993; NC Wildlife Resources Commission 2002) or are already extinct. Many of the same human-mediated and environmental stressors responsible for the declines of freshwater mussels throughout North America have also contributed to the declines in North Carolina. Principally, the stressors associated with human development and urbanization in almost all of the State's 17 river basins has hastened these declines over the past 20 to 50 years.

The primary focus of this project has been to generate a robust set of toxicological information on the sub-lethal endocrine and reproductive effects of fluoxetine on adults (both male and female) of the eastern elliptio (*Elliptio complanata*) mussel in laboratory tests. The intended pharmacologic action of fluoxetine in human therapy as an anti-depressant is to act as a selective serotonin reuptake inhibitor (SSRI) and increase serotonin levels at nerve synapses. Serotonin (5-hydroxytrypamine; 5-HT), an important neurotransmitter in vertebrate and

invertebrate systems, has been used to artificially induce spawning in freshwater bivalves for aquaculture purposes (Cunha and Machado 2001) and has been investigated as a potential chemical control mechanism (i.e., disruptor of reproduction) for exotic bivalve species like the zebra mussel (*Dreissena polymorpha*; Fong et al. 1994, Ram et al. 1992; 1996). Prior to this work funded by WRRI in the 2005-2006 cycle, there was limited evidence that fluoxetine and other SSRIs may exert reproductive effects on bivalves (Cunha and Machado 2001) similar to serotonin (Gibbons and Castagna 1984), making environmental exposures from this class of pharmaceuticals to native freshwater mussels and other aquatic biota through discharge of pharmacologically active compound in treated wastewater to surface waters an imminent concern. The specific objectives of this project were to:

1. Conduct a 96-hour laboratory toxicity test with gravid female eastern elliptio mussels and a range of concentrations of fluoxetine, serotonin (used as a positive control), and methiothepin (an inhibitor of serotonin pathways) to assess effects on reproductive endpoints such as time to parturition (or spontaneous abortion) of mussel larvae (glochidia) and viability of released glochidia.
2. Conduct a 96-hour laboratory toxicity test with ripe male eastern elliptio mussels and a range of concentrations of fluoxetine, serotonin (used as a positive control), and methiothepin (an inhibitor of serotonin pathways) to assess effects on reproductive endpoints such as time to spawning (or premature release of sperm) and viability of released sperm.
3. Quantify exposure concentrations of fluoxetine in the test chambers during the 96-h toxicity tests by analyzing samples of water and the novel passive sampling device simultaneously deployed in the test chambers with the mussels.
4. Quantify concentrations of fluoxetine accumulated in novel passive sampling devices deployed at sites in Crabtree Creek of the Neuse River Basin immediately downstream of the City of Cary Wastewater Treatment Plant effluent discharge.
5. Compare the results of the mussel toxicity tests with fluoxetine to any available toxicity data for standard aquatic test organisms such as *Ceriodaphnia dubia* and rainbow trout to assess relative risk of exposure.
6. Compare the results of the mussel toxicity tests with fluoxetine to measured environmental concentrations from the Neuse River Basin in this study, to the peer-reviewed literature, or to predicted environmental concentrations, if they exist, to assess relative risk of adverse effects of fluoxetine.
7. Conduct a 96-hour laboratory toxicity test with gravid female *Lampsilis* spp. mussels and a low range of concentrations of fluoxetine, and serotonin (used as a positive control) to assess the reproductive behavioral effects on mantle flap (fish lure) display, time used, and relative action (e.g., beats per minute)

Methods, Procedures, and Facilities

Adult eastern elliptio mussels were collected from several relatively uncontaminated, rural forested streams in the central Piedmont of North Carolina. The mussels were transported (methods in Cope et al. 2003) to the Aquatic Toxicology Laboratory at North Carolina State University, where they were maintained in reconstituted hard water (ASTM 1993) at 18-20°C for at least 24 h prior to beginning any experiments to ensure that spawning or release of glochidia is not a result of handling or transport stress. For testing, mussels that had not released gametes or

glochidia were placed in 3.75-L glass aquaria containing 2 L of reconstituted hard water and aerated with compressed air to ensure dissolved oxygen concentrations greater than 60% of saturation at all times (ASTM 1993). The mussels were exposed to five fluoxetine treatments (0, 0.3, 3.0, 30, 300, or 3000 μg/L), with 3 replicates per treatment and 3 mussels per replicate in static renewal tests for 96 h. In addition, a serotonin treatment was included as a positive control and another treatment included mussels that had been briefly exposed to a serotonin inhibitor, methiothepin (Fong et al. 1994), and then exposed to serotonin or fluoxetine to demonstrate that fluoxetine is acting as an SSRI in *E. complanata*. All mussels were monitored continuously for the first 6 h for release of gametes (available literature indicates that serotonin and SSRI action is relatively rapid), then at 24 h intervals over the remaining exposure duration. Relevant endpoints quantified during the exposure included time to release of sperm or glochidia (larvae); in the case of parturition (females), glochidia were examined for viability and recorded as either immature or mature and a likewise assessment of sperm viability from males. Water quality conditions (dissolved oxygen, temperature, conductivity, hardness, and alkalinity) were measured with standard methods (ASTM 1993) in samples taken from the test chambers at time 0, 24, 48, 72, and 96 h of the test. A 100% renewal of fluoxetine concentrations was done at 24 h intervals on replicates of a treatment in which mussels had not released to ensure target test concentrations were maintained. Water samples were taken from each test chamber at the time mussels were initially placed in fluoxetine treatments and again at the time of first release for analysis of fluoxetine concentrations by liquid chromatography/mass spectroscopy (LC-MS), with methods already developed by R.M. Heltsley (in our laboratory) and modified from Brooks et al. (2003a).

The novel PSDs were deployed in triplicate at four sites in Crabtree Creek of the Neuse River Basin immediately downstream of the City of Cary Wastewater Treatment Plant effluent discharge. The PSDs were retrieved approximately 30 d after deployment, transported to the Analytical Toxicology Laboratory at NCSU, extracted, and analyzed for fluoxetine and a suite of other polar and non-polar contaminants by LC/MS and/or gas chromatography/mass spectroscopy methods.

For the behavioral test, adult female *Lampsilis fasciola* were collected from a relatively uncontaminated, rural forested portion of the Little Tennessee River in the mountain region of North Carolina. The mussels were transported (methods in Cope et al. 2003) to the Aquatic Toxicology Laboratory at North Carolina State University, where they were maintained in reconstituted hard water (ASTM 1993) at 18-20°C for at least 48 h prior to beginning any experiments to ensure that spawning or release of glochidia was not a result of handling or transport stress and that the gravid females were displaying their mantle flaps as normal behavior. For testing, mussels that had not released glochidia and were displaying normally were placed into 3.75-L glass aquaria containing 2 L of reconstituted hard water and aerated with compressed air to ensure dissolved oxygen concentrations greater than 60% of saturation at all times (ASTM 1993). The mussels were exposed to five fluoxetine treatments (0, 0.3, 3.0, 30, 300, or 3000 μg/L), with 3 replicates per treatment and 5 mussels per replicate in static renewal tests for 96 h. In addition, a serotonin treatment was included as a positive control. All mussels were monitored continuously for the first 6 h for release of gametes and the occurrence, frequency and duration of mantle flap display, and then at selected hourly intervals over the remaining exposure duration. Relevant endpoints quantified during the exposure included time to release of glochidia (larvae); in the case of parturition, glochidia were examined for viability and recorded as either immature or mature. The occurrence, frequency, and duration of mantle flap display were categorized as follows: shell closed, shell gaped-no mantle exposed, shell

gaped-mantle extended, shell gaped-mantle extended with fish lure out, and shell gaped-mantle extended with fish lure out and beating (if lure was out, the number of beats/min was quantified). Water quality conditions (dissolved oxygen, temperature, conductivity, hardness, and alkalinity) were measured with standard methods (ASTM 1993) in samples taken from the test chambers at time 0, 24, 48, 72, and 96 h of the test. A 100% renewal of fluoxetine concentrations was done at 24 h intervals on replicates of a treatment in which mussels had not released to ensure target test concentrations were maintained. Water samples were taken from each test chamber at the time mussels were initially placed in fluoxetine treatments and again at the time of first release of behavioral effect for analysis of fluoxetine concentrations by liquid chromatography/mass spectroscopy (LC-MS).

Principal Findings and Significance

Toxicity tests with gravid female adult eastern elliptio (*Elliptio complanata*) mussels and a range of fluoxetine concentrations were completed. These tests evaluated the potential for fluoxetine to cause pre-mature release (spontaneous parturition) of glochidia and the viability of the glochidia that were released. We found that fluoxetine does indeed cause the pre-mature release of non-viable and viable larvae (glochidia) in native freshwater mussels in < 48 h of exposure (Fig. 1). The major threshold concentration for significant effects appears to be between 150 and 223 µg/L, a concentration range greater than those measured in environmental samples.

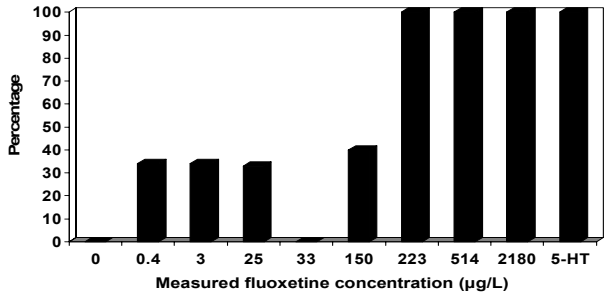

Fig. 1. Percentage of gravid female mussels pre-maturely releasing glochidia with 48 h of fluoxetine exposure in two laboratory tests.

Therefore, our study has confirmed that fluoxetine (and possibly other SSRIs) may exert reproductive effects on bivalves similar to serotonin (5-HT, the positive control in our tests), making environmental exposures from this class of pharmaceuticals to native freshwater mussels and other aquatic biota through discharge of pharmacologically active compound in treated wastewater to surface waters an imminent concern. We have also successfully completed the behavioral test of mantle flap display and have found that fluoxetine alters display behavior at the highest concentration tested. The analytical chemistry results of PSDs deployed in Crabtree Creek are forthcoming.

The ecological effects of an ill-timed release of larval mussels or gametes caused by environmental fluoxetine exposure could be potentially devastating to localized mussel

populations. Likewise, the inability of a female mussel to attract her obligate fish host through reduced or non-existent mantle flap (fish lure) display behavior such that she would not be able to successfully infest a fish with glochidia could also result in total reproductive failure and devastate local mussel populations. Because the mode of action of fluoxetine is to alter behavior through neuroendrocrine pathways, this scenario is biologically plausible and warrants further investigation.

References

ASTM (American Society for Testing and Materials). 1993. Standard guide for conducting acute toxicity tests with fishes, macroinvertebrates, and amphibians. E 729-88a, American Society for Testing and Materials, Philadelphia, PA, pp 102-121.

Augspurger, T. P., A. E. Keller, M. C. Black, W. G. Cope, and F. J. Dwyer. 2003. Water quality guidance for protection of freshwater mussels (Unionidae) from ammonia exposure. *Environmental Toxicology and Chemistry* 22:2569-2575.

Bogan, A. E. 1993. Freshwater bivalve extinctions (Mollusca: Unionoida): a search for causes. *American Zoologist* 33:599-609.

Bogan, A. E. 2002. A Workbook and Key to the Freshwater Mussels of North Carolina. NC Museum of Natural Sciences, Raleigh, NC.

Brim Box, J. and Mossa, J. 1999. Sediment, land use, and freshwater mussels: prospects and problems. *Journal of the North American Benthological Society*, 18:99-117.

Brooks, B. W., Foran, C. M., Richards, S. M., Weston, J., Turner, P. K., Stanley, J. K., Solomon, K. R., Slattery, M., and La Point, T. W. 2003a. Aquatic ecotoxicology of fluoxetine. *Toxicology Letters* 142:169-183.

Brooks, B. W., Turner, P. K., Stanley, J. K., Weston, J., Glidewell, E. A., Foran, C. M., Slattery, M., La Point, T. W., and Huggett, D. B. 2003b. Waterborne and sediment toxicity of fluoxetine to select organisms. *Chemosphere* 52:135-142.

Code of Federal Regulations. 1993. Part 17--Endangered and threatened wildlife and plants, *Section 17.11, Endangered and threatened wildlife*, U.S. Government Printing Office, 50, 108-110.

Colborn, T., vom Saal, F. S., and Soto, A. M. 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environmental Health Perspectives* 101:378-384.

Cope, W. G., M. C. Hove, D. L. Waller, D. J. Hornbach, M. R. Bartsch, L. A. Cunningham, H. L. Dunn and A. R. Kapuscinski. 2003. Evaluation of relocation of unionid mussels to *in situ* refugia. *Journal of Molluscan Studies*, 69:27-34.

Cunha, E. M. and Machado, J. 2001. Parturition in *Anodonta cygnea* induced by selective serotonin reuptake inhibitors (SSRIs). *Canadian Journal of Zoology* 79:95-100.

Daughton, C. G. and Ternes, T. A. 1999. Pharmaceuticals and personal care products in the environment: agents of subtle change? *Environmental Health Perspectives* 107:907-938.

Desbrow, C., Routledge, E. J., Brighty, G. C., Sumpter, J. P., and Waldock, M. 1998. Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening. *Environmental Science and Technology* 32:1549-1558.

Fong, P. P., Duncan, J., and Ram, J. L. 1994. Inhibition and sex specific induction of spawning by sertonergic ligands in the zebra mussel *Dreissena polymorpha* (Pallas). *Experientia* 50:506-509.

Foran, C. M., Weston, J., Slattery, M., Brooks, B. W., and Huggett, D. B. 2004. Reproductive assessment of Japanese medaka (*Oryzias latipes*) following a four-week fluoxetine (SSRI) exposure. *Archives of Environmental Contamination and Toxicology* 46:511-517.

Fuller, S. L. H. 1974. Clams and mussels (Mollusca: Bivalvia). In: *Pollution Ecology of Freshwater Invertebrates*, (C. W. Hart, Jr. and S. L. H. Fuller, eds), 215-273. Academic Press, New York.

Gibbons, M. C. and M. Castagna. 1984. Serotonin as an inducer of spawning in six bivalve species. *Aquaculture* 40:189-191.

Goudreau, S. E., Neves, R. J. and Sheehan, R. J. 1993. Effects of wastewater treatment plant effluents on freshwater mollusks in the upper Clinch River, Virginia, USA. *Hydrobiologia*, 252: 211-230.

Henry, T. B., Kwon, J-W., Armbrust, K. L., and Black, M. C. 2004. Acute and chronic toxicity of five selective serotonin reuptake inhibitors in *Ceriodaphnia dubia*. *Environmental Toxicology and Chemistry* 23:2229-2233.

Johnson, D. J., Sanderson, H., Brain, R. A., Wilson, C. J., Bestari, K. T., and Solomon, K. R. 2005. Exposure assessment and microcosm fate of selected selective serotonin reuptake inhibitors. *Regulatory Toxicology and Pharmacology* 42:313-323.

Kolpin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B., and Buxton, H. T. 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: A national reconnaissance. *Environmental Science and Technology* 36:1202-1211.

Kolpin, D. W., Skopec, M., Meyer, M. T., Furlong, E. T., and Zaugg, S. D. 2004. Urban contribution of pharmaceuticals and other organic wastewater contaminants to streams during differing flow conditions. *Science of the Total Environment* 328:119-130.

Levine, J. F., A. E. Bogan, K. H. Pollock, H. A. Devine, L. L. Gustafson, C. B. Eads, P. P. Russel, and E. F. Anderson. 2003. Distribution of freshwater mussel populations in relationship to crossing structures. Final completion report to the NC Department of Transportation, NC State University, College of Veterinary Medicine, Raleigh, NC.

McMahon, R. F. and A. E. Bogan. 2001. Mollusca: Bivalvia. In: J. H. Thorpe & A. P. Covich, editors. *Ecology and classification of North American freshwater invertebrates*. New York: Academic Press. pp. 331-429.

NC Wildlife Resources Commission. 2002. List of State Threatened and Endangered Mussel Species web resource: http://216.27.49.98/fs_index_07_conservation.htm.

Neves, R. J. 1997. A national strategy for the conservation of native freshwater mussels. In: *Conservation and management of freshwater mussels II: initiatives for the future*, (K. S. Cummings, A. C. Buchanan, C. A. Mayer & T. J. Naimo, eds), 1-10. Proceedings of a UMRCC Symposium, 16-18 October 1995, St. Louis, Missouri. Upper Mississippi River Conservation Committee, Rock Island, Illinois.

Ram, J. L., Fong, P., Croll, R. P., Nichols, S. J., and Wall, D. 1992. The zebra mussel (*Dreissena polymorpha*), a new pest in North America: reproductive mechanisms as possible targets of control strategies. *Invertebrate Reproduction and Development* 22:77-86.

Ram, J. L., Fong, P. P., and Garton, D. W. 1996. Physiological aspects of zebra mussel reproduction: maturation, spawning, and fertilization. *American Zoologist* 36:326-338.

Routledge, E. J., Sheahan, D., Desbrow, C., Brighty, G. C., Waldock, M., and Sumpter, J. P. 1998. Identification of estrogenic chemicals in STW effluent. 2. In vitro responses in trout and roach. *Environmental Science and Technology* 32:1559-1565.

Sanderson, H.Y., Johnson, D. J., Reitsma, T., Brain, R. A., Wilson, C. J., and Solomon, K. R. 2004. Ranking and prioritization of environmental risks of pharmaceuticals in surface waters. *Regulatory Toxicology and Pharmacology* 39:158-183.

Vaughn, C. C. and Taylor, C. M. 1999. Impoundments and the decline of freshwater mussels: a case study of an extinction gradient. *Conservation Biology* 13:912-920.

Williams, J. D., Warren, Jr., M. L., Cummings, K. S., Harris, J. L. and Neves, R. J. 1993. Conservation status of freshwater mussels of the United States and Canada. *Fisheries* 18:6-22.