Evaluation of the relationships between watershed-scale land use and contaminants in aquatic environments and the use of freshwater snails as indicators of impairment

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ABSTRACT

The use of manure from animal feeding operations (AFOs) as fertilizer on agricultural land may introduce contaminants to aquatic environments that can negatively affect the health of aquatic organisms. This study utilized a landscape-scale regression-based design to assess the effects of AFOs on contaminant concentrations and resident populations of a pleurocerid snail, *Leptoxis carinata,* in streams within the Shenandoah River watershed (Virginia, USA). Individual characteristics of *L. carinata* were also evaluated to provide further understanding of observed population characteristics. In streambed sediment and mollusk tissue, concentrations of the trace element arsenic, used as an additive in poultry feed, were not directly related to watershed densities of AFOs. In-stream concentrations of dissolved nutrients and estrogenic compounds, measured as estrogenic activity, were directly related to watershed densities of AFOs. Population sex ratios of *L. carinata* varied across study sites, from balanced to female-biased, but were not related to concentrations of estrogenic compounds. However, the spatial variation in population sex ratios, coupled with little variation in site-specific sex ratios across seasons and generations, suggest an influence of site-specific environmental conditions. Individual-level studies of *L. carinata* revealed that there is an eight month lag between hatching and gametogenesis which could allow disruption of sexual differentiation by environmental contaminants, but further study of the effects of specific contaminants on sexual differentiation in this species is needed. Population densities of *L. carinata* were related to in-stream nutrient concentrations and landscape sources of nutrients, including AFOs, but none of these factors were directly related to the infection prevalence of digenetic trematodes in *L. carinata* populations. Although trematode infection rates in *L. carinata* populations do not appear to be viable indicators of the influence of eutrophic conditions on disease incidence in aquatic organisms, the identification of five types of trematodes in *L. carinata* populations highlights the utility of this snail species for further investigation of transmission dynamics of trematode parasites in lotic systems.

To my husband, Michael Brennan, for his love and support

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CHAPTER 1

Introduction

Alteration of natural landscapes by human activities can negatively impact aquatic environments through several pathways. The cumulative effects of diffuse sources of contaminants within landscapes, i.e. nonpoint sources, are receiving increasing regulatory attention due to their recognized effects on water quality. Agricultural land use is a well-recognized nonpoint source of contaminants to streams and rivers, primarily due to activities related to crop production and cattle grazing (USEPA, 2009). In recent decades, industrialization of animal agriculture has led to large numbers of animals maintained on small land areas (Mallin and Cahoon, 2003). Manure from these animal feeding operations (AFOs) is collected and applied on nearby cropland or pastureland as fertilizer. While this is seemingly an effective method of waste disposal and nutrient utilization, it has been suggested that AFOs effectively decouple animals and the land area used to support them, which results in nutrient production in excess of what the surrounding landscape can assimilate (Mallin and Cahoon, 2003).

The introduction of excess nutrients in manure from AFOs to aquatic ecosystems is suspected as one of the primary contemporary causes of eutrophication, which is the most widespread water quality problem in the United States (Carpenter et al., 1998). Excess algal biomass can ultimately result in physical, biological, and chemical changes that cause losses of biodiversity and ecosystem services (Smith et al., 1999), and there is increasing evidence that nutrient enrichment in aquatic environments can also lead to an increased risk of parasitic and infectious disease in humans and wildlife (McKenzie and Townsend, 2007). In addition to nutrients, manure from AFOs contains fecal bacteria, natural hormones produced by the animals, synthetic hormones that may be given to the animals to regulate growth and reproduction, and feed additives (e.g. trace elements, antibiotics) that are administered to maintain the health of the animals while in confinement (Brock et al., 2006; Gupta and Gardner, 2005; Hanselman et al., 2003; USEPA, 2004). These substances can ultimately enter aquatic environments after land-application of manure and may have detrimental effects on aquatic organisms. For example, the presence of natural hormones and compounds that mimic their action in aquatic environments can interfere with endocrine system function in fishes, causing a disruption in sexual development, and ultimately, impaired reproductive function (Jobling et al., 1998). Certain trace elements can bioaccumulate in the tissues of aquatic organisms and at high concentrations, cause chronic toxicological effects in several organ systems (Hamilton and Hoffman, 2003). Although several studies have investigated local effects of AFOs on contaminant concentrations in aquatic environments (e.g. Brown et al., 2005; Matthiessen et al., 2006) few have examined the cumulative effects of many

AFOs across the landscape (e.g. Weldon and Hornbuckle, 2006). Landscape-scale studies are necessary in order to effectively manage manure from AFOs to protect aquatic environments while maintaining agricultural productivity.

The need for landscape-scale assessments of the effects of AFOs on aquatic environments is particularly relevant to the Shenandoah River watershed (Virginia, USA). The 7,600 km^2 watershed is 39% agricultural land, which receives manure from approximately 1200 AFOs and 300 farms that maintain grazing beef cattle (VADCR, 2010; VADEQ, 2006). The Shenandoah River discharges to the Potomac River, which is a major tributary of Chesapeake Bay, a critically imperiled ecosystem due, in part, to eutrophication (Boesch et al., 2001). Excess nutrients in the Shenandoah River are a recognized problem. For the Shenandoah and Potomac Rivers, it has been estimated that nitrogen and phosphorous loadings must be reduced by 44% and 29%, respectively in order to adequately protect the health of Chesapeake Bay (Commonwealth of Virginia, 2005). In addition to eutrophication, seasonal fish kills have occurred in the Shenandoah River since 2004. The cause of these kills remains unknown, but one theory is that multiple environmental stressors are causing immunosuppression in the fish, increasing their susceptibility to opportunistic pathogens (Ripley et al., 2008). Surviving fish show symptoms of chronic immune stress, including: skin lesions, inflammation and presence of macrophage aggregates in the spleen and head kidney, and parasitic infections in different organs (Blazer et al., 2010). Smallmouth bass from the Shenandoah River also have a high incidence of intersex, a condition in which oocytes are present in the testes of male fish, compared to other local drainages (Blazer et al., 2007). Intersex suggests exposure to endocrine-disrupting compounds (EDCs) and these compounds, as well as trace elements and other contaminants, can affect the immune function of fishes (Lage et al., 2006; Iwanowicz and Ottinger, 2009). Thus far, studies of Shenandoah River fish have failed to establish relationships between responses, stressors, and their sources. This can be attributed to inherent difficulties in sampling fish populations, including: inability to collect large numbers of targeted species, habitat limitations of larger species, and uncertainty regarding exposure history of the fish due to their mobility.

Biological monitoring (biomonitoring) with freshwater benthic macroinvertebrates is commonly used to identify impacted sites in aquatic environments (Norton et al., 2000; Richards et al., 1993) and is well-suited to landscape-scale assessments. The sessile nature of benthic macroinvertebrates offers a distinct advantage in their use as sentinel organisms compared to fish because it allows determination of relationships between organism responses and site-specific or watershed-specific environmental variables. In addition, ease of collection of organisms facilitates statistically valid sampling. Biomonitoring with freshwater benthic macroinvertebrates at the

assemblage or community organizational level is emphasized for tracking trends and determining overall impairment for regulatory purposes, but this approach is often not effective for elucidating causes of impairment. Alternative approaches to biomonitoring incorporate endpoints at lower organizational levels in order to provide information about environmental stressors that may not elicit known or predictable responses in macroinvertebrate communities (Bonada et al., 2006). Freshwater snails are a promising group of organisms for biomonitoring conducted at the individual and population level. Unlike aquatic insects, mollusks are aquatic for their entire life cycle, which increases their exposure to contaminants. In addition, mollusks are obligate first intermediate hosts for digenetic trematode parasites (parasitic flatworms) and appear to be susceptible to endocrine-disrupting effects of natural and synthetic hormones (Matthiessen, 2008). Therefore, relationships between environmental conditions and biological effects, such as parasitic infection and endocrine disruption, can potentially be investigated using freshwater snails. However, a major disadvantage to using freshwater snails in biomonitoring is that their population characteristics are generally not well studied, especially in lotic environments (Lysne et al., 2008).

The freshwater snail *Leptoxis carinata* (superorder Caenogastropoda: order Sorbeoconcha: superfamily Cerithioidea: family Pleuroceridae) is widely distributed in the Shenandoah River watershed, with densities reaching 3,000 individuals/m^2 (Orth et al., 2009). Pleurocerid snails are operculate, feed on attached algae and bacteria, and are all dioecious (separate sexes) with internal fertilization. The general life cycle of *Leptoxis spp.* has been described (Aldridge, 1982; Hendrix 1986; Miller-Way and Way, 1989), but variation in population characteristics over a large spatial scale and responses of these characteristics to environmental stressors have not been previously studied.

The main objective of this dissertation research was to investigate the relationships between land use, environmental stressors, and individual and population characteristics of *L. carinata* in the Shenandoah River watershed in order to address the following questions, which are illustrated by a conceptual model in Figure 1-1.

1) Are land use practices introducing contaminants that can impact the health of aquatic organisms?
2) Are population characteristics of pleurocerid snails suitable indicators of biological effects related to these contaminants and/or land use practices?
3) Do characteristics of individual snails explain some of the observed population-level responses, and does measurement of these individual-level responses have potential applications in biomonitoring?

The specific objectives of this study and corresponding chapter numbers are listed below. An expanded conceptual model, incorporating specific study objectives, is presented in Figure 1-2.

Specific study objectives:

1) Develop an appropriate study design to assess the impacts of land use on contaminant concentrations in streams within the Shenandoah River watershed and to assess the potential direct effects of land use on population characteristics of *L. carinata* (Chapter 2)
2) Evaluate the potential contribution of manure from AFOs to trace element concentrations in streambed sediment and resident mollusks relative to other sources (Chapter 3)
3) Assess the effects of AFOs on aqueous concentrations of nutrients and endocrine-disrupting compounds (EDCs) (Chapter 4)
4) Examine the variability in sex ratios of *L. carinata* populations within the Shenandoah River watershed and evaluate the use of population sex ratios as indicators of exposure to endocrine-disrupting compounds (Chapter 5)
5) Evaluate parasitic infection rates in *L. carinata* populations within the Shenandoah River watershed and their relationships with nutrient concentrations and sources (Chapter 6)
6) Describe gamete development in *L. carinata* in order to further understand and assess the potential effects of contaminants on sexual differentiation in this species (Chapter 7)
7) Develop an immune function assay for *L. carinata* in order to further understand and assess the potential effects of contaminants on susceptibility to parasitic infection in this species (Appendix A)

Literature cited

Aldridge, D. W. (1982). Reproductive tactics in relation to life-cycle bioenergetics in three natural populations of the freshwater snail, *Leptoxis carinata*. *Ecology, 63*(1), 196-208.

Blazer, V. S., Iwanowicz, D. D., Starliper, C. E., Iwanowicz, D. D., Barbash, P., Hedrick, J. D., et al., (2010). Mortality of centrarchid fishes in the Potomac drainage: Survey results and overview of potential contributing factors. *Journal of Aquatic Animal Health, 22*, 190-218.

Blazer, V. S., Iwanowicz, L. R., Iwanowicz, D. D., Smith, D. R., Young, J. A., Hedrick, J. D., et al., (2007). Intersex (testicular oocytes) in smallmouth bass from the potomac river and selected nearby drainages. *Journal of Aquatic Animal Health, 19*, 242-253.

Boesch, D. F., Brinsfield, R. B., & Magnien, R. E. (2001). Chesapeake Bay eutrophication: Scientific understanding, ecosystem restoration, and challenges for agriculture. *Journal of Environmental Quality, 30*, 303-320.

Bonada, N., Prat, N., Resh, V. H., & Statzner, B. (2006). Developments in aquatic insect biomonitoring: A comparative analysis of recent approaches. *Annual Review of Entomology, 51*, 495-523.

Brock, E. H., Ketterings, Q. M., & McBride, M. (2006). Copper and zinc accumulation in poultry and dairy manure-amended fields. *Soil Science, 171*, 388-399.

Brown, B. L., Slaughter, A. D., & Schreiber, M. E. (2005). Controls on roxarsone transport in agricultural watersheds. *Applied Geochemistry, 20*, 123-133.

Carpenter, S. R., Caraco, N. F., Correll, D. L., Howarth, R. W., Sharpley, A. N., & Smith, V. H. (1998). Nonpoint pollution of surface waters with phosphorus and nitrogen. *Ecological Applications, 8*, 559-568.

Commonwealth of Virginia. (2005). Chesapeake Bay nutrient and sediment reduction strategy for the Shenandoah and Potomac River basins. http://www.dcr.virginia.gov/soil_and_water/documents/tsshenpoall032805.pdf.

Gupta, G., & Gardner, W. (2005). Use of clay mineral (montmorillonite) for reducing poultry litter leachate toxicity (ec_{50}). *Journal of Hazardous Materials, B118*, 81-83.

Hamilton, S. J., & Hoffman, D. J. (2003). Trace element and nutrition interactions in fish and wildlife. In D. J. Hoffman (Ed.), *Handbook of ecotoxicology, second edition* (pp. 1198-1225). Boca Raton: Lewis Publishers.

Hanselman, T. A., Graetz, D. A., & Wilkie, A. C. (2003). Manure-borne estrogens as potential environmental contaminants: A review. *Environmental Science and Technology, 37*, 5471-5478.

Hendrix, S. S. (1986). Population biology of the pleurocerid snail, *Leptoxis carinata* (Brug.) in Marsh Creek, Adams County, PA. *American Malacological Union Bulletin, 4*(1), 119.

Iwanowicz, L. R., & Ottinger, C. A. (2009). Estrogens, estrogen receptors, and their role as immunoregulators in fish. In G. Zaccone, J. Meseguer, A. Garcia-Ayala, & B. G. Kapoor (Eds.), *Fish defenses, volume 1: Immunology* (pp. 277-322). Enfield, NH: Science Publishers.

Jobling, S., Nolan, M., Tyler, C. R., Brighty, G., & Sumpter, J. P. (1998). Widespread sexual disruption in wild fish. *Environmental Science & Technology, 32*, 2498-2506.

Lage, C. R., Nayak, A., & Kim, C. H. (2006). Arsenic ecotoxicology and innate immunity. *Integrative and Comparative Biology, 46*, 1040-1054.

Lysne, S. J., Perez, K. E., Brown, K. M., Minton, R. L., & Sides, J. D. (2008). A review of freshwater gastropod conservation: Challenges and opportunities. *Journal of the North American Benthological Society, 27*, 463-470.

Mallin, M. A., & Cahoon, L. B. (2003). Industrialized animal production - a major source of nutrient and microbial pollution to aquatic ecosystems. *Population and Environment, 24*, 369-385.

Matthiessen, P. (2008). An assessment of endocrine disruption in mollusks and the potential for developing internationally standardized mollusk life cycle test guidelines. *Integrated Environmental Assessment and Management, 4*, 274-284.

Matthiessen, P., Arnold, D., Johnson, A. C., Pepper, T. J., Pottinger, T. G., & Pulman, K. G. T. (2006). Contamination of headwater streams in the United Kingdom by oestrogenic hormones from livestock farms. *Science of the Total Environment, 367*, 616-630.

McKenzie, V. J., & Townsend, A. R. (2007). Parasitic and infectious disease responses to changing global nutrient cycles. *EcoHealth, 4*, 384-396.

Miller-Way, C. A., & Way, C. M. (1989). The life history of *Leptoxis dilatata* (Conrad) (Prosobranchia: Pleuroceridae) from the Laurel Fork River, West Virginia. *American Midland Naturalist, 122*, 193-198.

Norton, S. B., Cormier, S. M., Smith, M., & Jones, R. C. (2000). Can biological assessments discriminate among types of stress? A case study from the Eastern Corn Belt Plains ecoregion. *Environmental Toxicology and Chemistry, 19*, 1113-1119.

Orth, D. J., Alexander, K. A., Ciparis, S., Frimpong, E., Henley, W., & Voshell, J. R. (2009). Investigation into smallmouth bass mortality in Virginia's rivers. http://www.dgif.virginia.gov/fishing/fish-kill/Smallmout-Bass-Mortality-Final-Rpt-VT-2009.pdf.

Richards, C., Host, G. E., & Arthur, J. W. (1993). Identification of predominant environmental factors structuring stream macroinvertebrate communities within a large agricultural catchment. *Freshwater Biology, 29*, 285-294.

Ripley, J., Iwanowicz, L. R., Blazer, V. S., & Foran, C. (2008). Utilization of protein expression profiles as indicators of environmental impairment of smallmouth bass (*Micropterus dolmieu*) from the Shenandoah River, Virginia, USA. *Environmental Toxicology and Chemistry, 27*, 1756-1767.

Smith, V. H., Tilman, G. D., & Nekola, J. C. (1999). Eutrophication: Impacts of excess nutrient inputs on freshwater, marine, and terrestrial ecosystems. *Environmental Pollution, 100*, 179-196.

USEPA (2009). National water quality inventory: Report to congress. 2004 reporting cycle. Washington, DC: Office of Water. EPA 841-R-08-001.

USEPA/USDA (2004). Regional science workshop: Animal feeding operations (AFOs) science and technical support needs. Washington, DC: Office of Research and Devleopment. http://www.epa.gov/osp/regions/afo_rep.pdf.

VADCR (2010). Animal feeding operations database. http://webdat.dcr.virginia.gov/cfprog/dswc/afoprm.cfm.

VADEQ (2006). Final 2006 305(b)/303(d) water quality assessment integrated report. http://www.deq.state.va.us/wqa/ir2006.htm

Weldon, M. B., & Hornbuckle, K. C. (2006). Concentrated animal feeding operations, row crops, and their relationships to nitrate in eastern Iowa rivers. *Environmental Science and Technology, 40*, 3168-3173.

Figure 1- 1. Conceptual model of the three components of the main objective of the dissertation.

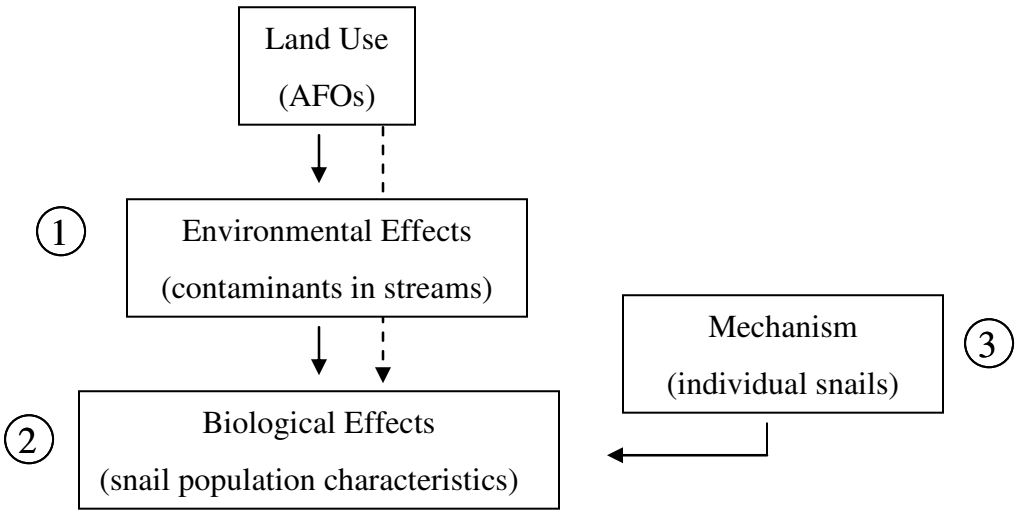
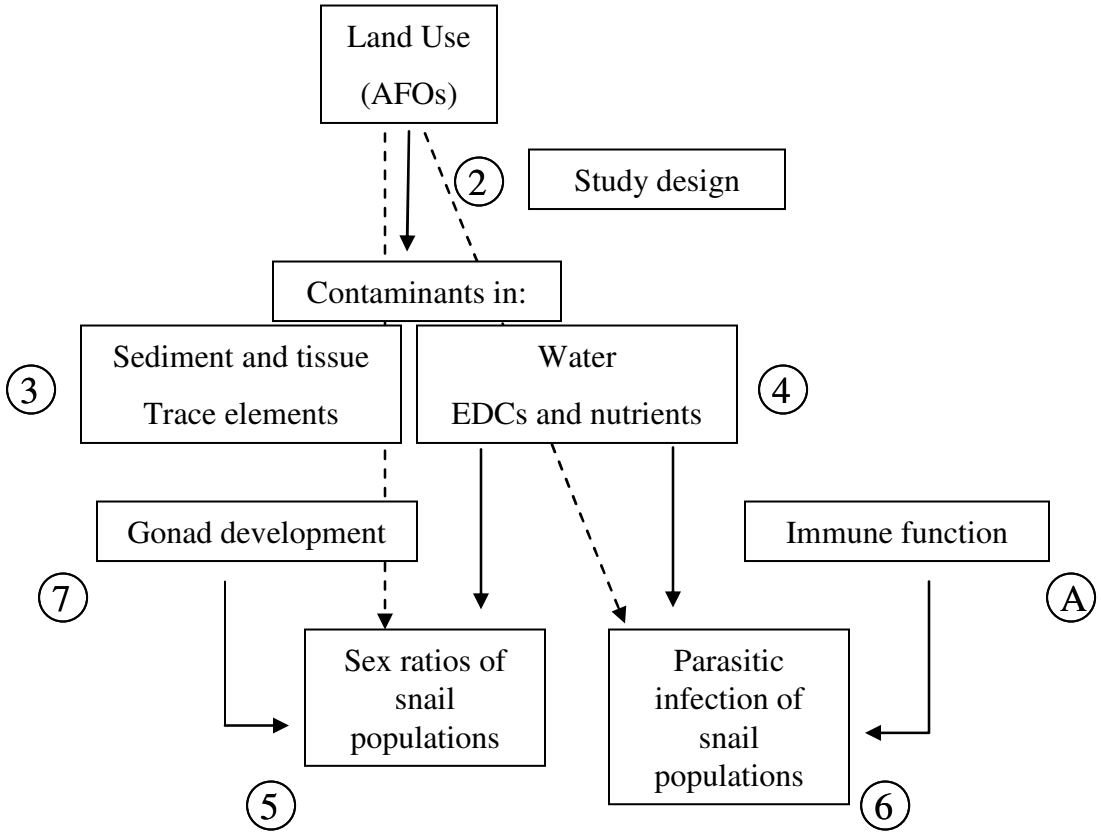


Figure 1- 2. Conceptual model of the individual objectives of the dissertation and corresponding chapters or appendix.



CHAPTER 2

Study design

The study design for the majority of research in this dissertation was based on the need to quantify the effects of landscape characteristics on site-specific measurements in individual streams within the Shenandoah River watershed (Virginia, USA). The U.S. Geological Survey has established watershed boundaries for streams and rivers within larger drainage basins in a nested, hierarchical system of hydrologic units (USDA National Resources Conservation Service, 2007). Each hydrologic unit is identified by a code, and both the digits and unit levels increase as the size of the delineated watershed decreases. The smallest delineated units are known as "subwatersheds", 12-digit, 6th level Hydrologic Unit Code (HUC 6), and range from 40-160 km^2 in size. Within the Shenandoah River watershed, there are 78 HUC 6 subwatersheds. Study sites were located near the outlets of these subwatersheds so that upstream land uses could be quantified.

Initial study site selection was performed using a geographic information system (ArcGis 9.3, ESRI, Redlands, CA). Flowlines for the entire Shenandoah River watershed were obtained from the National Hydrography Dataset (http://www.nhd.com) and the 78 HUC 6 subwatersheds were obtained from the U.S. Department of Agriculture National Resources Conservation Service (http://datagateway.nrcs.usda.gov/). Subwatersheds that did not contain portions of the Shenandoah River or drain land areas within one of the three cities in the region (Staunton, Waynesboro, or Harrisonburg) were considered eligible for sampling (45 eligible subwatersheds). Sites were initially selected to represent different levels of influence from animal feeding operations (AFOs) that use land-application as a method of manure disposal (poultry and dairy operations) and wastewater treatment plants (WWTPs) since effluent can contain similar contaminants as manure (endocrine-disrupting compounds, fecal bacteria, trace elements, pharmaceuticals). Locations of poultry, dairy, and beef AFOs were obtained from the Virginia Department of Environmental Quality (VADEQ) and the Virginia Department of Conservation and Recreation (VADCR) and locations of WWTP effluent discharges were obtained from VADEQ. The numbers of each type of AFO and WWTPs were quantified in each eligible subwatershed. Quartiles were used to assign categories to each subwatershed for each type of AFO: low (<25th percentile), moderate (25th-75th percentile) or high (>75th percentile). A factorial-type design was used to select sites with high or moderate poultry, combined with either high or low/moderate dairy, further separated by either the presence or absence of WWTP discharges. Twenty subwatersheds within these eight possible categories were selected. Five additional subwatersheds with low poultry, low dairy and no permanent WWTP discharges were selected as reference sites. Of the 25 selected subwatersheds, four drained to larger tributaries downstream of other HUC 6 subwatersheds, and the design was adjusted so that an

upstream (US) sampling site was included in the primary subwatershed in addition to the downstream (DS) site in the receiving subwatershed. Thus, 25 sites in 21 tributaries were initially selected for sampling (Figure 2-1, Table 2-1). Eleven sampling sites could not be located near the outlet of the delineated HUC 6 subwatershed due to limited tributary access. For these 11 tributaries, the watershed area was recalculated using Digital Elevation Model (DEM) data (30 m resolution). The new delineation was used to quantify upstream land use variables.

Although the initial sampling site selection was based on categorical methods, a regression-based design was desired in order to capture the effects of gradients in numbers of AFOs and WWTPs and to accommodate the co-occurrence or different types of AFOs and their relationships with the larger landscape. For each sampling site, the number of each type of AFO located upstream was converted to a density (number/1000 acres). Beef cattle operations were not included during study site selection, but densities were included in the final study design. For this study, the 278 farms that maintain grazing beef cattle in the Shenandoah River watershed (VADCR, 2010) were included as beef AFOs. In addition to densities of individual types of AFOs, the total density of upstream AFOs was also calculated. For each watershed with a WWTP discharge, the WWTP permit information was used to calculate the total permitted effluent discharge (in millions of gallons per day; MGD) upstream of sampling sites. In a complex landscape, AFOs and WWTPs do not exist in isolation. Therefore, land cover data for the Shenandoah River watershed (30 m resolution) were obtained from the 2001 National Land Cover Database (NLCD; Homer et al., 2007). The 2001 NLCD data are derived from Landsat 5 and 7 imagery. The database includes 29 land cover class descriptions, 13 of which pertain to the Shenandoah River watershed. Reclassification was used to combine the four types of developed land into one "developed" class, three forest types into one "forest" class, and two types of wetlands into one "wetlands" class. The resulting ArcGis layer had seven land use classifications: open water, wetland, barren land, forest, developed, pasture/hay, and cultivated crops. The area within each study watershed devoted to each land use classification was tabulated and converted to a percentage of total watershed area. The percentages of open water, barren land, and wetlands were negligible (< 1%) in each watershed of interest. Therefore, land use categories used for further analyses included the percentage of forest, pasture/hay, cropland, and developed land upstream of each sampling site (watershed-scale). For each study site, a total of nine watershed-scale land use variables were quantified and investigated as possibly predictive of in-stream contaminant concentrations and snail population characteristics (Table 2-1).

Observational scale has been identified as an important factor in the analysis of relationships between landscape data and contaminant concentrations (Hollister et al., 2008). Therefore, the percentages of forest, pasture/hay, cropland and developed land were calculated at two additional spatial scales: within a 300-m riparian zone along the entire length of the tributary(ies) upstream of each sampling site (riparian-scale), and within the 300-m riparian zone extending only 1000 m upstream of the sampling site (local-scale). Riparian- and watershed-scale land cover percentages were highly correlated, and because of this similarity, riparian-scale variables were not included in data analysis. Local-scale land cover percentages were not as strongly correlated with watershed-scale land use percentages and were included in data analysis. However, local-scale land cover was not predictive of in-stream contaminant concentrations or snail population characteristics and is generally not discussed further in this dissertation.

Initial sampling of sediment and mollusks for analysis of trace elements was conducted in 2007 at the 25 selected sampling sites. At this time, it was determined that 19 sites supported populations of *Leptoxis carinata*. These sites were retained for studies of aqueous contaminants and snail population characteristics beginning in 2008. Between the initial sampling period in spring 2008 and the second sampling period for aqueous contaminants in summer 2008, a barbed wire fence was erected across one stream at the sampling location (Christians Creek). This fence inhibited access to the riffle for sampling and the cattle contained by the fence trampled the riffle, so this site was subsequently dropped from the study. During the second snail population sampling period in fall 2008 it was determined that three sampling sites had unstable snail populations due to substrate instability (Smith Creek US), cattle trampling (Mill Creek; MCSF), and fluctuating water levels (Meadow Run). Sampling at these sites continued for aqueous contaminants, but snail population sampling was discontinued. Therefore, 25 sites were sampled for trace element in sediment and biota (Chapter 3), 18 sites were sampled for aqueous contaminants (Chapter 4), and 15 sites were sampled to assess snail population characteristics (Chapters 5 and 6). Two of the 15 sites were sampled further to describe gonad development in *L. carinata* (Chapter 7). This information is summarized in Table 2-1 and illustrated in Figure 2-1.

Literature cited

Hollister, J.W., August, P.V, & Paul, J.F. (2008). Effects of spatial extent on landscape structure and sediment metal concentration relationships in small estuarine systems of the United States' Mid-Atlantic Coast. *Landscape Ecology, 23*, 91-106.

Homer, C., Dewitz, J., Fry, J., Coan, M., Hossain, N., Larson, C., et al., (2001). Completion of the 2001 National Land Cover Database for the conterminous United States. *Photogrammetric Engineering and Remote Sensing, 73*, 337-341.

USDA National Resources Conservation Service (2007). Watersheds, hydrologic units, hydrologic unit codes, watershed approach, and rapid watershed assessments. http://www.nrcs.usda.gov/programs/rwa/watershed_hu_huc_WatershedApproach_defined_6-18-07.pdf

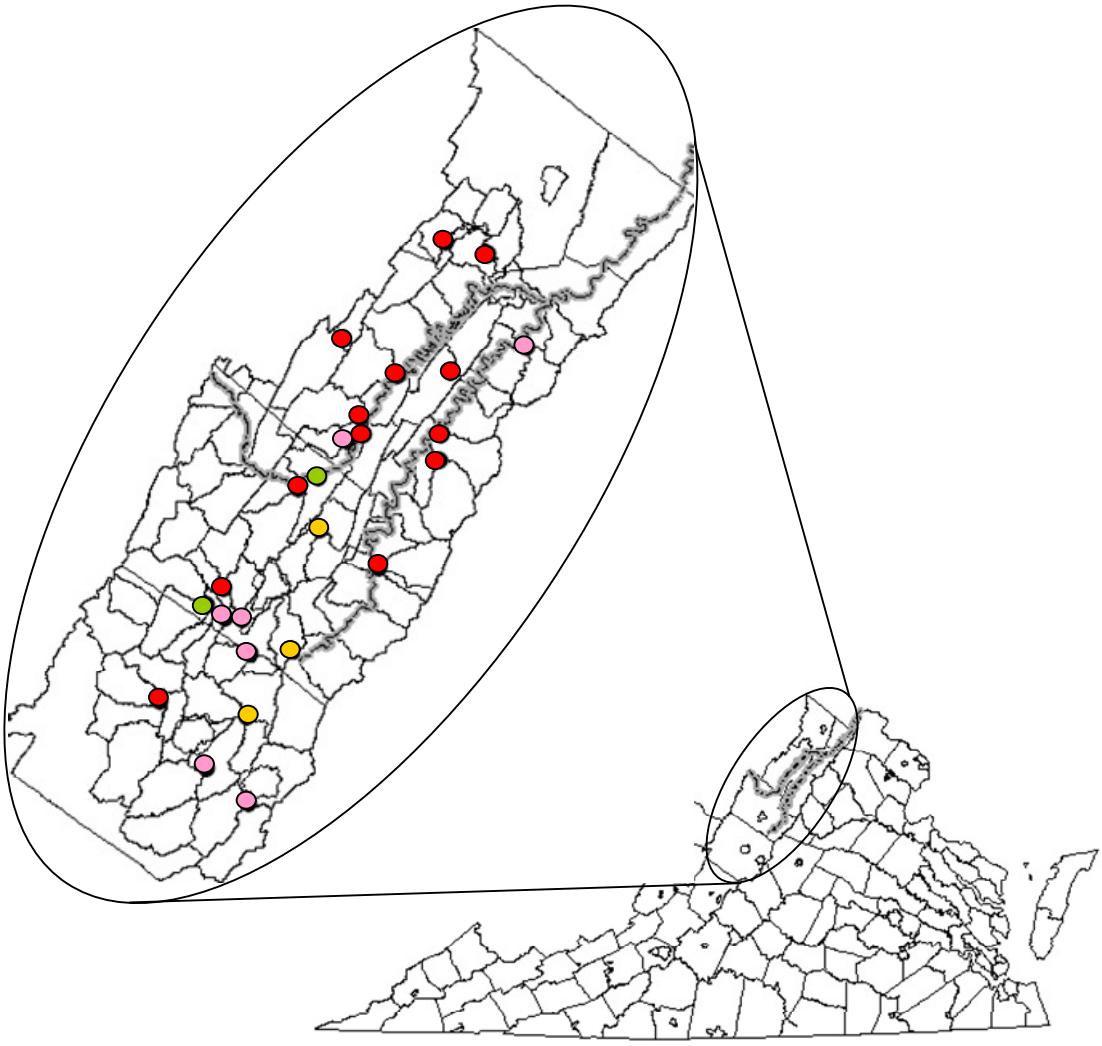
VADCR (2010). Animal feeding operations database. http://webdat.dcr.virginia.gov/cfprog/dswc/afoprm.cfm.

Table 2- 1. Characteristics of each sampling site. These characteristics include: watershed area, watershed percentages of land cover classes, watershed densities (#/1000 Acres) of animal feeding operations, and total upstream permitted wastewater treatment plant (WWTP) effluent discharge in millions of gallons per day (MGD). Chapters in which each study site was utilized are indicated. Underlined sites were considered reference sites. Tributaries with upstream (US) and downstream (DS) sampling sites are indicated, as well as the total number of subwatersheds located upstream of DS sites.

Site Code	Tributary Name	Area km^2	Forest %	Past./ Hay %	Crop %	Devel %	Poultry Dens.	Beef Dens.	Dairy Dens.	Total AFOs Dens.	WWTP MGD	Chapter #
BACK	Back Cr.	108	85.2	5.2	1.3	7.9	0	0.150	0.038	0.188	0	3
BRIR	Briery Br.	128	82.5	13.2	1.9	2.4	0.601	0.316	0.348	1.265	0	3, 4, 5, 6, 7
CEHW	Cedar Cr. (US)	120	92.9	5.0	0.2	1.9	0	0	0	0	0	3, 4, 5, 6
CEDS	Cedar Cr. (DS; 5)	335	79.7	15.7	0.5	4.1	0	0.012	0	0.012	0[1]	3, 4, 5, 6
CHRIS	Christians Cr.	138	32.4	50.7	5.1	11.7	0.146	0.468	0.293	0.907	0.25	3
COOK	Cooks Cr.	58	10.7	57.9	12.6	18.6	2.29	0.765	5.77	8.830	0	3
GOON	Gooney R.	71	80.8	14.7	0.3	3.9	0	0	0	0	0	3
HAWK	Hawksbill (US)	124	58.8	29.2	2.8	9.1	0.980	0.425	0	1.405	0.2	3, 4, 5, 6
HADS	Hawksbill (DS; 2)	175	55.8	29.1	2.5	12.4	0.717	0.324	0	1.041	1.8	3, 4, 5, 6
HOLM	Holmans Cr.	48	28.8	63.0	3.5	4.1	0.676	0.253	0	0.929	0.008	3
JENN	Jennings Br.	92	73.6	17.0	1.2	8.2	0.132	0.220	0.044	0.395	0	3, 4, 5, 6
LGCR	Long Glade Cr.	48	18.5	70.7	4.6	6.3	0.761	0.507	0.845	2.113	0	3
LINV	Linville Cr.	120	21.8	63.7	4.2	9.8	1.28	0.473	0.709	2.464	0.03	3, 4, 5, 6
LOMR	Long Meadow R.	40	19.3	69.1	5.1	6.5	2.44	1.01	0.509	3.966	0	3, 4, 5, 6, 7
MCNF	Mill Cr.	121	52.3	39.1	2.2	6.3	0.605	0.235	0.134	0.974	0	3, 4, 5, 6
MCSF	Mill Cr.	37	15.8	65.8	7.1	10.7	0.540	0.540	0.757	1.837	0	3,4
MEAD	Meadow R.	50	15.1	61.3	4.3	19.2	0.082	0.327	0.491	0.900	0.015	3,4
MUDD	Muddy Cr.	81	37.1	47.9	7.3	7.7	1.29	0.845	2.08	4.227	0.005	3, 4, 5, 6
NAAU	Naked Cr.	59	28.8	58.8	3.1	9.2	0.613	0.273	0.341	1.226	0	3
NAPA	Naked Cr.	114	84.5	9.9	0.6	4.9	0.250	0	0	0.250	0	3, 4, 5, 6
PASS	Passage Cr.	105	90.3	6.0	0.2	3.4	0.039	0	0	0.039	0[1]	3, 4, 5, 6
SMHW	Smith Cr. (US)	55	59.5	34.8	2.7	2.9	0.512	0.438	0.365	1.315	0.03	3,4
SMDS	Smith Cr. (DS; 4)	265	48.6	40.2	3.4	7.7	0.504	0.443	0.245	1.192	0.091	3, 4, 5, 6
STHW	Stony Cr. (US)	140	84.9	8.0	0.3	6.5	0.174	0	0	0.174	0.639	3, 4, 5, 6
STDS	Stony Cr. (DS; 3)	294	67.5	23.3	2.0	7.0	0.221	0.138	0.041	0.400	0.814	3, 4, 5, 6

[1]No permanent municipal WWTP discharge is present, but WWTPs for camps operate during the summer only

Figure 2- 1. Locations of sampling sites in streams within the Shenandoah River watershed. The enlargement shows the watershed in the context of counties in the state of Virginia. In the Shenandoah River watershed, the two forks of the river are indicated by the thin grey line and the 12-digit hydrologic unit code subwatersheds are outlined. Sites in green were utilized in Chapters 3-7, sites in red were utilized in Chapters 3-6, sites in yellow were utilized in Chapters 3 and 4, and sites in pink were utilized in Chapter 3 only.



CHAPTER 3

Using watershed characteristics, sediment, and tissue of resident mollusks to identify potential sources of trace elements to streams in a complex agricultural landscape

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Abstract

Trace elements used in animal feed additives can be introduced to aquatic environments through application of manures from animal feeding operations to agricultural land as fertilizer. The use of poultry feed additives containing arsenic (As) is of particular concern in the Shenandoah River watershed (Virginia, USA), an agricultural landscape with a high density of poultry operations. This study investigated the relationship between watershed characteristics of Shenandoah River tributaries and trace element concentrations in streambed sediment and tissue of resident mollusks, including: Asian clams (*Corbicula fluminea*), which are commonly used biomonitors, and pleurocerid snails (*Leptoxis carinata*), which are generally understudied. Results failed to support the primary hypothesis of a predictive relationship between watershed densities of poultry operations and As concentrations in sediment and mollusk tissue. However, there were statistical relationships between land use in tributary watersheds and other trace elements in sediment (Cu, Mn, Pb, Zn) and tissue (Cd, Hg, Pb). Principal components analysis of the sediment data suggested a possible geologic source of As at some sites. Factors affecting As bioavailability in the region require further research, since tissue concentrations of As were elevated compared to other studies. Tissue concentrations of As were significantly higher (paired t-test, $t=2.26$, $p=0.04$) in snails (2.5-10 µg/g dry weight) than in clams (1.2-6.0 µg/g dry weight), but clams accumulated higher concentrations of other trace elements (Cd, Cr, Hg, Pb, Se). Differential bioaccumulation of trace elements by snails and clams is likely due to their different feeding strategies. Snails may be useful biomonitors of environmental As, but appear to be less suitable than clams for identification of landscape sources of other trace elements.

Introduction

Land-application of animal waste as fertilizer is a potential mechanism for introduction of trace elements to streams in agricultural landscapes. Trace elements in animal waste, such as Cu, Mn, Se, and Zn, originate from growth promoting feed additives which are excreted by the animals (Brock et al., 2006; Gupta and Gardner, 2005). Arsenic is of particular concern in poultry litter because of the addition of roxarsone (3-nitro-4-hydroxyphenylarsonic acid) to some poultry feeds as a growth promoter. This compound is excreted by the birds, is highly soluble in poultry litter (71%), and can be transformed to more toxic inorganic forms of As (Brown et al., 2005; Jackson et al., 2003).

In the Shenandoah River watershed (Virginia, USA), over 700 poultry operations generate more than 450,000 tons of litter annually (Pelletier and Kenyon, 2000). The poultry litter, along with manure from over 400 dairy operations, is land-applied to cropland and pastures or hayfields, which comprise approximately 39% of the 7,600 km^2 total watershed area (VADEQ, 2006). In 2005, sampling for Virginia's fish tissue monitoring program included the Shenandoah River and select tributaries, and As concentrations were above state screening values (0.072 µg/g wet wt.) in 25% of fish sampled from these areas (VADEQ, 2005). These results, coupled with recurrent seasonal fish kills in the Shenandoah River, have increased concerns about land-application of poultry litter in the watershed and potential introduction of trace elements, particularly As, to aquatic environments (Ripley et al., 2008).

Contemporary agricultural practices are not the only potential source of As and other trace elements to aquatic environments in the Shenandoah River watershed. Historically, commercial apple orchards were present in all counties in the watershed, and lead arsenate many have been applied in many areas given the extensive usage of this pesticide between 1890 and 1950 (Schooley et al., 2008). The geology of the Shenandoah River watershed is primarily carbonate and clastic rocks; Devonian sedimentary rocks, including black shale, comprise a portion of the clastic rocks (Yager et al., 2008). Due to deposition under anaerobic conditions, black shale can contain sulfide minerals enriched in trace elements which may be released during oxidative weathering (Ogendi et al., 2007; Tuttle et al., 2009). Atmospheric deposition is another potential source of trace elements to aquatic environments in the Shenandoah River watershed (Kolker et al., 2008; Tuncel et al., 1985). Although the watershed is only 19% developed land (urban and residential), there are numerous municipal wastewater treatment plants (WWTPs), and effluents may introduce trace elements to streams (Gagnon and Saulnier, 2003). Studies of the effects of these sources on trace element concentrations in streams in the Shenandoah River watershed have yet to be conducted over a broad spatial scale.

Different environmental matrices have distinct advantages and disadvantages for landscape-scale assessment of sources of trace elements to aquatic environments. Analysis of streambed sediment has been used to identify watershed sources of trace elements (Comelo et al., 1996; N'guessan et al., 2009), but disadvantages include variable residence time of streambed sediment and effects of sediment properties on trace element partitioning (N'guessan et al., 2009). Tissues of resident organisms accumulate trace elements over time and provide an indication of bioavailability. However, selective uptake and metabolism may complicate the use of tissue

concentrations in source identification studies. The use of fish tissue is further complicated by fishes' mobility (Short et al., 2008). Freshwater mollusks are relatively sessile, and their entirely aquatic life cycle and relatively high tissue biomass increase their utility as biomonitors of inputs of trace elements to aquatic environments. The invasive Asian clam (*Corbicula fluminea*) is commonly used for biological monitoring of trace elements (Angelo et al., 2007; Leland and Scudder, 1990; Luoma et al., 1990; Peltier et al., 2008; Shoults-Wilson et al., 2009). Unlike filter-feeding Asian clams, freshwater snails in the family Pleuroceridae primarily feed on periphyton (Brown et al., 2008), and may preferentially accumulate trace elements bioconcentrated by algae. However, concentrations of trace elements in pleurocerid snails have not been previously used for landscape-scale source evaluation.

The purpose of this study was to examine the relationship between watershed land use and trace element concentrations in stream sediment and biota within the larger Shenandoah River watershed, which is a complex landscape with multiple potential sources of trace elements. Increasing concentrations of As in sediment and biota with increasing watershed densities of poultry operations and a greater proportion of watershed area occupied by agricultural land were expected because of the use of As in poultry feed additives and the intensity of manure application. Due to the complexity of the landscape, concentrations of trace and major elements in sediment were used to evaluate other potential sources of As and the overall influence of land use on element concentrations in the Shenandoah River watershed. Concentrations of As and other bioaccumulative trace elements monitored by the state of Virginia were measured in two resident mollusks, the Asian clam (*Corbicula fluminea*) and a pleurocerid snail (*Leptoxis carinata).* Tissue concentrations were utilized to test expected relationships between As and agricultural land use, to compare relative bioaccumulation between two mollusks with different feeding strategies, and to evaluate their utility as biomonitors of trace elements in landscape-scale studies of potential sources.

Methods

Selection and characterization of study sites

Sampling sites in Shenandoah River tributaries were selected using a geographic information system (ArcGis 9.3, ESRI, Redlands, CA). Sites were initially selected to represent a gradient of influence from animal feeding operations (AFOs) that use land application as a method of manure disposal (poultry and dairy) and WWTP discharges. In Virginia, AFOs are defined as facilities that confine and feed animals for at least 45 days in a 12-month period and preclude growth of vegetation. Locations of AFOs and WWTPs were obtained from Virginia state

agencies, and their numbers were quantified within delineated 12-digit, 6th level Hydrologic Unit Code (HUC 6) subwatersheds (40-160 km^2 in size). Twenty-five sites, located near outlets of the delineated HUC 6 subwatersheds, were selected. Four of the tributaries drained multiple subwatersheds, and an upstream (US) sampling site was located in the primary subwatershed as well as a downstream (DS) site draining multiple subwatersheds. Thus, 25 sites in 21 tributaries were selected for sampling (Figure 1; Table 1). Eleven sampling sites could not be located near the outlet of the delineated HUC 6 subwatershed due to limited tributary access. For these 11 tributaries, the watershed area was recalculated using Digital Elevation Model (DEM) data (30 m resolution). The new delineation was used to quantify upstream land use variables. For each sampling site, the number of each type of AFO located upstream was converted to a density (number/1000 acres) and the total density of upstream AFOs (all types) was calculated. For each watershed with a WWTP discharge, the WWTP permit information was used to calculate the total permitted effluent flow (in millions of gallons per day; MGD).

In addition to watershed densities of AFOs and WWTP total permitted effluent flow, land cover percentages were also quantified upstream of each sampling site. Land cover data (30 m resolution) were obtained from the 2001 National Land Cover Database (Homer et al., 2007), and reclassification and areal tabulation were used to quantify the watershed percentages of forest, developed land, pasture/hay, and cultivated crops upstream of each sampling site. Percentages of all other land use types (i.e. open water, barren land, etc.) were negligible (<1%) in each watershed of interest. For each study site, a total of nine watershed-scale land use variables were quantified and investigated as possibly predictive of trace element concentrations in sediment and tissues (Table 3-1).

Maps of regional geology were obtained from the USGS National Geologic Map Database catalog (scale 1:250,000; Dicken et al., 2005). Rock formations are mapped as polygons with the following attributes: unit age, "rock type 1" (Type 1), and "rock type 2" (Type 2). Type 1 indicates the dominant lithology (> 50% of the unit) and Type 2 indicates second most dominant lithology, using a standardized data dictionary (Dicken et al., 2005). Definitions are based on the draft Geologic Map Classification version 6.1 (http://ngmdb.usgs.gov/www-nadm/dmdt/pdf/lithclass61.pdf). The maps were used to identify general geologic characteristics of the tributary watersheds; watersheds dominated by limestone and/or dolomite formations, and watersheds with black shale present as either Type 1 or Type 2 (Table 3-2). These geologic characteristics were used to aid interpretation of trace element concentrations in sediment.

Field sampling

Periods immediately after initial spring high flow events were targeted for sample collection. It was hypothesized that spring high flow would represent maximum potential input of trace elements from the surrounding landscape due to intensive springtime application of animal wastes and increased runoff. In 2007, significant spring rain events and associated high flows occurred during early and mid-March, and sampling was conducted from 24 March to 23 April. In 2008, significant spring rain events did not occur until early and mid-May, and sampling took place from 14 May to 24 May. Discharge data from a gauge at Mount Jackson, VA, located on the North Fork of the Shenandoah River, illustrate these trends (http://nwis.waterdata.usgs.gov/va/nwis/dv?cb_00060=on&format=gif_default&begin_date=2007-01-01&end_date=2008-06-30&site_no=01633000&referred_module=sw).

Sediment was collected from depositional areas of the 25 tributary sampling sites in 2007. Ten individual surficial (5-10 cm) sediment samples were collected with a stainless steel scoop fitted with a moveable cap to minimize the loss of fine-grained material. The 10 individual samples were composited and homogenized in a stainless steel pot after removal of large debris (leaves and sticks). A subsample of the composite was placed in a pre-cleaned glass jar, held on ice, and shipped overnight to an analytical laboratory.

Asian clams (*Corbicula fluminea*) were collected from 21 of the 25 tributaries in 2007 (no clams at BACK, BRIR, NAPA, STHW). Clams were collected from gravelly substrate in riffle/run areas at each study site using a trowel or D-frame net. Clams were placed in a plastic sorting pan and 15 - 20 similarly sized adults (>8 mm) were placed in site water in pre-cleaned glass jars. Clams were held in chilled site water for 24 h to clear their gut contents and were then blotted dry and immediately frozen.

Pleurocerid snails (*Leptoxis carinata*) were collected from 19 of the 25 tributaries in 2008 as part of a larger study of snail population characteristics (no snails at BACK, GOON, COOK, HOLM, LGCR, NAAU). *Leptoxis carinata* have a two-year life history (Aldridge, 1982) and adults (2-year olds) are easily distinguished from 1-year old juveniles in the spring due to differences in shell lengths. Approximately 50 adult snails were hand-collected from cobble substrate in riffle/run areas (same as clams) and placed in site water in pre-cleaned glass jars. Snails were held in chilled site water for 24 h to clear their gut contents, and were then blotted dry and immediately frozen.

Prior to chemical analysis, clams and snails were briefly thawed, soft tissue was removed from the shells using stainless steel forceps, and the tissue was freeze-dried and homogenized to form one composite sample. Clam shell lengths were measured with a digital caliper (nearest 0.01 mm) to confirm similarity in sizes. Snails were not measured due to collection of only the 2-year old cohort.

Chemical analysis

Sediment samples were analyzed by Hampton-Clarke/Veritech (HC-V) Laboratories, Inc. (Fairfield, NJ). Sediment was acid digested following USEPA SW-846 standard method 3050B for analysis of 22 of the TAL elements (Al, Sb, As, Ba, Be, Ca, Cd, Cr, Co, Cu, Fe, Pb, Mn, Mg, Ni, K, Na, Se, Ag, Tl, V, Zn). These elements were quantified in the digests using inductively coupled plasma atomic emission spectrometry (Perkin Elmer, Waltham, MA) following USEPA SW-846 standard method 6010B. Sediment was digested and analyzed for total Hg following USEPA SW-846 standard method 7471A with quantification by cold vapor atomic absorption spectrophotometry (Perkin Elmer FIMS100, Waltham, MA). Practical quantitation limits (reporting limits) for the elements Hg, Be, Sb, Cd, Se, Ag, and Tl were 0.05, 0.6, 2.0, 0.6, 1.8, 1.5, and 1.2 μg/g dry weight, respectively. The reporting limits (RLs) for Na and K were 500 μg/g dry weight and RLs for all other elements are listed in Table 2. All analyses followed HC-V's standard QA/QC protocols. Analytical method blanks were analyzed with each digestion batch. For each sample batch, initial and continuing calibration verification was performed as well as analysis of interference check samples. Reproducibility was checked using a duplicate subsample and serial dilution of a sample digest; the acceptable relative percent difference (RPD) was $\leq$20% for duplicate subsamples and $\leq$10% for serial dilutions. Method recoveries were assessed for all analytes using laboratory control samples, recoveries ranged from 88% (Al) to 104% (Hg).

Clam and snail tissues were analyzed at the College of William and Mary (Williamsburg, VA). Freeze-dried tissue samples (0.5 g) were microwave digested in trace metal grade nitric acid following USEPA SW-846 standard method 3052. Solutions were diluted to a final volume of 100 ml. The elements As, Cd, Cr, Pb, and Se were analyzed using graphite furnace atomic absorption spectrophotometry (Perkin Elmer AAnalyst 700, Waltham, MA) using prescribed instrument, furnace, and matrix modifier parameters. Total Hg was determined using atomic fluorescence spectrophotometry (Brooks Rand, Seattle, WA) following thermal release after reduction with $SnCl_2$, purging, and amalgamation onto gold traps. The RLs for As, Cd, Cr, Hg, Pb, and Se were 0.05, 0.01, 0.05, 0.01, 0.1, and 0.5 μg/g dry weight, respectively. Analytical method blanks were analyzed with each digestion batch.

Instrument calibrations were determined from 4-6 point standard curves. Calibrations were checked for instrument drift using a standard solution (every 10 samples), and instrument response was recalibrated if deviations from expected values exceeded 10%. Reproducibility was checked using duplicate subsamples (every five samples). Method recoveries were assessed through analysis of a standard reference material (SRM; oyster tissue), concentrations of all elements were within 5-10% of certified values.

Data analysis

Concentrations of individual trace elements in sediment and tissue were only included in statistical analyses if they were above the RL at more than 60% of the sampling sites. For trace elements that met this criterion, a value equal to half of the RL was substituted for any concentrations below the RL. All statistical analyses were performed using SAS 9.2 (SAS Institute, Cary, NC), with a significance level of α=0.05.

Independent (land use) and dependent (concentrations) variables were examined for potential outliers using quartile ranges of the data. The quartile range (Q3-Q1) was multiplied by 1.5 and the resulting value was added to Q3 (75^{th} percentile) and subtracted from Q1 (25^{th} percentile) to determine critical values. Outliers were flagged and their effect on statistical analyses was tested. Only influential outliers were removed from the dataset (indicated in the results). Independent and dependent variables were tested for normality using the Shapiro-Wilk W test. Non-normal data were transformed to minimize deviations from normality for univariate statistics, or to obtain more symmetrical distributions for multivariate analyses. When necessary, $\log_{10}$ (concentration data) and square root (density data) transformations were used.

Principal components analysis (PCA) was used to examine relationships between correlated variables via singular value decomposition of the correlation matrix (centered and scaled data). The resulting orthagonal eigenvectors or principal components (PCs) represent weighted composites of the correlated data, and weights >0.20 were considered influential to the component. The proportion of variance explained by each PC (Scree plot) and eigenvalues (>1) were used to determine the number of PCs retained for further analysis. The value of the PC (score) for each observation (site) was calculated for application to other statistical analyses.

Pearson product moment correlation analysis was used to assess relationships between: individual land use variables, land use variables and individual element concentrations or PC scores (for correlated concentrations), concentrations of different trace elements in tissue, concentrations of trace elements in different mollusk tissue types, and concentrations of trace elements in sediment and tissue. Differences in mean concentrations between

tissue types were assessed using paired t-tests. Regression analysis and examination of scatterplots were used to assess relationships of watershed densities of poultry AFOs and percentages of pasture/hay and cropland with concentrations of As in sediment and tissue.

Partial least squares (PLS) regression was used to examine the covariance between correlated watershed-scale land use variables and correlated element concentrations in sediment through simultaneous singular value decomposition of predictor (X) and dependent variable (Y) matrices. The PLS method accommodates multicolinearity by forming new predictor variables (X score vector; t) as linear combinations of the old predictor variables, and uses these new t's as predictors of the dependent variables (Y score vector, u) (Eriksson et al., 1995). The PLS method accommodates a low number of observations relative to predictor variables. Each dimension (factor) of the PLS model incorporates an X loading vector (p), which illustrates how the Xs combine to form t, an X weight vector (w), and a Y weight vector (c). The vectors w and c are used to interpret which X variables are important for predicting the Y variables (Eriksson et al., 1995), variables with weights >0.20 were considered influential. For PLS analysis all watershed-scale variables, excluding percentage of forest and total density of AFOs, were included as independent (X) variables and sediment concentrations of 12 elements were included as dependent variables (Y). All data were centered and scaled for the analysis. Cross-validation, using 10-fold exclusion of random subsets, was used to examine the predictive capability of models incorporating different numbers of factors.

Principal components regression (PCR) was used to examine relationships between correlated watershed-scale land use variables and individual trace element concentrations in tissue (uncorrelated). A PCA was conducted on all watershed-scale land use variables (excluding percentage of forest and total density of AFOs) as described above, and resulting PC scores were used as independent variables in a regression analysis.

Results

Sediment

All element concentrations in sediment are reported on a dry weight basis. Six potentially toxic trace elements (Hg, Sb, Cd, Se, Ag, Tl) and the elements Na and K were below RLs (see methods section for values) in sediment from all 25 tributaries. Sediment concentrations of 12 elements were above RLs in the majority of tributaries sampled (Table 3-2), and were included in further statistical analyses. Nickel was above the RL at ten sites (Table 2), and Be was only above the RL at NAAU, SMHW and STHW (1.1, 0.94, and 0.89 µg/g,

respectively). Vanadium was quantifiable at nine sampling sites, including the five sites (BACK, GOON, HAWK, HADS, NAPA) in streams draining the westward facing slopes of Shenandoah National Park (Table 3-2).

Sediment concentrations of Cr, Cu, Pb, and Zn did not approach screening concentrations for freshwater sediment published in the National Oceanic and Atmospheric Administration (NOAA) Screening Quick Reference Tables (Table 3-2; Buchman, 1999). These values are guidelines for preliminary assessment of the potential for adverse ecotoxicological effects. Threshold effects levels (TEL) and probable effects levels (PEL) were derived from a compilation of benthic community metrics and results of toxicity tests; the TEL represents a concentration below which adverse effects are expected to occur only rarely and the PEL is a concentration above which adverse effects are frequently expected (Buchman, 1999). Concentrations of Ni were above the TEL but well below the PEL at two of the 10 tributary sites where this element was quantifiable (Table 3-2). At more than half of the sites where As was quantifiable, sediment concentrations exceeded the TEL, but were well below the PEL (Table 3-2).

Concentrations of several of the 12 elements included in statistical analysis were correlated and PCA was conducted to reduce the dimensionality of the data and investigate relationships between different elements. The final correlation matrix and PCA are derived from 24 sites because outlying concentrations of Al, Fe, and Ba were measured at one site (HAWK; Table 3-2), and these concentrations influenced the results of the PCA. This was the only site located directly upstream of a low-head dam, which may have influenced the composition of the sample through greater accumulation of fine sediment and organic matter or possibly through activities at the adjacent former mill. The first three PCs from the final PCA had eigenvalues greater than one and cumulatively explained 77.2% of the variance in the data set. These three PCs were retained for further analysis (Table 3-3).

For PC I, eigenvector weights were positive and greater than 0.20 for all 12 elements (Table 3-3). Increasing PC I scores for individual sites reflect overall increasing element concentrations (Figure 3-2). For PC II, eigenvector weights were positive (>0.23) for Al, Cu, Ba, Ca, and Mg and negative (<-0.33) for As, Cr, Fe and Co (Table 3-3). The trace elements Pb, Zn, and Mn were not represented by PC II, and the five sites with positive PC I scores and PC II scores $\leq$0.5 had elevated concentrations of one or more of these elements (particularly Pb) and relatively high concentrations of elements in both PC II groups (positive and negative weights) (Figure 3-2, Table 3-2). For PC III, eigenvector weights were positive (>0.24) for Co, Cu, Zn, and Ba and negative (<-0.21) for Pb, Cr, Ca, and Mg (Table 3-3). This component accounted for a minor proportion of variance in the data, and most sites had intermediate PC III scores. Four sites had scores >1 (BRIR, SMDS, SMHW, STHW) and four sites had scores

<-1 (HOLM, NAAU, CHRIS, MCNF), reflecting elevated concentrations of one or two PC III elements relative to other elements (Table 3-2).

Comparisons of sampling sites with geologic maps illustrated that all sites with positive scores for PC II (except BRIR) have watersheds dominated by limestone or dolomite (Table 3-2). Where limestone and/or dolomite are not predominant, the bedrock is either shale and sandstone, or for watersheds draining Shenandoah National Park: meta-basalt, quartzite, granitic gneiss, and granulite. Sites with negative scores for PC II are either located in tributaries that drain Shenandoah National Park (negative PC I scores) or have black shale listed as rock Type 1 in at least part of the watershed (Figure 3-2, Table 3-2). The sites NAAU, LGCR, and SMHW are an exception; these sites have watersheds dominated by limestone or dolomite, but have black shale listed as rock Type 2 within the watershed (Table 3-2). Sediments from these three sites, along with CEDS, had measurable concentrations of V, and were all relatively enriched in element concentrations, favoring As, Cr, Fe and Co (Figure 3-2).

Scores for PC I and II were significantly correlated (Pearson, $p<0.05$) to some of the watershed-scale land use variables (Table 3-4). One tributary (COOK) had outlying values for watershed percentage of crops and density of dairies; these values were highly influential to the correlations with PC scores and were not included in the final analysis. Scores for PC I were negatively correlated with watershed percentage of forest and positively correlated with all other land use variables except watershed density of poultry AFOs and total permitted WWTP flow. Scores for PC II were significantly correlated with only percentages of forested land (negative), pasture/hay, and crops in tributary watersheds, with lower correlation coefficients than PC I (Table 3-4). Scores for PC III were not significantly correlated with any of the land use variables.

Watershed percentage of forest was negatively correlated with all other land use variables, including individual and total densities of AFOs ($r\leq-0.64$, $p\leq0.0006$), and explained 37% of the variance in PC I scores and 18% of the variance in PC II scores. Multicolinearity between the watershed-scale land use variables confounded the use of multiple regression to investigate effects of a combination of the positively correlated land uses on PC scores. The strength of correlation was $r\geq0.66$ ($p\leq0.0004$) between watershed percentage of pasture/hay, crops, and the densities of each type of AFO. The percentage of developed land was less strongly correlated with the other variables (r: 0.3–0.6, p: 0.0006–0.1). Only total permitted WWTP discharge was uncorrelated with any other land use variable. Therefore, PLS regression was used to assess the effects of a combination of all watershed-scale land use variables, excluding percentage of forest and total density of AFOs, on sediment concentrations of the 12

elements included in the PCA. The two previously mentioned sites with multivariate outliers (HAWK and COOK) were not included in the PLS regression.

Inclusion of all land use variables in a three-factor PLS regression model did not result in a strong predictive relationship between land use and sediment trace element concentrations. The model explained 88% of the variance of the predictor (land use) variables (Table 3-5a), but the cumulative proportion of variance in element concentrations explained by the model was only 26% (Table 3-5b). Three factors were retained in the model in order to include permitted WWTP discharge as a potential predictor, as this variable was the primary component of the third factor, even though cross-validation analysis indicated that the predictive capability of the model was not substantially improved beyond inclusion of only the first factor. For the three-factor model, the variable importance in the projection (VIP) was >0.8 for all land use variables. All land use variables, with the exception of WWTP discharge, contributed to the first factor, and predictive weights were greatest for the watershed percentages of pasture/hay and crops (Table 3-5a). The elements with the greatest weights for this factor were Al, Ca, Pb, Mn, Mg, Ba, and Cu (Table 3-5b). Factor two distinguished element concentrations that were better predicted by the density of poultry and dairy AFOs (negative; Cu), relative to the percentage of developed land, percentage of pasture/hay, and total permitted WWTP discharge (Al, Mn, Mg, Ca, Co, Cr, Fe), but this factor only accounted for 3.6% of the total variance (Tables 3-5a and b). The third factor further distinguished elements better predicted by the percentage of pasture/hay (positive; Ca, Mn) than total permitted WWTP discharge and the density of dairies (negative; Zn and Cu) (Tables 5a and b). Negative weights were greatest for WWTP discharge and Zn concentrations. Although the third factor only accounted for 4.2% of the total variance, scatterplot analysis indicated that its inclusion improved the ability of the model to predict Zn concentrations. Examination of factor weights and scatterplots of predicted values indicated that the model was a poor predictor of sediment concentrations of As, Cr, Co, and Fe. Analysis of univariate scatterplots confirmed that there was no mathematical relationship between As concentrations in sediment and land use variables, including the density of poultry AFOs.

Tissue

All tissue concentrations of trace elements are reported on a dry weight basis. Concentrations of As, Cd, Cr, Hg, Pb, and Se were above RLs in *C. fluminea* tissue at all sites where clams were collected (n=21). Clam tissue trace element concentrations were uncorrelated, except for Hg and Cd (r=0.72, p=0.0002). Therefore, correlations between concentrations of individual trace elements and individual land use variables were examined. Similar to

sediment data, outlying watershed percentage of crops and density of dairies at COOK were highly influential to these correlations and were not included in the final analysis.

Concentrations of As in clam tissue ranged from 1.2-6.0 μg/g (mean 3.5 μg/g; Figure 3-3). Clam tissue concentrations of As were uncorrelated with all land use variables ($p \geq 0.16$). Graphical analysis of the relationship between watershed density of poultry AFOs and concentrations of As in clam tissue showed higher concentrations at moderate densities. A third-order polynomial regression best described this relationship ($R^2=0.59$, $p=0.001$; Figure 4), but it is not likely a predictive model because of the sinuosity of the mathematical function.

Concentrations of Cr in clam tissue ranged from 1.0-4.1 μg/g (mean 2.1 μg/g; Figure 3-3). The highest Cr concentration (4.1 μg/g) was measured in clams collected from HADS, located downstream of a former tannery, which is a potential source of Cr (USEPA and VADEQ 2001). Clam tissue concentrations of Se ranged from 1.6-6.1 μg/g (mean 3.5 μg/g; Figure 3-3). Clam tissue concentrations of both Cr and Se were uncorrelated with all land use variables ($p \geq 0.14$).

Concentrations of Pb in clam tissue ranged from 0.60-3.2 μg/g (mean 1.5 μg/g; Figure 3). This range in concentrations excludes one outlying concentration, 8.1 μg/g, measured in clams collected from the site GOON. Clams collected from this site also had the second highest measured concentration of Cr (3.6 μg/g). Excluding GOON, clam tissue Pb concentrations were negatively correlated with watershed percentage of forest and positively correlated with percentages of pasture/hay, crops, and developed land (Table 3-6).

Concentrations of Cd in clam tissue ranged from 0.091-3.8 μg/g (mean 0.67 μg/g; Figure 3-3). Clam tissue Cd concentrations were positively correlated with watershed percentage of forest and negatively correlated with all other land use variables except WWTP discharge (Table 3-6). Concentrations of Hg in clam tissue ranged from 0.055-0.64 μg/g (mean 0.16 μg/g; Figure 3-3). Correlations between clam tissue Hg concentrations and land use variables were similar to those determined for Cd (Table 3-6).

Principal components regression (PCR) was used to examine the relationship between clam tissue concentrations of individual trace elements and the combination of all land use variables, excluding percentage of forest and total AFO density. Two PCs explained 78.9% of the variance in the land use data for sites where clams were collected. The PC I eigenvector weights were positive and greater than 0.30 for all land use variables except total permitted WWTP discharge (-0.05). The PC II eigenvector weights were 0.44 and 0.88 for watershed percentage of developed land and total permitted WWTP discharge, respectively and were <0.15 for all other land

use variables. Scores for PC II were not statistically related to tissue concentrations of any of the trace elements. There were no relationships between PC I scores and clam tissue concentrations of As, Cr, or Se. There were significant linear relationships between PC I scores and clam tissue concentrations of Pb (positive, p=0.02), Hg (negative, p=0.0002), and Hg (negative, p=0.003). However, the proportions of variance in clam tissue concentrations of these trace elements explained by PC I were similar to the proportions explained by relationships with the watershed percentage of forest (5% lower for Pb, 7% lower for Cd, 1% higher for Hg). Thus, the combination of all human activities in the watershed did not have a cumulative impact on clam tissue concentrations of these trace elements.

Concentrations of As in snail tissue were above RLs at all sites where snails were collected, ranging from 2.5-10 µg/g (mean 4.9 µg/g). Concentrations of As in snail tissue were significantly higher (paired t-test, t=2.26, p=0.04) than clam tissue concentrations (Figure 3-3). Snail tissue As concentrations were not correlated with any land use variables ($p \geq 0.38$). Unlike clam tissue, the relationship between As concentrations in snail tissue and watershed density of poultry AFOs was not well described by mathematical functions (Figure 3-4), including polynomial regression (p=0.46). Concentrations of As in snail tissue were correlated with clam tissue As concentrations (r=0.72, p=0.004), but only when concentrations measured at two sites (JENN and LOMR) were excluded from the analysis (Figure 3-5). Both sites had unusual snail population characteristics relative to the other sampling sites; snails at JENN had the highest incidence of parasitism by digenetic trematodes (69.5%) and there was no evidence of egg-laying at LOMR during the sampling period, whereas egg-laying had begun at all other sampling sites (S. Ciparis, unpublished data).

Concentrations of Pb, Cr, Se, Cd, and Hg in *L. carinata* tissue were lower than concentrations measured in clam tissue. Concentrations of Pb in snail tissue were below RLs at all sites where snails were collected (n=19). Concentrations of Cr were above RLs at five sampling sites (LOMR, HADS, SMDS, NAPA, and MEAD), and ranged from 0.53-2.8 µg/g. Concentrations of Se were above reporting limits at four sampling sites (LOMR, MCNF, SMHW, and STDS) and ranged from 0.85-2.2 µg/g. Concentrations of Cd in snail tissue were above RLs at 17 sampling sites, and ranged from 0.036-1.5 µg/g (mean 0.39 µg/g). Similar to results for clam tissue, snail tissue Cd concentrations were positively correlated with watershed percentage of forest and negatively correlated with watershed percentages of pasture/hay and crops, and densities of beef and dairy AFOs (Table 3-6). At the 16 sites where both snails and clams were collected, snail tissue Cd concentrations were significantly lower (paired t-test, t=-

3.17, p=0.006) than clam tissue concentrations (Figure 3-3), and site-specific correlations of Cd concentrations in the two types of mollusks were not significant (p=0.19). Concentrations of Hg in snail tissue were above RLs at 16 sampling sites, and ranged from 0.021-0.45 µg/g (mean 0.08 µg/g). Similar to Cd, snail tissue Hg concentrations were significantly lower (paired t-test, t=-4.01, p=0.001) than clam tissue concentrations (Figure 3-3), and site-specific correlations of Hg concentrations in the two types of mollusks were not significant (p=0.38). However, snail tissue Hg concentrations were not correlated with any land use variables ($p \geq 0.63$).

Results of PCA of land use variables for sites where snails were collected were applied to a PCR with snail tissue trace elements concentrations. Two PCs explained 80.4% of the variance in the land use data. The PC I eigenvector weights were positive and greater than 0.24 for all land use variables except total permitted WWTP discharge (-0.05). The PC II eigenvector weights were 0.61 and 0.75 for watershed percentage of developed land and total permitted WWTP discharge, respectively, -0.23 for the watershed density of dairies, and <0.10 for all other variables. Only the relationship between snail tissue Cd concentrations and PC I scores was significant (negative, p=0.004). The proportion of variance in snail tissue Cd concentrations explained by the relationship with PC I (40%) was similar to the proportion explained by the relationship to watershed percentage of forest (41%).

Tissue concentrations of As (clams and snails), Cr (clams) and Pb (clams) were not correlated with sediment concentrations ($p \geq 0.29$). Tissue and sediment concentrations of Cd, Hg, and Se could not be compared because these elements were below their RLs in sediment.

Discussion

Sediment

Overall, trace elements in streambed sediment from the 25 study sites were well below concentrations expected to cause ecotoxicological effects. Arsenic was the only trace element above the NOAA TEL at the majority of sites where it was quantifiable. However, As concentrations were generally within the range of average streambed sediments (5-8 µg/g; (Smedley and Kinniburgh, 2002) and were 10-100 times lower than in sediment contaminated by mining (Farag et al., 2007) or disposal of coal fly ash (Unrine et al., 2007). There was no apparent relationship between concentrations of As in streambed sediment and the quantified land uses, particularly the density of poultry AFOs or percentages of pasture/hay and cropland. Sediment concentrations of Cr, Co, and Fe were also poorly

predicted by land use variables, and PCA revealed a strong relationship between these elements and As at some sampling sites. The PCA results suggest that element concentrations in sediment at these sites are influenced by something other than limestone/dolomite geology, given the opposite PC II eigenvector weights of As, Cr, Co, and Fe compared to Ca and Mg. This was generally supported by examination of geologic maps (Dicken et al., 2005). In the Shenandoah River watershed most human activities are concentrated in the Shenandoah Valley, where limestone/dolomite is predominant. This interaction provides a mechanism for the positive relationship of Ca and Mg with agricultural and other activities, and for a lack of relationship between human activities and elements with a geologic source other than limestone/dolomite.

A correlation between As and Fe suggests possible association of As with Fe oxyhydroxides, which has been observed in many other studies (e.g. Smedley and Kinniburgh, 2002; Tuttle et al., 2009). Weathering and oxidation of pyrite (FeS_2) in black shale can release dissolved ferrous iron, sulfate, and associated acid-soluble trace elements such as As. Ferrous iron subsequently oxidizes and precipitates as Fe-oxyhydroxides due to interaction with O_2, and acid-soluble trace metals can adsorb to or co-precipitate with the oxyhydroxides (Smedley and Kinniburgh, 2002; Tuttle et al., 2009). Thus, pyrite in the Devonian black shale present in these watersheds may be a source of As and other trace elements in stream sediment. Orndorff (2001) found pyrite in various forms in Devonian black shale (Marcellus and Millboro) exposed by roadcuts in counties immediately south and west of the study area, but As concentrations were not analyzed. Sediment concentrations of As in the current study were generally higher than concentrations in streams flowing over Mississippian Fayetteville black shale (1.6-6.2 μg/g; Ogendi et al., 2007) but were much lower than concentrations in streams affected by weathering of Devonian New Albany black shale (38-98 μg/g; Tuttle et al., 2009).

In addition to As, Tuttle et al. (2009) found that Cd, Co, Cu, Mn, Ni, Zn, Cr, Mo, Pb, Sb, Tl and V were mobilized from New Albany Shale during oxidative weathering. Chromium and V were primarily associated with clay components of the shale (vs. pyrite) and were mobilized in particulate form. In the current study, V was quantifiable at four of the six sites with strong associations between Cr, Co, Fe and As, which could be due to black shale weathering. Vanadium is also strongly associated with S-containing atmospheric fine particulate matter (Lake et al., 2004) and has been measured in Shenandoah Valley air samples (Tuncel et al., 1985). Atmospheric deposition of S has caused acidification of streams draining Shenandoah National Park (Sullivan et al., 2008), and could represent a source of V to these streams, which had low overall metals concentrations (PC I scores) and no

quantifiable As. However, distinguishing between potential diffuse sources such as atmospheric deposition and geologic formations was not possible with the sediment data in the current study. Further analysis of geologic formations in watersheds with black shale would be required to support or refute their potential contribution of As and other trace metals to streams, as the geologic maps utilized in this study only provide information on the presence or absence of Devonian black shale over a large spatial scale, and not depth of the formations or possible exposure to weathering.

Another potential source of As to streams in the Shenandoah River watershed unrelated to poultry AFOs is the historic use of lead arsenate. Soil disturbance through agricultural activities in historic orchards that used lead arsenate could mobilize As and Pb, resulting in elevated concentrations in streambed sediment (Renshaw et al., 2006; Robinson et al., 2007). However, Pb was not represented by the PC II eigenvector, whereas As had a negative weight. The sites with relatively high sediment Pb concentrations had relatively high sediment As concentrations, but are also underlain by limestone/dolomite (Ca and Mg). These sites generally had higher densities of AFOs and higher percentages of crops, pasture/hay, and developed land, and the relationship between Pb and these land uses was indicated by the PLS regression. In addition to lead arsenate, potential sources of Pb to these sites include atmospheric deposition, WWTP discharges, and geologic formations (Harlavan et al., 2010; van der Perk, 2006). Schooley et al. (2008) indicated the historic intensity of apple production in the Shenandoah Valley, but determining specific locations of commercial apple orchards that used lead arsenate was beyond the scope of this study and would be required to assess potential impacts on streambed sediment.

Unlike As, sediment concentrations of Cu were positively related to agricultural activities as expected. The negative weight of Cu in the second factor of the PLS regression suggests that Cu is better predicted by watershed densities of poultry and dairy AFOs than the other elements generally correlated with agriculture, which may be related to land application of manure. Copper is present in poultry litter (55-1196 μg/g; Jackson et al., 2003) and dairy manure (18-1100 μg/g; McBride and Spiers 2001), with elevated concentrations in dairy manure partially attributed to use of $CuSO_4$ hoof dips. Jackson et al., (2003) found that 49% of Cu in poultry litter was water soluble, possibly due to formation of complexes with dissolved organic carbon (DOC). Thus, leaching after manure application could transport Cu to streams. In addition, the strong affinity of Cu for organic matter (Horowitz, 1991) may lead to increased Cu accumulation in sediment of streams draining agriculturally dominated watersheds if the amount of organic matter input increases through either runoff of manure, soil erosion, or grazing cattle.

Both PCA and PLS regression showed no relationship of Zn with agricultural land use, which was unexpected. Zinc is present in poultry litter and dairy manure at similar concentrations as Cu (Jackson et al., 2003; McBride and Spiers, 2001), but compared to Cu, Zn has a lower affinity for DOC and is considerably less soluble from poultry litter (6%; Jackson et al., 2003; McBride and Spiers, 2001). The PLS regression did indicate a relationship between sediment Zn concentrations and total permitted WWTP discharge; their weights in the third factor were much greater than all other elements and types of land use. Previous studies of municipal WWTP effluent have shown that Zn is present in higher concentrations than other trace elements, and the majority is associated with particulate matter (Gagnon and Saulnier, 2003). In the current study, WWTPs appeared to have a greater influence on sediment concentrations of Zn than agriculture.

Other elements positively related to agricultural land use and other human activities in the studied watersheds included Mn, Ba, and Al. Manganese is present as a minor constituent of limestone/dolomite (van der Perk, 2006), and is also present in poultry litter with low solubility (Jackson et al., 2003) which supports the association with agricultural land use. However, Mn is also present in black shale (Tuttle et al., 2009) and Mn-oxyhydroxides can adsorb multiple trace elements, which may explain the failure of Mn to associate with either group of PC II elements. Barium is likely associated with limestone/dolomite, as calcite can be a host mineral (Reimann and de Caritat 1998). Aluminum is considered to be a conservative element, with a uniform flux from crustal sources, but this depends on the source rocks (Horowitz, 1991; N'guessan et al., 2009). Sediment concentrations of Al could potentially be enhanced by soil disturbance from development, crop cultivation, and cattle grazing, which may result in higher transport of fine clay particles that both host and bind Al (van der Perk, 2006).

Sediment concentrations of all trace elements generally increased with the amount of human activity in the studied watersheds, as indicated by the positive relationship of PC I with all watershed-scale activities, except percentage of forest (negative relationship) and total permitted WWTP discharge (no relationship). However, the effects were not additive; combining all watershed-scale human activities in the PLS regression did not increase the explained proportion of variance in the data. This is likely due, at least in part, to multiple sources of many of the elements which were not all quantified in this study. Second, possible associations between the elements and with mineral surfaces add complexity to interpretations of the sediment data. Metal (Fe, Al, and Mn) oxyhydroxides and clays adsorb As, Pb, Cu and Zn under oxic conditions through surface complexation processes (McBride, 1994;

Stollenwerk, 2003). These elements are relatively stable as adsorbed species, but if redox conditions change, they can be released (Smedley and Kinniburgh, 2002). Changes in pH can also trigger release of adsorbed trace elements. A decrease in pH can result in release of trace elements that occur as cations in aqueous solution (Cu, Zn, Pb, etc). For trace elements that occur as oxyanions in solution (As, Se, V, etc.), the adsorption edge is opposite to that of cations; an increase in pH can trigger release of the element to solution. Competitive adsorption interactions between the oxyanions can also result in mobilization (e.g. Manning and Goldberg, 1996). Finally, as bulk concentrations were the target of this study, grain size and organic matter content of the sediment were not analyzed, which may have introduced additional variability into the dataset as both properties can affect the sorption of trace elements.

Tissue

Clam tissue As concentrations were relatively high compared to other studies. The mean clam tissue As concentration in this study (3.5 µg/g) was greater than the mean concentration in *C. fluminea* from forested and agricultural sites in the Chattahoochee River basin (2.7 µg/g) and was similar instead to the mean concentration in clams from sites impacted by coal fired power plants (3.6 µg/g) (Peltier et al., 2008). However, clam tissue As concentrations in the current study were lower than in *C. fluminea* from a wetland in the Chattahoochee River basin affected by coal fly ash disposal (11 µg/g; Unrine et al., 2007). Compared to tributaries in the Boulder River watershed, clam tissue As concentrations at many sites in the current study were higher than concentrations in invertebrates from pooled reference sites (3.0 µg/g), but were much lower than concentrations in invertebrates from sites impacted by mining operations (7.5-80 µg/g; Farag et al., 2007). In the current study, eight sites had clam tissue As concentrations within the range of concentrations measured in *C. fluminea* from the San Joaquin River (4.2-6.1 µg/g), which is impacted by agricultural drainage (Leland et al., 1990). While clam tissue As concentrations generally did not approach concentrations measured at contaminated sites, there appears to be a source(s) of bioavailable As that results in concentrations greater than reference conditions in other studies.

A dominant source of bioavailable As was not evident from the land use variables quantified in this study. Watershed density of poultry AFOs or watershed percentages of pasture/hay and cropland were not predictive of clam tissue As concentrations as evidenced by the lack of a linear correlation. However, higher tissue As concentrations at moderate densities of poultry AFOs fails to eliminate the possibility that land application of poultry litter is a source of bioavailable As to aquatic organisms. Arsenic has been shown to leach into soil water from fields receiving litter applications in the Shenandoah Valley (Brown et al., 2005), which could lead to eventual

transport to stream channels. It is also possible that land application of poultry litter is not a source of bioavailable As, and other sources of As predominate in watersheds with moderate densities of poultry AFOs. Arsenic from black shale remains strongly associated with Fe (Tuttle et al., 2009), and may not be readily bioavailable, as clams from reference sites with black shale present (CEHW, CEDS, PASS) had relatively low tissue concentrations of As. Erosion of soil in orchards treated with lead arsenate results in As strongly sorbed to sediment (Renshaw et al., 2006), which may limit bioavailability. However, mobility of As can be affected by PO_4^{3-}, which competes with As for mineral sorption sites. Addition of PO_4^{3-} to As-contaminated surface soils can increase As concentrations in pore water and leachate (Lee and Kim, 2008; Peryea and Kammereck, 1997). Application of animal wastes and other fertilizers on historic orchard sites contaminated with lead arsenate, and possibly on fields with underlying black shale, could potentially increase mobility of As, leading to groundwater and surface water transport, and subsequent uptake by aquatic organisms. Thus, the nonlinear relationship between clam tissue As concentrations and watershed density of poultry AFOs may result from interactions between contemporary agricultural practices and historic land use or geology.

There are several possibilities for the apparent lower bioavailability of As in streams with the highest densities of poultry AFOs (and other agricultural activities). Shoults-Wilson et al. (2009) found that *C. fluminea* in the Altamaha River basin accumulated higher concentrations of As at sites with higher dissolved oxygen (DO). Eutrophic conditions could lower DO in tributaries that have a high watershed density of agricultural land use, particularly at the sediment-water interface where *C. fluminea* reside, and reduce As bioavailability. If As exists primarily in the dissolved phase, high PO_4^{3-} concentrations could inhibit As uptake by organisms through competition for binding sites on membrane carrier proteins (Lee and Kim, 2008). Conversely, higher loadings of inorganic suspended solids from soil disturbance in the watershed could sorb As and reduce bioavailability to *C. fluminea*, which is a particle selective feeder (Leff et al., 1990). Further analysis of these environmental factors would be required to determine the influence of instream processes and site-specific conditions on As bioavailability relative to watershed-scale land use.

Similar to clams, *L. carinata* accumulated relatively high concentrations of As and there was no linear correlation between snail tissue As concentrations and watershed density of poultry AFOs, or other land uses. At almost all sites where snails and clams were collected, snails accumulated higher concentrations of As, which may be due to differences in feeding strategies between the two mollusks. *Leptoxis spp*. and other pleurocerid snails feed

primarily on periphyton, including algal cells (diatoms) and associated bacteria and detritus (Brown et al., 2008). Due to the structural similarity between PO_4^{3-} and arsenate, algal cells (including periphytic algae) actively take up As (Kuwabara et al., 1990; Levy et al., 2005) and inorganic matter incorporated in periphyton mats sorbs As (Newman et al., 1985). These processes result in elevated concentrations of As in periphyton relative to overlying water (Farag et al., 2007; Kuwabara, 1990). Asian clams are primarily detritivores (Unrine et al., 2007), and while some detritus likely originates from sloughed periphyton, sediment organic matter and allochthonous inputs also contribute to this food source. In addition to differences in feeding strategy, temporal variations in environmental As concentrations may have contributed to differences in bioaccumulation between the two types of mollusks because samples were collected in different years. However, the significant linear relationship between As concentrations in clam and snail tissue suggests temporal consistency in the relative bioavailability of As between the study sites.

The linear relationship between tissue concentrations of As in the two types of mollusks was only significant with the removal of two sites with large discrepancies in bioaccumulation. While anomalous snail population characteristics at these two sites, including delayed egg-laying and a high proportion of parasitized individuals, supported their removal from the analysis, the actual effects of these characteristics on bioaccumulation of As and other trace elements is unknown. Studies of terrestrial snails have demonstrated that accumulation of trace elements is greater in the gonad/digestive gland than in the head/foot region of the body (Gimbert et al., 2008). In *L. carinata*, developing oocytes in the gonad/digestive gland are rich in C and protein (Aldridge, 1982), and egg-laying could result in substantial depuration of associated trace elements, similar to the effects of spawning on trace element body burdens of marine mussels (Chernova, 2010; Simpson, 1979). The gonad/digestive gland is also the site of proliferation of digenetic trematode parasites in freshwater snails. Few studies have been conducted on the potential effects of parasitism on bioaccumulation of trace elements in snails, but several studies have documented alterations in metallic ion concentrations in trematode-infected compared to uninfected snails (Mostafa, 2008, and references therin). Pleurocerid snails appear to be viable biomonitors for As, but potential effects of egg-laying and parasitism on bioaccumulation require further study and should be considered during study design and tissue processing.

Asian clams accumulated higher concentrations of Hg, Cd, Pb, Cr, and Se than *L. carinata*. Filter-feeding by *C. fluminea* likely results in greater uptake of dissolved contaminants and contaminants associated with colloids and organic particles relative to *L. carinata*, as individual Asian clams can filter >1 L per day (Leff et al., 1990).

Clam tissue concentrations of Se and Cr were not correlated with any quantified land uses, but significant relationships between clam tissue concentrations of Pb, Hg, and Cd and land uses support the use of Asian clams in landscape-scale studies of the influence of different human activities on trace element concentrations in small streams. The negative correlation of clam tissue Pb concentrations with watershed percentage of forest is likely due to the various anthropogenic sources of Pb discussed previously, including the possible influence of disturbance of historic orchards. Tissue concentrations of Pb were generally similar to concentrations in *C. fluminea* from waterbodies unaffected by Pb contamination (1.6-2.8 µg/g; Angelo et al., 2007; Luoma et al., 1990). The reason for the exceptionally high Pb concentration in clam tissue from the site GOON was unclear. A former carbon disulfide manufacturing plant and a closed county landfill are located in an adjacent watershed (USEPA and VADEQ, 2001), which could be hydrologically connected by groundwater, but their potential impacts on GOON are unknown.The positive correlation between clam tissue concentrations of Hg and Cd and watershed percentage of forest is likely related to atmospheric deposition of these elements, concentration of atmospheric particulates by the forest canopy, and resulting enrichment of throughfall in Hg and Cd relative to wet deposition (Kolka et al., 1999; Mason et al., 2000). Peltier et al. (2008) observed a similar correlation between Hg in *C. fluminea* tissue and watershed percentage of forest within the Chattahoochee River basin.

Concentrations of trace elements in mollusk tissue were uncorrelated with sediment concentrations, which was not unexpected. Neither *C. fluminea* nor *L. carinata* utilize sediment as a primary food source or reside in the depositional areas where sediment was collected. In addition, as bulk sediment samples were collected and analyzed for trace elements, the potentially bioavailable fraction was not targeted. Several sediment properties, including concentrations of particulate sulfides, organic carbon, and Fe hydroxides, can affect bioavailability of trace elements. The purpose of collection of both sediment and mollusk tissue was to gain further insight regarding potential sources, rather than to define relationships between the abiotic and biotic compartments. Measurement of bulk concentrations of trace and major elements in sediment appears to be particularly useful for identifying geological sources of trace elements while measurement of trace concentrations in tissue appears to be more suitable for identification of more diffuse sources (e.g. atmospheric deposition).

Conclusions

Results of this study showed positive associations between agricultural land use and other human activities and concentrations of several trace and major elements in sediment and mollusks collected from Shenandoah River tributaries. Contrary to expectations, there were no direct relationships between As concentrations and watershed densities of poultry AFOs or agricultural land receiving waste from these operations. However, As concentrations in clam tissue were relatively high compared to other studies indicating the presence of bioavailable As. Geologic sources and historic orchard sites, as well as their interactions with contemporary agricultural practices, require further investigation as potential sources of As in the Shenandoah River watershed. Site specific studies may be more appropriate to identify factors affecting bioavailability of As due to its complex geochemistry.

Pleurocerid snails may be viable sessile biomonitors of As concentrations, but potential effects of seasonal reproduction and parasitism on contaminant bioaccumulation require further consideration. Compared to snails, Asian clams appear to be better biomonitors of landscape sources of other trace elements (Cd, Hg, Pb, Cr, Se) due to greater bioaccumulation. Differential bioaccumulation of trace elements by snails and clams is likely due to their different feeding strategies.

Literature cited

Aldridge, D. W. (1982). Reproductive tactics in relation to life-cycle bioenergetics in three natural populations of the freshwater snail, *Leptoxis carinata*. *Ecology, 63*(1), 196-208.

Angelo, R. T., Cringan, M. S., Chamberlain, D. L., Stahl, A. J., Haslouer, S. G., & Goodrich, C. A. (2007). Residual effects of lead and zinc mining on freshwater mussels in the Spring River Basin (Kansas, Missouri, and Oaklahoma, USA). *Science of the Total Environment, 384*, 467-496.

Brock, E. H., Ketterings, Q. M., & McBride, M. (2006). Copper and zinc accumulation in poultry and dairy manure-amended fields. *Soil Science, 171*, 388-399.

Brown, B. L., Slaughter, A. D., & Schreiber, M. E. (2005). Controls on roxarsone transport in agricultural watersheds. *Applied Geochemistry, 20*, 123-133.

Brown, K. M., Lang, B., & Perez, K. E. (2008). The conservation ecology of North American pleurocerid and hydrobiid gastropods. *Journal of the North American Benthological Society, 27*(2), 484-495.

Buchman, M. F. (1999). NOAA screening quick reference tables. NOAA HAZMAT report 99-1, Seattle, WA, Coastal Protection and Restoration Division, National Oceanic and Atmospheric Administration.

Chernova, E. N. (2010). Changes in trace metal concentrations in the tissues of the White Sea mussel *Mytilus edulis* over the reproductive cycle. *Russian Journal of Marine Biology, 36*, 63-69.

Comelo, R. L., Paul, J. F., August, P. V., Copeland, J., Baker, C., Hale, S. S., et al., (1996). Relationships between watershed stressors and sediment contamination in Chesapeake Bay estuaries. *Landscape Ecology, 11*, 307-319.

Dicken, C. L., Nicholson, S. W., Horton, J. D., Kinney, S. A., Gunther, G., Foose, M. P., et al., (2005). Preliminary integrated geologic map databases for the United States: Delaware, Maryland, NewYork, Pennsylvania, and Virginia. United States Geological Survey. http://pubs.usgs.gov/of/2005/1325

Eriksson, L., Hermens, J. L. M., Johansson, E., Verhaar, H. J. M., & Wold, S. (1995). Multivariate-analysis of aquatic toxicity data with PLS. *Aquatic Sciences, 57*, 217-241.

Farag, A. M., Nimick, D. A., Kimball, B. A., Church, S. E., Harper, D. D., & Brumbaugh, W. G. (2007). Concentrations of metals in water, sediment, biofilm, benthic macroinvertebrates, and fish in the Boulder

River watershed, Montana, and the role of colloids in metal uptake. *Archives of Environmental Contamination and Toxicology, 52*, 397-409.

Gagnon, C., & Saulnier, I. (2003). Distribution and fate of metals in the dispersion plume of a major municipal effluent. *Environmental Pollution, 124*, 47-55.

Gimbert, F., Vijver, M. G., Coeurdassier, M., Scheifler, R., Pelinenburg, W. J. G. M., Badot, P.-M., et al., (2008). How subcellular partitioning can help to understand heavy metal accumulation and elimination kinetics in snails. *Environmental Toxicology and Chemistry, 27*, 1284-1292.

Gupta, G., & Gardner, W. (2005). Use of clay mineral (montmorillonite) for reducing poultry litter leachate toxicity (EC_{50}). *Journal of Hazardous Materials, B118*, 81-83.

Harlavan, Y., Almogi-Labin, A., & Herut, B. (2010). Tracing natural and anthropogenic Pb in sediments along the Mediterranean coast of Israel using Pb isotopes. *Environmental Science and Technology*, 44, 6576-6582.

Homer, C., Dewitz, J., Fry, J., Coan, M., Hossain, N., Larson, C., et al., (2001). Completion of the 2001 National Land Cover Database for the conterminous United States. Photogrammetric Engineering and Remote Sensing, 73, 337-341.

Horowitz, A. J. (1991). *Sediment-trace element chemistry*. Chelsea, Michigan: Lewis Publishers, Inc.

Jackson, B. P., Bertsch, P. M., Cabrera, M. L., Camberato, J. C., Seaman, J. C., & Wood, C. W. (2003). Trace element speciation in poultry litter. *Journal of Environmental Quality, 32*, 535-540.

Kolka, R. K., Nater, E. A., Grigal, D. F., & Verry, E. S. (1999). Atmospheric inputs of mercury and organic carbon into a forested upland/bog watershed. *Water Air and Soil Pollution, 113*, 273-294.

Kolker, A., Engle, M. A., Orem, W. H., Bunnell, J. E., Lerch, H. E., Krabbenhoft, D. P., et al., (2008). Mercury, trace elements and organic constituents in atmospheric fine particulate matter, Shenandoah National Park, Virginia, USA: A combined approach to sampling and analysis. *Geostandards and Geoanalytical Research, 32*(3), 279-293.

Kuwabara, J. S., Chang, C. C. Y., & Pasilis, S. P. (1990). Effects of benthic flora on arsenic transport. *Journal of Environmental Engineering, 116*, 394-409.

Lake, D. A., Tolocka, M. P., Johnston, M. V., & Wexler, A. S. (2004). The character of single particle sulfate in Baltimore. *Atmospheric Environment, 38*, 5311-5320.

Lee, B.-T., & Kim, K.-W. (2008). Arsenic accumulation and toxicity in the earthworm *Eisenia fetida* affected by chloride and phosphate. *Environmental Toxicology and Chemistry, 27*, 2488-2495.

Leff, L. G., Burch, J. L., & McArthur, J. V. (1990). Spatial distribution, seston removal, and potential competitive interactions of the bivalves *Corbicula fluminea* and *Elliptio complanata*, in a coastal plain stream. *Freshwater Biology, 24*, 409-416.

Leland, H. V., & Scudder, B. C. (1990). Trace elements in *Corbicula fluminea* from the San Joaquin River, California. *Science of the Total Environment, 97/98*, 641-672.

Levy, J. L., Stauber, J. L., Adams, M. S., Maher, W., Kirby, J. K., & Jolley, D. F. (2005). Toxicity, biotransformation, and mode of action of arsenic in two freshwater microalgae (*Chlorella* sp. And *Monoraphidium arcuatum*). *Environmental Toxicology and Chemistry, 24*, 2630-2639.

Luoma, S. N., Dagovitz, R., & Axtmann, E. (1990). Temporally intensive study of trace metals in sediments and bivalves from a large river-estuarine system: Suisun Bay/delta in San Francisco Bay. *The Science of the Total Environment, 97/98*, 685-712.

Manning, B. A., & Goldberg, S. (1996). Modeling competitive adsorption of arsenate with phosphate and molybdate on oxide minerals. *Soil Science Society of America Journal, 60*, 121-131.

Mason, R. P., Laporte, J. M., & Andres, S. (2000). Factors controlling the bioaccumulation of mercury, methylmercury, arsenic, selenium, and cadmium by freshwater invertebrates and fish. *Archives of Environmental Contamination and Toxicology, 38*, 283-297.

McBride, M. (1994). *Environmental chemistry of soils*. New York: Oxford University Press.

McBride, M. B., & Spiers, G. (2001). Trace element content of selected fertilizers and dairy manures as determined by ICP-MS. *Communications in Soil Science and Plant Analysis, 32*, 139-156.

Mostafa, O. M. S. (2008). Effects of *Fasicola gigantica* experimental infection on some inorganic elements in the snail host *Lymnaea natalensis*. *Experimental Parasitology, 118*, 468-471.

N'guessan, Y. M., Probst, J. L., Bur, T., & Probst, A. (2009). Trace elements in stream bed sediments from agricultural catchments (Gascogne region, S-W France): Where do they come from? *Science of the Total Environment, 407*, 2939-2952.

Newman, M. C., Alberts, J. J., & Greenhut, V. A. (1985). Geochemical factors complicating the use of aufwuchs to monitor bioaccumulation of arsenic, cadmium, chromium, copper, and zinc. *Water Research, 19*, 1157-1165.

Ogendi, G. M., Brumbaugh, W. G., Hannigan, R. E., & Farris, J. L. (2007). Effects of acid-volatile sulfide on metal bioavailability and toxicity to midge (*Chironomus tetans*) larvae in black shale sediments. *Environmental Toxicology and Chemistry, 26*, 325-334.

Orndorff, Z. W. (2001). *Evaluation of sulfidic materials in Virginia highway corridors*. Ph.D. Dissertation, Virginia Polytechnic Institute and State University.

Pelletier, B. A., & Kenyon, D. (2000). Poultry litter for corn exchange program for Virginia. http://ideas.repec.org/s/ags/vturp.html.

Peltier, G. L., Meyer, J. L., Jagoe, C. H., & Hopkins, W. A. (2008). Using trace element concentrations of *Corbicula fluminea* to identify potential sources of contamination in an urban river. *Environmental Pollution, 154*, 283-290.

Peryea, F. J., & Kammereck, R. (1997). Phosphate-enhanced movement of arsenic out of lead arsenate-contaminated topsoil and through uncontaminated subsoil. *Water Air and Soil Pollution, 93*, 243-254.

Reimann, C., & de Caritat, P. (1998). *Chemical elements in the environment: Factsheets for the geochemist and environmental scientist*. Berlin: Springer.

Renshaw, C. E., Bostick, B. C., Feng, X., Wong, C. K., Winston, E. S., Karimi, R., et al., (2006). Impact of land disturbance on the fate of arsenical pesticides. *Journal of Environmental Quality, 35*, 61-67.

Ripley, J., Iwanowicz, L. R., Blazer, V. S., & Foran, C. (2008). Utilization of protein expression profiles as indicators of environmental impairment of smallmouth bass (*Micropterus dolomieu*) from the Shenandoah River, Virginia, USA. *Environmental Toxicology and Chemistry, 27*, 1756-1767.

Robinson, G. R., Larkins, P., Boughton, C. J., Reed, B. W., & Sibrill, P. L. (2007). Assessment of contamination from arsenical pesticide use on orchards in the Great Valley region, Virginia and West Virginia, USA. *Journal of Environmental Quality, 36*, 654-663.

Schooley, T., Weaver, M. J., Mullins, D., & Eick, M. (2008). The history of lead arsenate use in apple production: Comparison of its impact in Virginia with other states. *Journal of Pesticide Safety Education, 10*, 22-53.

Short, T. M., Deweese, L. R., & Dubrovsky, N. M. (2008). Discrimination of trait-based characteristics by trace element bioaccumulation in riverine fishes. *Canadian Journal of Fisheries and Aquatic Sciences, 65*, 1087-1100.

Shoults-Wilson, W. A., Peterson, J. T., Unrine, J. M., Rickard, J., & Black, M. C. (2009). The Asian clam C*orbicula fluminea* as a biomonitor of trace element contamination: Accounting for different sources of variation using a hierarchical linear model. *Environmental Toxicology and Chemistry, 28*, 2224-2232.

Simpson, R. D. (1979). Uptake and loss of zinc and lead by mussels (*Mytilus edulis*) and relationships with body weight and reproductive cycle. *Marine Pollution Bulletin, 10*, 74-78.

Smedley, P. L., & Kinniburgh, D. G. (2002). A review of the source, behaviour and distribution of arsenic in natural waters. *Applied Geochemistry, 17*, 517-568.

Stollenwerk, K. (2003). Geochemical processes controlling transport of arsenic in groundwater: A review of adsorption. In K. Stollenwerk, & A. Welch (Eds.), *Arsenic in groundwater* (pp. 67-100). Boston: Kluwer Academic Publishers.

Sullivan, T. J., Cosby, B. J., Webb, J. R., Dennis, R. L., Bulger, A. J., & Deviney Jr., F. A. (2008). Streamwater acid-base chemistry and critical loads of atmospheric sulfur deposition in Shenandoah National Park, Virginia. *Environmental Monitoring and Assessment, 137*, 85-99.

Tuncel, S. G., Olmez, I., Parrington, J. R., & Gordon, G. E. (1985). Composition of fine particle regional sulfate component in Shenandoah Valley. *Environmental Science and Technology, 19*, 529-537.

Tuttle, M. L. W., Breit, G. N., & Goldhaber, M. B. (2009). Weathering of the New Albany Shale, Kentucky: II. Redistribution of minor and trace elements. *Applied Geochemistry, 24*, 1565-1578.

Unrine, J. M., Hopkins, W. A., Romanek, C. S., & Jackson, B. P. (2007). Bioaccumulation of trace elements in omnivorous amphibian larvae: Implications for amphibian health and contaminant transport. *Environmental Pollution, 149*, 182-192.

United States Environmental Protection Agency (USEPA) & Virginia Department of Environmental Quality (VADEQ) (2001). Development of Shenandoah River PCB TMDL. Final report. http://www.epa.gov/reg3.

VADEQ (2005). Fish tissue analysis for metals 2005. http://www.deq.virginia.gov/fishtissue/fishtissue.html.

VADEQ (2006). Final 2006 305(b)/303(d) water quality assessment integrated report. http://www.deq.state.va.us/wqa/ir2006.htm.

van der Perk, M. (2006). *Soil and water contamination from molecular to catchment scale*. Philadelphia: Taylor and Francis.

Yager, R. M., Southworth, S., & Voss, C. I. (2008). Simulation of ground-water flow in the Shenandoah Valley, Virginia and West Virginia, using variable-direction anisotropy in hydraulic conductivity to represent bedrock structure. United States Geological Survey Scientific Investigations Report 2008-5002.

Table 3- 1. Summary statistics for watershed land use characteristics of the 25 sampling sites in Shenandoah River tributaries. The range and mean with one standard deviation are in parentheses. Watershed land use characteristics were quantified as follows: land cover classes as percentages, individual and total animal feeding operations (AFOs) as densities (# AFOs/1000 acres), and wastewater treatment plant (WWTP) flow as total upstream permitted effluent discharge in millions of gallons per day (MGD).

Land Use	Range	Mean
% Forest	11 – 93	53 (28)
% Past./Hay	5.0 - 71	36 (23)
% Crops	0.2 – 13	3.2 (2.8)
% Developed	1.9 – 19	7.7 (4.4)
Dens. Poultry	0 – 2.4	0.59 (0.66)
Dens. Beef	0 – 1.0	0.33 (0.28)
Dens. Dairy	0 – 5.8	0.52 (1.2)
Tot. Dens. AFOs	0 – 4.2	1.2 (1.2)
WWTP flow (MGD)	0 – 1.8	0.16 (0.40)

Table 3- 2. Concentrations (µg/g dry weight) of the 14 elements above reporting limits (RLs) in sediment collected from 25 sampling sites in the Shenandoah River watershed. Blank cells indicate concentrations below the RL. Concentrations of As and Ni in bold exceeded the threshold effects level (TEL) published in the NOAA Screening Quick Reference Tables (Buchman 1999). All concentrations were below the probable effects level (PEL). Watersheds dominated by limestone/dolomite and watersheds with black shale present as either rock Type 1 or Type 2 are indicated. Underlined tributaries were considered reference sites. Tributaries with upstream (US) and downstream (DS) sampling sites are indicated, including the total number of subwatersheds upstream of DS sites.

Tributary Name	Site Code	Al	As	Ba	Ca	Cr	Co	Cu	Fe	Mg	Mn	Ni	Pb	V	Zn	Li/Do Maj.	Bl. S. Pres.
Back Cr.	BACK	3,300	2.9	62		9.3	11	7	17,000		450	8.9		20	24		
Briery Br.	BRIR	2,400		52			4.3	21	8,100	930	280	**20**			24		
Cedar Cr. (DS; 5)	CEDS	4,200	**7.6**	48	11,000	19	7.7	9.6	21,000	1,300	370	14	8.6	16	40		T1
Cedar Cr. (US)	CEHW	5,400	**6.4**	66	19,000	14	9.9	16	18,000	1,600	630				47		T1
Christians Cr.	CHRIS	7,300	5.4	81	140,000	11	8.2	13	14,000	3,400	710		14		35	X	T2
Cooks Cr.	COOK	8,900	5.3	70	58,000	17	9.9	19	18,000	2,200	620		19		70	X	T2
Gooney R.	GOON	4,100		31		12	4.7		11,000	750	170			20	27		
Hawksbill (DS; 2)	HADS	6,200		58	2,900	18	6.3	9.4	18,000	1,500	290			21	48		
Hawksbill (US)	HAWK	14,000		140	8,300	14	11	19	30,000	2,400	390		16	46	72		
Holmans Cr.	HOLM	5,400		79	160,000			9.8	8,500	2,600	410		10		26	X	T2
Jennings Br.	JENN	3,500	**6.3**	43		11	6.4	9	13,000	1,100	240	9.5	13		28		T1
Long Glade Cr.	LGCR	5,100	**10**	44	30,000	22	11	11	22,000		740		17	21	44	X	T2
Linville Cr.	LINV	6,700		73	150,000		6.8	12	12,000	2,700	620		14		41	X	T2
Long Meadow R.	LOMR	7,500	**6.9**	79	42,000	19	6.4	13	12,000	1,300	430		19		39	X	T2
Mill Cr.	MCNF	5,500	**7.8**	80	71,000	14	8.1	10	17,000	2,800	570	9.8	21		44	X	T1,T2
Mill Cr.	MCSF	5,900		63	190,000			16	12,000	1,800	410				36	X	T2
Meadow R.	MEAD	9,900	**6.5**	86	34,000	17	11	17	20,000	2,100	850		18		46	X	T2
Muddy Cr.	MUDD	5,700	5.7	81	8,000	13	8.1	18	13,000	1,800	470		17		59	X	T1,T2
Naked Cr.	NAAU	4,400	**7.0**	39	85,000	24	6.6		20,000	1,500	740		13	24	20	X	T2
Naked Cr.	NAPA	3,600		53			7.5	8.1	17,000	1,400	310			24	25		
Passage Cr.	PASS	2,600	3.6	24		8.3	5.2		11,000		190	8.3			24		T1
Smith Cr. (DS; 4)	SMDS	8,500		90	72,000	13	8.7	15	18,000	2,400	670	13			52	X	T2
Smith Cr. (US)	SMHW	4,700	**8.6**	110	3,200	15	11	13	27,000	930	620	**22**	11	14	75	X	T2
Stony Cr. (DS; 3)	STDS	4,100	**6.5**	50	6,700	15	8.3	10	18,000	1,500	480	13	15		52		T1,T2
Stony Cr. (US)	STHW	3,700	5.9	70	4,300	10	8.2	11	15,000		270	16	7.9		55		T1,T2
RL		200	2.0	10	1,000	5.0	2.5	5.0	200	500	10	5.0	5.0	10	10		
TEL			5.9			37		28				18	35		123		
PEL			17			90		36				36	91		315		

Table 3- 3. Principal components (PCs) calculated from PCA of element concentrations in sediment. The eigenvalues, proportion of variance explained, and weighted eigenvector for each individual element are included for each PC. The first three PCs explained 77.2% of the variance in the data. Elements with eigenvector weights in bold are considered to be influential to the component.

	PC I	PC II	PC III
Eigenval.	5.10	2.80	1.35
Prop. Var.	0.425	0.234	0.113
Element	*Eigvct.*	*Eigvct.*	*Eigvct.*
Al	**0.334**	**0.262**	-0.049
As	**0.263**	**-0.374**	-0.177
Cr	**0.247**	**-0.371**	**-0.269**
Co	**0.281**	**-0.333**	**0.245**
Cu	**0.217**	**0.265**	**0.444**
Pb	**0.310**	-0.022	**-0.333**
Mn	**0.378**	0.032	-0.162
Zn	**0.316**	-0.056	**0.436**
Fe	**0.271**	**-0.364**	0.124
Ba	**0.304**	**0.233**	**0.347**
Ca (log_{10})	**0.287**	**0.305**	**-0.357**
Mg	**0.212**	**0.434**	**-0.216**

Table 3- 4. Correlation coefficients (Pearson's r) and their respective p-values (italics) between watershed-scale land use variables and PC scores. Only statistically significant (p<0.05) relationships are shown.

	PC I	PC II
Forest (%)	-0.61 *0.002*	-0.42 *0.04*
Past./Hay (%)	0.58 *0.003*	0.41 *0.04*
Crops (%)	0.49 *0.02*	0.49 *0.02*
Developed (%)	0.50 *0.01*	
Beef (√Density)	0.56 *0.004*	
Dairy (√Density)	0.50 *0.01*	
AFOs (√Density)	0.53 *0.008*	

Table 3- 5a. Results of partial least squares (PLS) regression analysis for predictor (X) variables. Results include model effect weights for individual land use variables (X weight vector, w), the proportion of variance explained by each factor, and the cumulative proportion of variance explained. Weights in bold are considered to be influential to the factor.

	Model effect weights							% Variation	
Factor	Past./ Hay (%)	Crops (%)	Devel (%)	Poultry (√Dens)	Beef (√Dens)	Dairy (√Dens)	WWTP (√MGD)	Indiv.	Total
1	**0.50**	**0.46**	**0.33**	**0.31**	**0.44**	**0.39**	0.02	61	61
2	**0.34**	-0.17	**0.73**	**-0.55**	-0.15	**-0.26**	**0.33**	16	77
3	**0.54**	-0.05	-0.06	-0.07	-0.19	**-0.26**	**-0.88**	11	88

Table 3-5b. Results of PLS regression analysis for dependent (Y) variables. Results include weights for individual element concentrations in sediment (Y weight vector, c), the proportion of variance explained by each factor, and the cumulative proportion of variance explained. Weights in bold are considered to be influential to the factor.

	Dependent variable weights												% Variation	
Factor	Al	As	Ba	Log_{10} Ca	Co	Cr	Cu	Fe	Mg	Mn	Pb	Zn	Indiv.	Tot.
1	**0.44**	0.07	**0.32**	**0.44**	-0.03	0.10	**0.31**	-0.01	**0.31**	**0.36**	**0.39**	0.17	18.3	18.3
2	**0.62**	-0.07	-0.03	**0.23**	**0.26**	**0.23**	**-0.24**	**0.33**	**0.33**	**0.34**	0.02	-0.01	3.6	21.9
3	0.17	-0.12	-0.19	**0.36**	-0.15	0.05	**-0.30**	-0.05	0.16	**0.37**	0.16	**-0.69**	4.2	26.1

Table 3- 6. Correlation coefficients (Pearson's r) and their respective p-values (italics) between watershed-scale land use variables and concentrations of trace elements measured in clam and snail tissue. Only statistically significant (p<0.05) relationships are shown.

	Clam tissue			Snail tissue
	Cd $Log_{10}(\mu g/g)$	Hg $Log_{10}(\mu g/g)$	Pb $Log_{10}(\mu g/g)$	Cd $Log_{10}(\mu g/g)$
Forest (%)	0.69 *0.0006*	0.70 *0.0004*	-0.57 *0.009*	0.64 *0.003*
Pasture/Hay (%)	-0.61 *0.003*	-0.62 *0.003*	0.51 *0.02*	-0.65 *0.002*
Crops (%)	-0.56 *0.009*	-0.61 *0.005*	0.52 *0.02*	-0.62 *0.004*
Developed (%)	-0.74 *0.0001*	-0.65 *0.001*	0.51 *0.02*	
Poultry (√Density)	-0.49 *0.02*	-0.67 *0.0009*		
Beef (√Density)	-0.54 *0.01*	-0.58 *0.006*		-0.48 *0.04*
Dairy (√Density)				-0.62 *0.004*
AFOs (√Density)	-0.57 *0.007*	-0.71 *0.0003*		-0.55 *0.01*

Figure 3- 1. Locations of the 25 study sites within the Shenandoah River watershed. The enlargement shows the 78 12-digit hydrologic unit code (HUC) subwatersheds of the Shenandoah River (indicated in grey) and the counties included in the entire watershed, relative to the state of Virginia.

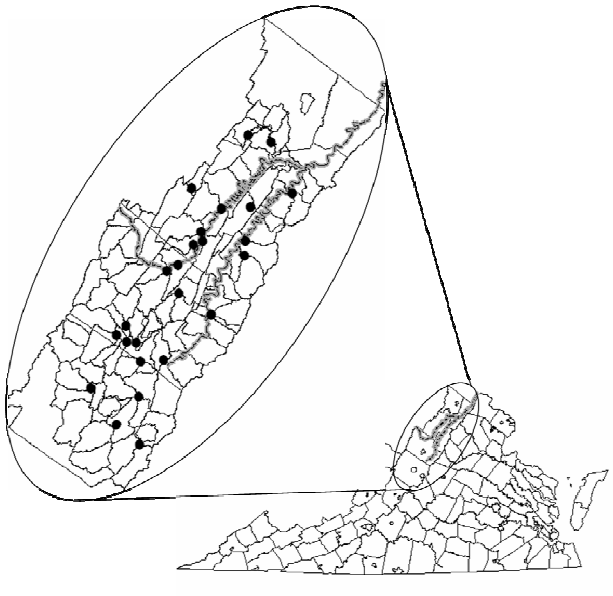


Figure 3- 2. Site-specific scores from the principal components analysis (PCA) of element concentrations in sediment (PC I vs. PC II). The proportion of variance explained by each component is listed in parentheses. Rays represent eigenvector weights for each element, scaled by a factor of five for visibility.

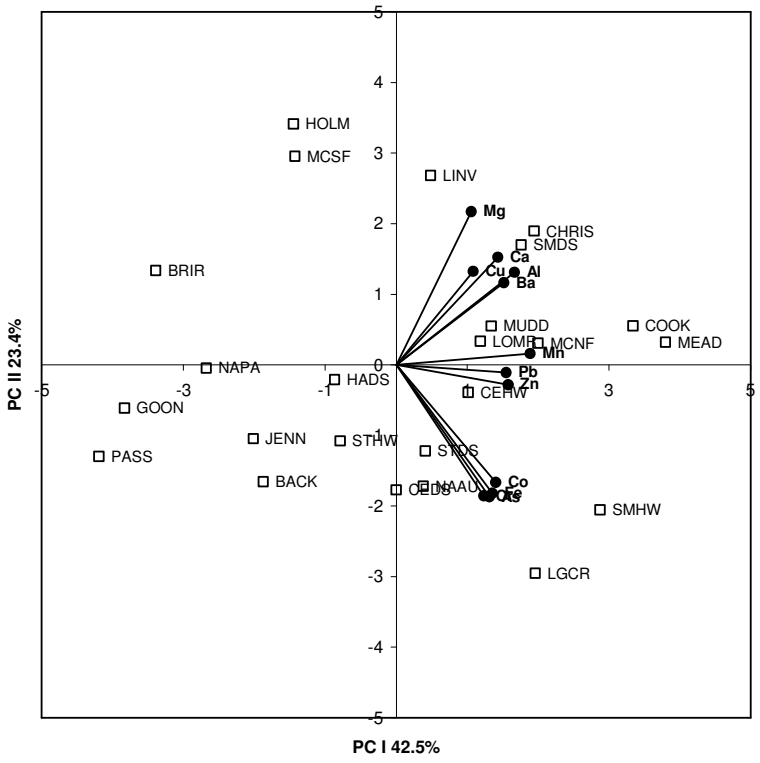


Figure 3- 3. Mean trace element concentrations in clam tissue (n=21) and snail tissue (n=19). Error bars represent standard error of the mean. For the 16 sites where both clams and snails were collected, concentrations of As, Cd, and Hg were significantly different between the two types of tissue (paired t-test, $|t|>2.26$, $p<0.04$), as indicated by an asterisk. Concentrations of Cr and Se in snail tissue were above reporting limits at <5 sites and Pb was not above reporting limits at any site where snails were collected.

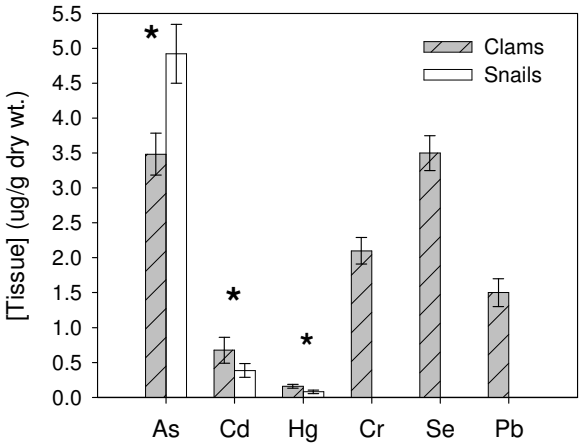


Figure 3- 4. Relationship between As concentrations in clam tissue (closed triangles), snail tissue (open triangles) and the watershed density of poultry AFOs (square-root transformed). The relationship between clam tissue As and the density of poultry AFOs was best described by a polynomial regression (spline curve; [As]=13.5x-15x2+2.9x3+1.7, R2=0.59, p=0.001), but this model is not necessarily predictive. The relationship between snail tissue As and poultry AFOs was not well-described by a mathematical relationship. At five sampling sites only clams were present, and at three sampling sites (flipped open triangles), only snails were present.

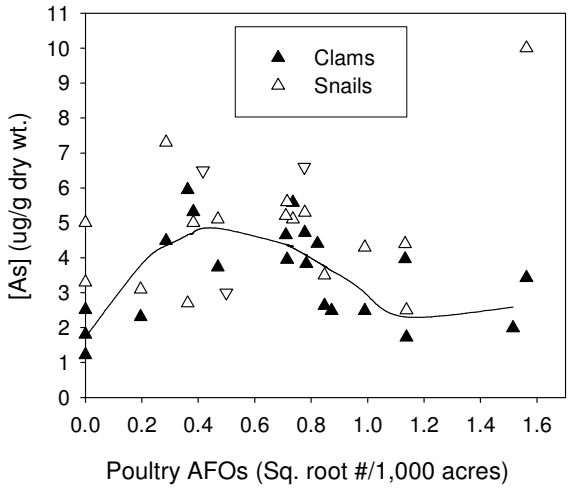
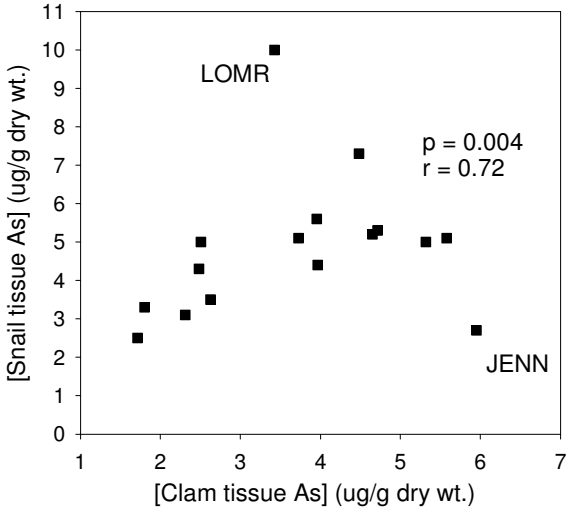


Figure 3- 5. Relationship between As concentrations in clam and snail tissue. The correlation was significant (r =0.72, p=0.004) when two sites (JENN and LOMR) with unusual snail population characteristics were excluded from the analysis.



CHAPTER 4

Effects of watershed densities of animal feeding operations on nutrient concentrations and estrogenic activity in agricultural streams

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Abstract

Application of manures from animal feeding operations (AFOs) as fertilizer on agricultural land can introduce nutrients and hormones (e.g. estrogens) to streams. A landscape-scale study was conducted in the Shenandoah River watershed (Virginia, USA) in order to assess the relationship between densities of AFOs in watersheds of agricultural streams and in-stream nutrient concentrations and estrogenic activity. The effect of wastewater treatment plants (WWTPs) on nutrients and estrogenic activity was also evaluated. During periods of high and low flow, dissolved inorganic nitrogen (DIN) and orthophosphate (PO_4-P) concentrations were analyzed and estrogens/estrogenic compounds were extracted and quantified as17β-estradiol equivalents (E2Eq) using a bioluminescent yeast estrogen screen. Estrogenic activity was measurable in the majority of collected samples, and 20% had E2Eq concentrations >1 ng/L. High concentrations of DIN (>1,000 µg/L) were also frequently detected. During all sampling periods, there were strong linear relationships between watershed densities of AFOs and in-stream concentrations of DIN (R^2=0.56-0.81) and E2Eq (R^2=0.39-0.75). Linear relationships between watershed densities of AFOs and PO_4-P were weaker, but were also significant (R^2=0.27-0.57). When combined with the effect of watershed AFO density, streams receiving WWTP effluent had higher concentrations of PO_4-P than streams without WWTP discharges, and PO_4-P was the only analyte with a consistent relationship to WWTPs. The results of this study suggest that as the watershed density of AFOs increases, there is a proportional increase in the potential for nonpoint source pollution of agricultural streams and their receiving waters by nutrients, particularly DIN, and compounds that can cause endocrine disruption in aquatic organisms.

Introduction

Livestock wastes contain high concentrations of nutrients and steroidal estrogens (Hanselman et al., 2003; Johnson et al., 2006; Mallin and Cahoon, 2003; USDA, 1992). These compounds may enter surface waters through runoff or leachate from agricultural land that has received applications of manure from animal feeding operations (AFOs) as fertilizer (Finlay-Moore et al., 2000; Kjaer et al., 2007; Matthiessen et al., 2006; Shore et al., 1995). Surface water contamination by nutrients and hormones in animal waste can also occur when grazing animals deposit waste into or directly adjacent to bodies of water (Kolodziej and Sedlak, 2007). The negative effects of excess nutrients in surface waters are well documented (e.g. Boesch et al., 2001) and many studies have shown that

estrogens can disrupt endocrine system function of aquatic organisms at concentrations less than 10 ng/L (Young et al., 2004). In regions with high densities of AFOs, there is increasing concern that high rates of manure application on local agricultural land will lead to eutrophication of surface waters and potential endocrine-related effects in aquatic biota (Kellog et al., 2000; Mallin and Cahoon, 2003; Yonkos et al., 2010).

Relationships between AFOs, nutrients, estrogens, and streams require further assessment in order to manage livestock waste effectively and protect the health of surface waters in agricultural landscapes. A recent study of Iowa rivers in watersheds receiving wastes from AFOs showed a strong relationship between nitrate concentrations and watershed densities of animal units within the AFOs (Weldon and Hornbuckle, 2006), but hormone concentrations were not evaluated. Most studies of estrogens or estrogenic activity in livestock wastes have focused on the potential for aquatic contamination, through measurement of concentrations in storage facilities (Hutchins et al., 2007; Raman et al., 2004) or concentrations in runoff and leachate from manure-treated fields (Finlay-Moore et al., 2000; Kjaer et al., 2007; Nichols et al., 1997). In-stream assessments have generally been conducted in headwater streams adjacent to individual feedlots or farms receiving waste applications (Matthiessen et al., 2006; Shore et al., 1995). These studies isolated the effects of animal wastes on concentrations of estrogens in streams, but did not assess the cumulative effects of animal wastes that may occur in streams with larger drainage areas.

The relationship between livestock production and water quality is of particular interest in the Shenandoah River watershed (Virginia, USA). The 7,600 km^2 watershed is 39% agricultural land, which receives manure from approximately 1200 AFOs and 300 farms that maintain grazing beef cattle (VADCR, 2010; VADEQ, 2006). Seasonal fish kills have occurred in the Shenandoah River since 2004 and resident smallmouth bass have impaired immune function (Ripley et al., 2008) and a high proportion of males with intersex compared to other basins (Blazer et al., 2007). Because estrogenic compounds can induce intersex (Hahlbeck et al., 2004; Lange et al., 2009) and affect the immune function of fishes (Iwanowicz and Ottinger, 2009; Robertson et al., 2009), assessment of their presence in the watershed and relationship to land use is warranted. Nutrient concentrations are of concern due to risk of local eutrophication and potential effects on the health of aquatic organisms (Camargo et al., 2005; Guillette and Edwards, 2005; Johnson et al., 2010). The relationship between nutrient concentrations and land use has management implications outside of the Shenandoah River watershed, as the Shenandoah discharges into the Potomac River, a major tributary of Chesapeake Bay. In order to adequately protect and restore the health of

Chesapeake Bay, estimated required reductions in nitrogen and phosphorous loadings for the Shenandoah and Potomac Rivers are 44% and 29%, respectively (Commonwealth of Virginia, 2005).

Comprehensive assessment of the potential effect of AFOs on concentrations of estrogens and nutrients in streams within agricultural landscapes requires consideration of additional sources of these compounds. The Shenandoah River watershed has 81 municipal wastewater treatment plants (WWTPs); the majority (71) are minor facilities discharging less than one million gallons of effluent per day. Effluent from WWTPs can be a significant source of natural and synthetic estrogens from human excretion, and synthetic xenoestrogens from household and industrial use, to surface waters (Lagana et al., 2004; Muller et al., 2008; Petrovic et al., 2002; Ying et al., 2008). Depending on the level of treatment, WWTPs can also contribute significant nutrient loads to receiving waters, and reducing these loads is a significant component of the strategy to protect and restore the health of Chesapeake Bay (Commonwealth of Virginia, 2005).

The main objective of this study was to evaluate relationships between watershed densities of AFOs and concentrations of nutrients and estrogenic activity in streams within the larger Shenandoah River watershed. A secondary objective was to examine the effect of WWTPs alone and in combination with AFOs on nutrients and estrogenic activity. Increasing watershed densities of AFOs and effluent from WWTPs were expected to increase concentrations of nutrients and estrogenic activity in streams. Estrogenic activity was selected over measurement of individual compounds in order to assess the overall potential for biological activity of estrogens and estrogenic compounds in stream water, and to evaluate the utility of screening techniques in large-scale water quality monitoring programs.

Methods

Selection and characterization of study sites

Land use in the Shenandoah River watershed was characterized using a geographic information system (ArcGis 9.3, ESRI, Redlands, CA). Delineated 12-digit, 6th level Hydrologic Unit Code (HUC 6) subwatersheds, 40-160 km^2 in size, were used to quantify all land uses within drainage areas of Shenandoah River tributaries. Locations and numbers of animal units for AFOs (poultry, dairy, and beef), farms maintaining grazing beef cattle, and locations and permit information for WWTPs were obtained from Virginia state agencies. In Virginia, AFOs are

defined as facilities that confine animals for at least 45 days and preclude the growth of vegetation, while concentrated AFOs maintain >300 animals (cattle or swine). In the Shenandoah River watershed, the 680 poultry AFOs maintain 10,000-200,000 birds and all hold Virginia Pollution Abatement (VPA) permits. Only 21 of 430 dairy AFOs and three of 110 beef AFOs are concentrated operations and require VPA permits. For this study, the 278 farms maintaining grazing beef cattle were included in beef AFO calculations, because of a similar risk of contamination of surface water from manure of grazing animals (Kolodziej and Sedlak, 2007; Soupir et al., 2006). Many pastures in the area have noticeably high concentrations of cow feces, particularly during winter/early spring, and cattle are allowed access to streams for water during the summer in many areas (personal observation).

Eighteen sampling sites were selected to represent a gradient of influence from AFOs combined with the presence or absence of WWTP discharges as described in Chapter 2. (Figure 4-1). Sampling sites were located in 14 Shenandoah River tributaries; four tributaries drained multiple subwatersheds and an upstream (US) sampling site was located in the primary subwatershed as well as a downstream (DS) site draining multiple subwatersheds (Table 4-1). Eight sampling sites could not be located near the outlet of the delineated HUC 6 subwatershed due to limited tributary access, and the watershed area was recalculated using U.S. Geological Survey Digital Elevation Model (DEM) data (30 m resolution) and the hydrology toolset in ArcGis. The new delineation was used to quantify upstream land use variables. The number of each type of AFO and each type of animal in each watershed was converted to a density (number/1000 acres) and the total watershed density of AFOs (all types) was calculated for each sampling site. For poultry, dairy, and beef AFOs, the watershed density of animals was strongly correlated with the watershed density of each type of operation ($r = 0.96$, 0.99, and 0.88, respectively), so only AFO density was used in data analysis. For each watershed with a WWTP discharge, the WWTP permit information was used to calculate the total permitted effluent discharge (in millions of gallons per day; MGD). Pasture/hay and cropland are primary application sites for manure from AFOs. Therefore, land cover data for the Shenandoah River watershed (30 m resolution) were obtained from the 2001 National Land Cover Database (Homer et al., 2007). Reclassification and areal tabulation were used to quantify the percentage of forest, developed land, pasture/hay, and cultivated crops upstream of each sampling site. Percentages of all other land use types (i.e. open water, barren land, etc.) were negligible (<1%) in each watershed of interest. For each study site, a total of nine land use variables were quantified (Table 4-1).

Field sampling and water quality analysis

Field sampling was conducted in late spring (14-24 May 2008, 21-30 May 2009), mid-summer (8-14 August 2008, 10-11 August 2009), and late winter (10-15 March 2009 only). Late spring represented a period of relatively high flow after the majority of spring manure applications had occurred and mid-summer represented near-baseflow conditions. Late winter was selected to represent a time period prior to spring manure applications, but due to low precipitation, this period also represented relatively low-flow conditions in 2009. Discharge data from the North Fork Shenandoah River (Mount Jackson, VA) illustrate these trends (http://nwis.waterdata.usgs.gov/va/nwis/dv?cb_00060=on&format=gif_default&begin_date=2008-03-01&end_date=2009-10-01&site_no=01633000&referred_module=sw). Discharge was calculated at each site for all 2009 sampling events using the midsection method (Gore, 2006), with velocity and depth measured using a Marsh-McBirney Model 2000 Flow-Mate meter (Marsh McBirney, Inc., Frederick, MD). Discharge data were used to calculate loadings of nutrients and estrogenic compounds for the 2009 sampling periods.

Temperature and specific conductivity were measured at each site using a calibrated YSI 30 Salinity/Conductivity/Temperature meter (YSI Incorporated, Yellow Springs, OH). Water samples collected for nutrient analysis were field-filtered using an acid-washed syringe filtration apparatus containing a pre-ashed GF/F filter (0.7 μm). Filtrate was collected in sterile 50 ml polypropylene tubes, preserved with 0.01% v/v chloroform, held on ice, and stored at 4 ^{o}C upon return to the laboratory. Within two weeks of collection, dissolved nutrients (NO_3-N, NH_4-N, and PO_4-P) were analyzed using flow injection analysis (Lachat QuickChem 8500, Hach Co., Loveland, CO) following standard methods (APHA 2005a, 2005b, 2005c). The mean relative percent differences (RPDs) for field duplicates (8 pairs) were 1.3%, 14%, and 8.0% for NO_3-N, NH_4-N, and PO_4-P, respectively. Concentrations of NO_3-N and NH_4-N were summed to total dissolved inorganic nitrogen (DIN) for data analysis. In the majority of samples, NH_4-N concentrations were <5% of total DIN.

Water samples were collected for analysis of estrogenicity using 900 ml pre-cleaned amber glass bottles (I-Chem, Rockwood, TN), acidified to pH 3 with concentrated HCl, held on ice, and stored at 4 ^{o}C upon return to the laboratory. Within one week of collection, the preserved water samples were filtered through a pre-weighed pre-ashed (500 ^{o}C) GF/F filter (0.7 μm) using a solvent rinsed all-glass apparatus. Methanol (1-1.5 ml) was used to wash

any estrogenic compounds off the retained suspended solids. One blank (HPLC water at pH 3) was filtered with every batch of 10 samples (two per sampling date). Filtered samples and blanks were subjected to solid phase extraction (SPE) using OASIS® HLB (200 mg) glass cartridges (Waters Corporation, Milford, MA), following a modified version of a method published by Lagana et al., (2004). All solvents were HPLC grade. Cartridges were sequentially pre-conditioned with 5 ml each of: ethyl acetate, 50:50 methanol:dichloromethane (DCM), methanol, and HPLC-grade water. Each sample (400 ml) was loaded onto the cartridge at a flow rate of 5-6 ml/minute (continuous vacuum). Elution solvents consisted of 6 ml methanol and 6 ml of 50:50 methanol:DCM, which were combined into fraction one. Ethyl acetate (6 ml) was the final elution solvent (fraction two). Eluates were reduced to dryness under ultra high purity N_2 and re-dissolved in 1 ml of methanol. Preliminary analyses of spiked samples indicated no estrogenic activity in fraction two. Therefore, only fraction one was analyzed.

The total concentration of estrogenic compounds in the water samples relative to 17β-estradiol (E2) was estimated using a bioluminescent yeast estrogen screen (BLYES). Yeast (*Saccharomyces cerevisiae*) was bioengineered to contain the human estrogen receptor, plasmid-bound estrogen response elements, and plasmid-bound luminescence reporters (Sanseverino et al., 2005). The BLYES assay was performed at the U.S. Geological Survey (USGS) National Fish Health Laboratory (Leetown, WV). Strain BLYES was a kind gift from Dr. John Sanseverino (University of Tennessee) and was maintained in a dormant stage at 4°C in modified Yeast Minimal Media (YMM leu-, ura-; Routledge and Sumpter, 1996). Preparation for the screening assay involved the expansion of strain BLYES to early stationary phase in YMM at 30°C on a rotary shaker to an approximate OD_{600} of 0.750. The BLYES assay was performed in sterile, clear-bottom, black polystyrene 96-well assay plates (Costar, Corning Incorporated, Corning, NY). Samples were diluted to 10% in YMM, and 100 µl of the diluted sample was added to each well in duplicate. All assay plates included a 12-point standard curve consisting of E2 ranging from 2.3×10^{-5}–0.50 µM and sample blanks containing YMM only. Strain BLYES was added to all preloaded wells at a volume of 100 µl, resulting in a final sample dilution of 5%. Plates (covered) were incubated in the dark at 30 °C for 6 h on an orbital shaker. Luminescence was quantified using a SpectraFluor Plus plate reader (Tecan Group Ltd, Durham, NC), in luminescence mode (1 s integration time/well, gain 180). Luminescence was read over two kinetic cycles separated by 159 s and orbital shaking (5 s). Data from the second kinetic cycle were analyzed.

The BLYES responds to E2 as a standard non-linear dose-response curve (Sanseverino et al., 2005), and produced maximum luminescence from 5.6×10^{-3}-0.050 µM E2 after 6 h of incubation (Figure 4-2). In order to

avoid potential errors in non-linear curve fitting at the lower end of the standard curve (where most sample concentrations were located), a linear calibration curve was created using $\log_{10}$ transformations of the five lowest standards (2.3×10^{-5}–2.1×10^{-4} μM E2) and their associated mean luminescence (Figure 4-2). Concentrations in samples with luminescence above this range were quantified using four points from the linear portion of the dose-response curve ($\log_{10}$[E2] vs. mean luminescence; 1.2×10^{-4}-1.9×10^{-3} μM E2) (Figure 4-2). Mean luminescence values of zero wells (YMM only) were subtracted from luminescence values of all E2 standards prior to calculation of standard curves. The quantitation limit (QL) of E2 was 2.3×10^{-5} μM (0.31 ng/L in samples), luminescence for this standard was always >600 units above that of YMM-only wells. Luminescence of blanks was never above that of the lowest standard, the difference in mean luminescence between blanks and YMM ranged from 82-282 units. Mean luminescence of the blanks was subtracted from the raw luminescence for all samples collected on that date (same plate) prior to calculation of sample concentrations. Concentrations were calculated from luminescence in each duplicate well, and the final reported concentration is the mean value. All calculated sample concentrations are reported as ng/L E2 equivalents (E2Eq). For E2Eq concentrations below the QL (<33% of samples during any sampling period), 0.156 ng/L (1/2 QL) was substituted for statistical analyses and load calculations.

Samples of HPLC water and samples of site water (from Cedar Creek) were spiked with E2 (100-295 ng) to assess method recoveries. Using the BLYES for quantification, the mean percent recovery of E2 was 93% in HPLC water and 91% in site water. The extracts were also analyzed for E2 at the Virginia Institute of Marine Science (Gloucester Point, VA) using the UPLC/MS^2 method developed by Rice and Hale (2009). The mean percent recovery of E2 was 91% in HPLC water and 90% in site water. Using BLYES, the mean RPD for field duplicates (6 pairs) was 13%. Variability of BLYES assay results was assessed by analyzing 10 samples on two different dates, and the mean RPD was 12%.

Data analysis

Due to co-occurrence of different types of AFOs within watersheds, the total watershed density of AFOs was examined as potentially predictive of concentrations and loadings of E2Eq, DIN, and PO_4-P. Relationships between AFO density and analytes were assessed using regression analysis. Independent and dependent variables were examined for potential outliers and tested for normality prior to statistical analyses. The interquartile range*1.5 added to or subtracted from upper and lower quartiles (25^{th} and 75^{th} percentiles) was used to identify outliers in the

dataset and their effect on statistical analyses was tested, as described below. Variables were tested for normality using the Shapiro-Wilk W test. Non-normal data were transformed as follows: $\log_{10}$ for concentration/loading data and square root for density and proportion data. The influence of outliers on the final regression model was assessed through calculation of Cook's D (Montgomery et al., 2006). If outliers had Cook's D values >0.25, they were removed from the model and the resulting changes in R^2 and slope were assessed. Concentrations of only one analyte from one site (PO_4-P at Muddy Creek) were excluded due to inflation of the R^2 (change >10%) and slopes (change >20%) of regression models if included (see results section).

The R^2 of the final models were compared to R^2 values for regressions of watershed percentages of the land cover variables and concentration/loading data in order to assess the relative effect of watershed density of AFOs. Colinearity between watershed density of AFOs and watershed percentages of pasture/hay and cropland ($r>0.81$ for all combinations) prohibited the use of multiple regression to assess their potential combined effects. Therefore, an "agricultural index" (AI) was calculated for each site, where AI = (proportion of pasture/hay + proportion of cropland) * density of AFOs. Relationships between the AI and analyte concentrations/loadings were also assessed using regression analysis and R^2 values were compared with other models.

Multiple methods were used to examine the potential influence of WWTPs on analyte concentrations and loadings. Discharge data from each sampling site and the total permitted flow of WWTP effluent in the watershed were used to estimate the proportion of stream flow as WWTP effluent. The relationship between this estimated proportion of WWTP effluent and analyte concentrations/loadings was assessed using univariate regression analysis and stepwise multiple regression in combination with total AFO density (variable entry criterion of $p\leq0.10$). Although discharge was not measured in 2008, seasonal flow conditions were similar between 2008 and 2009. Therefore, the estimated proportion of WWTP effluent in May and August 2009 were tested as predictors of analyte concentrations in May and August 2008. While discharge at each sampling site is unlikely to have been exactly the same between the two years, the relative proportions of stream flow as WWTP effluent between the different tributaries were expected to be consistent under similar flow conditions (high or low). Treating WWTP effluent as a continuous variable had multiple sources of error, including: potential differences in actual vs. permitted effluent flow, high temporal variability in effluent flow, and assumptions associated with extrapolation between 2009 and 2008. The potential effects of WWTPs on analyte concentrations/loadings were also assessed categorically; the

presence or absence of WWTPs in each watershed was utilized as a treatment variable in an analysis of covariance (ANCOVA), as described below.

A two-way ANCOVA was used to simultaneously test the effects of WWTP presence/absence and sampling period on analyte concentrations/loadings (y), while taking into account significant relationships with the watershed density of AFOs (covariate, x). Initially, all variables and possible interactions were included in the models. Non-significant interaction terms were sequentially removed until final models with only significant variables were produced (Milliken and Johnson, 1984). If there were no significant covariate-treatment interactions (equal slopes), pairwise comparisons (t-test) of least-squares adjusted means (X= $\bar{x}$) were used to quantify significant treatment effects. If significant covariate-treatment interactions were present (unequal slopes), regression models and least-squares adjusted means were compared at three different points: $\bar{x}$, mean of three lowest x values, and mean of three highest x values (Milliken and Johnson, 1984).

All correlations within independent and dependent variables were evaluated using Pearson product moment correlation analysis. All statistical analyses were performed using SAS 9.2 (SAS Institute, Cary, NC), with a significance level of α=0.05.

Results

Within the 90 water samples collected during this study, concentrations of DIN ranged from 5.0 – 7,588 µg/L (Table 4-2, Figure 4-3). Concentrations of E2Eq ranged from below the QL (0.31 ng/L; 21 samples) - 7.2 ng/L (Table 4-2, Figure 4-4). There were significant relationships between watershed density of AFOs and concentrations of DIN and E2Eq for all five sampling periods (Table 4-3). For both analytes, the proportion of variance explained by the relationship was greater during high flow (May; Table 4-3). As the watershed density of AFOs increased over 1 AFO/1000 acres, the frequency of E2Eq concentrations >1 ng/L and DIN concentrations >1,000 µg/L generally increased (Figures 4-3 and 4-4a). Concentrations of PO_4-P ranged from 1.5 – 1,150 µg/L over the five sampling periods (Table 4-2, Figure 4-5). The watershed density of AFOs predicted concentrations of PO_4-P for all sampling periods except March 2009 (Table 4-3), and the strength of these relationships and proportion of variance explained were less than those for DIN and E2Eq. Exceptionally high PO_4-P concentrations were measured at Muddy Creek in August 2008, March 2009, May 2009, and August 2009 (1.15×10^3, 349, 345, and 503 µg/L, respectively), and

these outliers were not included in regression analyses due to their influence on the results (inflation of R^2 values and slopes) and the multiple potential sources of PO_4-P at this site. The Muddy Creek watershed had the highest AFO density in this study, but the stream also receives effluent from one small municipal WWTP (0.005 MGD, estimated proportion of flow 0.21-0.17%), and a poultry processing facility (unknown MGD), which warrants further investigation as a potential source of PO_4-P.

When compared to relationships with watershed percentages of pasture/hay and crops, relationships with watershed density of AFOs explained a greater proportion of variance in E2Eq, DIN, and PO_4-P concentrations for all sampling periods (Table 4-3 and Table 4-4). Relationships between watershed percentage of developed land and the three analytes were not significant ($R^2 < 0.22$, $p \geq 0.06$), with the exception of one significant relationship with PO_4-P ($R^2=0.25$, $p=0.04$) in August 2008. The relationship between the watershed percentage of forest and concentrations of E2Eq, DIN, and PO_4-P were always negative, with similar R^2 (± 0.04) and p-values as the relationships between these analytes and watershed percentage of pasture/hay. The combination of watershed percentages of pasture/hay and crops with watershed density of AFOs into an agricultural index (AI) did not result in explanation of a greater proportion of variance in analyte concentrations than watershed density of AFOs alone (Table 4-3 and Table 4-4). Thus, watershed density of AFOs was the best predictor of concentrations of E2Eq, DIN, and PO_4-P during all sampling periods, with the exception of PO_4-P concentrations during March 2009. For this sampling period, only the estimated proportion of flow as WWTP effluent had a significant linear relationship with PO_4-P concentrations (Table 4-3). This was the only time period and analyte for which the estimated proportion of flow as WWTP effluent alone was predictive. For PO_4-P concentrations only, the combination of watershed density of AFOs and the estimated proportion of flow as WWTP effluent in a multiple regression explained a greater proportion of variance than either variable alone during three sampling periods: August 2008, March 2009, and May 2009 (Table 4-3).

Results of ANCOVA indicated no effect of sampling period ($p=0.40$) or WWTP presence/absence ($p=0.59$) on DIN concentrations with regard to the overall relationship with watershed AFO densities (Figure 4-3, Table 4-5). For E2Eq concentrations, ANCOVA indicated a significant effect of both sampling period ($p=0.02$) and WWTP presence/absence ($p=0.03$), with regard to the overall relationship with watershed densities of AFOs. Although the slope of the relationship between E2Eq concentrations and watershed density of AFOs appeared different in August 2008 compared to the other sampling periods (Figure 4-4a), there was no significant interaction between sampling

period and watershed density of AFOs (p=0.56), and the slopes were considered equal. There was also no significant interaction between sampling period and WWTP presence/absence (p=0.60), but there was a significant interaction between watershed densities of AFOs and WWTP presence/absence (p=0.02). Separate models of the main effects were created in order to evaluate differences in adjusted means (Figures 4-4a and 4-4b). For sampling period, pairwise comparisons of least-squares adjusted means indicated that amongst the low flow sampling periods, adjusted mean E2Eq concentrations were significantly lower ($p \leq 0.012$) in August 2008 than in March 2009 and August 2009 (Table 4-5). The only significant difference between high and low flow periods was higher adjusted mean E2Eq concentrations in March 2009 than in May 2009 (p=0.035, Table 4-5). Adjusted mean concentrations in May 2008 were not significantly different from any other sampling periods ($p \geq 0.073$). Because the equal slopes hypothesis was rejected for the effect of WWTP presence/absence (Figure 4-4b), regression models and least-squares adjusted means were evaluated at three points (x= 0.10, 0.94, and 1.87). This analysis indicated that the regression models were not equal at the lowest watershed AFO density (p=0.047), but were equal at the two higher AFO densities ($p \geq 0.11$). Consequently, least-squares adjusted means were only significantly different at the lowest AFO density (p=0.047), where the adjusted mean E2Eq concentration was higher at sampling sites with at least one upstream WWTP (0.27±0.05 ng/L) compared to sampling sites with no upstream WWTPs (0.16±0.04 ng/L). However, these concentrations should be interpreted with caution as both are below the QL for E2Eq (0.31 ng/L) as a result of ½ QL substitutions for statistical analyses.

For PO_4-P concentrations, ANCOVA did not include the March 2009 sampling period due to the lack of a significant univariate relationship with watershed density of AFOs. There was no effect of the other four sampling periods (p=0.42) on PO_4-P concentrations (Table 4-5), but there was a significant effect of WWTP presence/absence (p=0.0006), when the relationship with AFOs was taken into account (Figure 4-5), with no interaction between WWTP presence/absence and watershed density of AFOs (p=0.26). Least-squares adjusted mean PO_4-P concentrations were significantly higher (p=0.0008) at sampling sites with at least one WWTP located upstream (16.6±2.3 µg/L) compared to sampling sites with no upstream WWTPs (8.5±1.2 µg/L). Comparison of unadjusted mean concentrations of PO_4-P in March 2009 also indicated significantly higher (p=0.01) concentrations at sites with upstream WWTPs (12.0±4.2 µg/L) compared to sites without upstream WWTPs (2.95±1.0 µg/L). For sites with upstream WWTPs, the unadjusted mean March 2009 PO_4-P concentration was similar to the adjusted mean

concentration during the other sampling periods. However, for sites without upstream WWTPs, the unadjusted mean March 2009 PO_4-P concentration was lower than the adjusted mean concentration for the other sampling periods.

Estimated loadings (normalized to watershed area) of E2Eq ranged from 0.01-1.8 mg/km^2/d and loadings of DIN ranged from 0.6-5,841 g/km^2/d over the three sampling periods when discharge was measured (Table 4-2). Estimated loadings of PO_4-P ranged from 0.2-61 g/km^2/d over the three sampling periods, excluding loadings calculated from outlying concentrations measured in Muddy Creek (49, 377, 137 g/km^2/d in March, May, and August 2009, respectively), which were not included in statistical analyses. Loadings of all three analytes were higher during May 2009 compared to March and August 2009, following the pattern of relative discharge (Table 4-2). Similar to concentrations, there were significant relationships between watershed density of AFOs and loadings of DIN and E2Eq for all three sampling periods (Table 4-3), and watershed density of AFOs was the best predictor of loadings of all analytes relative to land cover variables. Watershed density of AFOs predicted loadings of PO_4-P in May and August 2009, but not in March (Table 4-3). Similar to concentrations, PO_4-P loadings in March were predicted by the estimated proportion of flow as WWTP effluent, and a combination of this measure and watershed density of AFOs improved prediction of PO_4-P loadings in May 2009 (Table 4-3).

For loadings of all analytes (excluding PO_4-P in March), ANCOVA indicated a significant effect of sampling period ($p \leq 0.0002$) when the relationship with watershed density of AFOs was taken into account, with no interaction between variables ($p \geq 0.18$), including WWTP presence/absence. Adjusted mean loadings of DIN and E2Eq were significantly higher in May ($p \leq 0.005$) than in March and August (Table 4-5). Adjusted mean loadings of DIN and E2Eq during March and August were not statistically different ($p > 0.093$). Adjusted mean loadings of PO_4-P were significantly higher in May than in August ($p < 0.0001$; Table 4-5). There was no significant effect of WWTP presence/absence on loadings of PO_4-P (excluding March), E2Eq, and DIN ($p = 0.064$, 0.10, and 0.41, respectively). Unadjusted mean loadings of PO_4-P in March were significantly higher ($p = 0.011$) at sites with at least one upstream WWTP (2.6 g/km^2/d) compared to sites with no upstream WWTPs (0.56 g/km^2/d).

Concentrations of DIN were correlated with concentrations of E2Eq ($r = 0.56$, $p < 0.0001$) and PO_4-P ($r = 0.51$, $p < 0.0001$), and there was a weaker correlation between concentrations of PO_4-P and E2Eq ($r = 0.35$, $p = 0.001$). Loadings of all three analytes were correlated, including DIN and E2Eq ($r = 0.69$, $p < 0.0001$), DIN and PO_4-P ($r = 0.63$, $p < 0.0001$), and PO_4-P and E2Eq ($r = 0.63$, $p < 0.0001$). Coefficients for correlations between analyte loadings were larger than those for correlations between analyte concentrations.

Discussion

Concentrations and sources of DIN and estrogenic activity

Results of this study suggest that as densities of AFOs increase in watersheds of Shenandoah River tributaries, the amount of manure applied in the local area increases, which directly affects in-stream concentrations of nutrients and estrogenic activity. Watershed densities of AFOs had the strongest relationships with in-stream DIN concentrations during all sampling periods. A similar relationship between NO_3-N concentrations and AFO densities was described by Weldon and Hornbuckle (2006) for Iowa watersheds that were larger (area >500 km^2) than watersheds in the current study (38-334 km^2). The similar effect of watershed AFO density on dissolved N concentrations between two different ecoregions and size classes of streams supports previous general hypotheses that the risk of nonpoint source nutrient pollution is greater for streams in regions with high densities of AFOs (Carpenter et al., 1998; Kellog et al., 2000; Mallin and Cahoon, 2003). In this study, over 50% of the water samples had DIN concentrations >1,000 µg/L and these samples were primarily collected from tributaries with watershed AFO densities >1 AFO/1000 acres. These concentrations indicate potential risk for local eutrophication; proliferation of benthic algae has been documented at DIN and total N concentrations less than 500 µg/L (Biggs, 2000; Dodds and Welch, 1997; Miltner, 2010). In addition, the ability of streams to remove NO_3-N through biotic uptake decreases rapidly between 150 and 1,500 µg/L (Mulholland et al., 2008), and the strength of the linear relationship between DIN concentrations at the watershed outlet and watershed AFO density suggests that these streams are exporting a large proportion of catchment-derived DIN. Downstream transport of DIN to the Shenandoah River and beyond is of concern due to the imperiled condition of Chesapeake Bay, where phytoplankton growth is N-limited in higher salinity areas and during the summer (Boesch et al., 2001). Mulholland et al. (2008) highlighted the importance of controlling nitrogen export by small streams in order to prevent downstream problems with eutrophication and hypoxia.

Relationships between watershed density of AFOs and in-stream estrogenic activity were similar to those for DIN. Estrogenic activity in Shenandoah River tributaries with high watershed densities of AFOs may present a risk to aquatic biota. Using a compilation of studies on endocrine disruption in fish, Young et al. (2004) derived 1 ng/L E2Eq as the predicted no-effect concentration of total estrogens on fish reproduction. In the current study, 18 samples from 10 sites had concentrations >1 ng/L E2Eq, and the majority of these sites had watershed AFO densities >1 AFO/1000 acres. Although land-applied manure from AFOs is a diffuse source of estrogens, the effect

of watershed application rates on estrogenicity in streams appears to be cumulative. The range in estrogenic activity observed during this study was similar to estrogen concentrations and total activity measured in streams with more concentrated sources, including: streams draining fields receiving waste from poultry AFOs (up to 5 ng/L E2; Shore et al., 1995), streams adjacent to pastures receiving waste from dairy and beef AFOs with or without grazing ewes (up to 9.2 ng/L E2Eq; Matthiessen et al., 2006), and rangeland creeks accessed by grazing cattle (up to 10.6 ng/L E2Eq assuming activity of 17α-estradiol=0.05*E2; Kolodziej and Sedlak, 2007). A similar range in estrogenic activity (up to 7 ng/L E2Eq) has also been measured in treated WWTP effluent (Salste et al., 2007) and in rivers receiving effluent from WWTPs (Vermeirssen et al., 2005). However, there was no consistent measurable effect of WWTPs on estrogenic activity in this study, and methods for quantifying this effect were validated by results for PO_4-P (as discussed below). The apparent effect of WWTPs on estrogenic activity at a low watershed density of AFOs was due to three samples; two streams had measurable estrogenic activity during summer sampling periods when camp WWTPs were operating and one stream with an AFO density < 1 AFO/1000 acres and a WWTP had estrogenic activity >1 ng/L during the late winter sampling period. At higher AFO densities, estrogenic activity was nearly identical between sites with and without WWTPs. The effect of watershed density of AFOs outweighs any effect of WWTPs on in-stream estrogenic activity in these tributaries, which may be due to the low effluent volume generated by the WWTPs.

Watershed density of AFOs explained the greatest proportion of variance in concentrations of both DIN and E2Eq during high flow conditions (May). Manure is applied almost year-round in Virginia (except mid-winter), but application rates are generally greatest in late spring/early summer (Marsh et al., 2009). The combination of high manure application rates and heavy rainfall during late spring may facilitate the transport of DIN and estrogens/estrogenic compounds to surface waters. High concentrations of inorganic and organic N (1-20 mg/L) and E2 (9-3,500 ng/L) have been measured in runoff from manure-treated fields (Finlay-Moore et al., 2000; Jenkins et al., 2006; Mishra et al., 2006; Nichols et al.,1997; Sistani et al., 2010; Soupir et al., 2006). Leaching into soil water and subsequent transfer through interflow is another pathway for nutrients and estrogens/estrogenic compounds in land-applied manure to enter surface waters (Daliparthy et al., 1995; Herman and Mills, 2003; Hyer et al., 2001; Kjaer et al., 2007). The high mobility of NO_3-N from animal manures is due to a combination of its solubility and production from mineralization and nitrification, which may occur in the soil after manure application. Unlike soluble DIN, colloid-mediated transport enhances the mobility of estrogens through the vadose zone (Steiner et al.,

2010; Thompson et al., 2009). Higher solubility of DIN compared to compounds contributing to estrogenic activity may account for the stronger relationships between DIN and watershed density of AFOs during all sampling periods. For both analytes, the lack of significantly higher concentrations during high flow conditions is likely due to the watershed-scale sampling design; runoff and interflow from areas with and without AFOs are combined in stream water sampled at the watershed outlet.

The presence of DIN and estrogens/estrogenic compounds in groundwater was indicated by their detection during summer low flow conditions (August) and the unusual late winter low flow condition in 2009. Significant relationships with watershed densities of AFOs were maintained during these periods, implying that land-applied manure is the source of DIN and estrogenicity in groundwater. An alternative explanation to analyte detection in stream water during summer sampling periods could be increased presence of grazing cattle in the streams, but this would not account for measured concentrations in late winter. Estrogens have been measured in springs from karst aquifers underlying areas of intensive poultry and cattle production (Peterson et al., 2000) and groundwater contamination by NO_3-N in aquifers underlying fields receiving animal manures is well documented (Andres et al., 1995; Gould et al., 1995; Karr et al., 2001; Lindsey et al., 2003). Many streams in the valley region of the Shenandoah River watershed are underlain by carbonate rocks (Yager et al., 2008). The valley region also contains the greatest concentration of AFOs, which creates a spatial correlation between karst features and potential sources of contaminants. Rapid linear transport (> 2 miles) and discharge of groundwater via springs (< 5 months) has been indicated in karst terrain of the Shenandoah River watershed (Wright, 1990). Thus, interactions between land application of manure and rainfall could potentially affect stream water contaminant concentrations a few months later. In contrast, land application of manure could affect contaminant concentrations in streams many years later if there are significant contributions of older groundwater to baseflow (Lindsey et al., 2003). The dynamics of estrogens and nutrients in groundwater require further study in this system, but the generally consistent DIN concentrations and estrogenic activity between high and low flow conditions is biologically relevant; organisms inhabiting streams draining watersheds with high densities of AFOs may experience continuous exposure to high concentrations.

Concentrations and sources of PO_4-P

The relationships between in-stream PO_4-P concentrations and watershed-scale land use were somewhat different than for DIN and estrogenic activity. Relative to the other analytes, relationships between watershed

density of AFOs and PO_4-P concentrations were weaker and there was no consistent change in the proportion of variance explained by these relationships during high flow conditions. These differences may be due to the tendency of phosphates to sorb to particles, resulting in lower solubility (Mueller and Spahr, 2006), and/or rapid biological uptake of PO_4-P (Dodds and Welch, 2000). However, linear relationships between PO_4-P and watershed density of AFOs during both spring high and summer low flow sampling periods suggest that P is moving from land applied manure into surface water and groundwater. Despite the tendency for sorption, a large proportion of the P lost from manure-treated fields can be in the dissolved form (Sistani et al., 2010; Withers and Jarvie, 2008).

Orthophosphate was the only analyte with a significant relationship to WWTPs, assessed as both the estimated proportion of stream flow as effluent and presence/absence. Streams receiving WWTP effluent had higher PO_4-P concentrations than those streams without WWTPs during all sampling periods, and during the late winter sampling period PO_4-P concentrations were directly proportional to the estimated relative amount of effluent received. Elevated concentrations of PO_4-P downstream of WWTPs have been previously observed (Mueller and Strahr, 2006), due to high P concentrations in the effluent and the effluent's highly soluble nature (Withers and Jarvie, 2008).

The combined effect of watershed AFO density and WWTPs on PO_4-P concentrations, quantified using both regression and ANCOVA, has important implications for management of the nutrient that typically limits primary production in freshwater environments. Upgrading the WWTPs to a level of treatment that includes nutrient removal would limit a direct source of PO_4-P to the studied tributaries, but streams would still receive PO_4-P from manure-treated fields. In addition, a significant portion of P may be sorbed to suspended particles or sediment in streams (Withers and Jarvie, 2008), particularly in agricultural areas (Klotz, 1985; Palmer-Felgate et al., 2009). Rapid mineralization of the sorbed P can lead to eutrophic conditions (Dodds and Welch, 2000). The P-based risk of eutrophication in Shenandoah River tributaries was likely underestimated by the measured PO_4-P concentrations, and minimization of this risk would require further understanding of in-stream P cycling.

Loadings of all analytes

Loadings of all analytes had linear relationships to watershed density of AFOs and other land uses similar to concentrations, but the proportion of variance in analyte loadings explained by these linear relationships was generally less than that of analyte concentrations. This may be due to effect of stream discharge, which is unrelated

to land use, on loading calculations. Weldon and Hornbuckle (2006) saw a similar pattern when comparing relationships of NO_3-N concentrations and loadings with watershed density of AFOs. Unlike concentrations, there were significant seasonal differences in loadings of all analytes. Higher loadings during the late spring sampling period indicate that larger amounts of nutrients and estrogens/estrogenic compounds are entering and being exported by Shenandoah River tributaries during high flow conditions. This increases the risk to receiving waters in terms of aquatic organism exposure to endocrine-disrupting compounds and delivery of excess nutrients. Fish kills in the Shenandoah River have generally occurred in late spring (Blazer et al., 2010), and while far from causative, there is an apparent temporal link between the fish kills and higher loadings of landscape-derived contaminants. The moderate correlations between concentrations of DIN, PO_4-P, and estrogenic activity are likely due to differences in in-stream processing of the compounds including: solubility, potential for biological uptake (nutrients), and degradation (estrogens). However, the stronger correlations between loadings of DIN, PO_4-P, and estrogens/estrogenic compounds suggest that the amounts of these contaminants entering Shenandoah River tributaries are related to each other and to landscape sources.

Additional sources of variability

Although the results of this study implicate AFOs as a primary contributor of nutrients and estrogens/estrogenic compounds to Shenandoah River tributaries, there are recognized limitations within the dataset. First, all potential sources of these contaminants were not included in the study design. For example, septic fields are a potential source of nutrients and estrogens/estrogenic compounds to groundwater and the use of inorganic N fertilizers on crops is a potential source of DIN (Lindsey et al., 2003; Swartz et al., 2006). These sources did not appear to be overly influential to in-stream analyte concentrations, given their weaker linear relationships with watershed percentages of cropland compared to watershed density of AFOs, and generally insignificant relationships with watershed percentage of developed land. However, confirmation of a lack of influence would require knowledge of locations and usage within each watershed. Second, the SPE methodology and use of the BLYES were designed to capture total estrogenicity in Shenandoah River tributaries, as an indication of potential endocrine-disrupting effects on aquatic organisms. The relative contribution of steroid estrogens, their conjugates, phyto- and myco-estrogens, as well as synthetic xenoestrogens to the measured estrogenic activity would require analysis by chromatography and mass spectrometry, which was beyond the scope of this study. The presence of estrogen

receptor antagonists also requires further study as the BLYES indicates the presence of compounds that can bind to the estrogen receptor, but does not distinguish between estrogen agonists and antagonists (Sanseverino et al., 2005). Third, the spatial overlap of different types of AFOs in the Shenandoah River watershed complicates the association of nutrients and estrogenic activity with specific animal sources. Poultry houses are the numerically dominant type of AFO, but most watersheds with high densities of poultry houses also have high densities of dairy AFOs and/or grazing beef cattle. The relative impact of one particular source of manure compared to the synergistic effect of all types of manure within the watersheds of Shenandoah River tributaries requires further study before more effective manure management practices can be implemented.

Conclusions

Results of this study indicate that as watershed densities of AFOs increase, concentrations of nutrients and compounds with endocrine-disrupting potential also increase in agricultural streams. In watersheds with high agricultural intensity and low development, the influence of AFOs on in-stream concentrations of DIN and estrogenic activity appears to outweigh the effects of small WWTPs. Minimizing the cumulative effect of AFOs on water quality may require implementation of watershed-scale manure management plans for nutrients. The apparent cumulative effects of AFOs on estrogenicity suggest that endocrine-disrupting potential in agricultural streams should be monitored at the landscape scale. Biologically-based screening methods such as the BLYES may offer a cost-effective approach to monitor estrogenicity on a broad spatial scale and identify at-risk areas for further assessment of the potential effects of estrogenic compounds on aquatic organisms in agricultural streams.

Literature cited

Andres, A. S. (1995). Animal waste management and edge of field losses. In K. Steele (Ed.), *Animal waste and the land-water interface* (pp. 57-68). New York: Lewis Publishers.

APHA (2005a). Nitrogen (ammonia) flow injection analysis. Method 4500-NH_3-H. In A. D. Eaton, L. S. Clesceri, E. W. Rice, & A. E. Greenberg (Eds.), *Standard methods for the examination of water and wastewater 21st edition* (pp. 4116-4117). Washington, DC: American Public Health Association.

APHA (2005b). Nitrogen (nitrate) cadmium reduction flow injection method. Method 4500 NO_3 I. In A. D. Eaton, L. S. Clesceri, E. W. Rice, & A. E. Greenberg (Eds.), *Standard methods for the examination of water and wastewater 21st edition* (pp. 4127-4129). Washington, DC: American Public Health Association.

APHA (2005c). Flow injection analysis for orthophosphate. Method 4500-P G. In A. D. Eaton, L. S. Clesceri, E. W. Rice, & A. E. Greenberg (Eds.), *Standard methods for the examination of water and wastewater 21st edition* (pp. 4156-4157). Washington, DC: American Public Health Association.

Biggs, B. J. F. (2000). Eutrophication of streams and rivers: Dissolved nutrient-chlorophyll relationships for benthic algae. *Journal of the North American Benthological Society, 19*, 17-31.

Blazer, V. S., Iwanowicz, D. D., Starliper, C. E., Iwanowicz, D. D., Barbash, P., Hedrick, J. D., et al., (2010). Mortality of centrarchid fishes in the Potomac drainage: Survey results and overview of potential contributing factors. *Journal of Aquatic Animal Health, 22*, 190-218.

Blazer, V. S., Iwanowicz, L. R., Iwanowicz, D. D., Smith, D. R., Young, J. A., Hedrick, J. D., et al., (2007). Intersex (testicular oocytes) in smallmouth bass from the Potomac River and selected nearby drainages. *Journal of Aquatic Animal Health, 19*, 242-253.

Boesch, D. F., Brinsfield, R. B., & Magnien, R. E. (2001). Chesapeake Bay eutrophication: Scientific understanding, ecosystem restoration, and challenges for agriculture. *Journal of Environmental Quality, 30*, 303-320.

Camargo, J. A., Alonso, A., & Salamanca, A. (2005). Nitrate toxicity to aquatic animals: A review with new data for freshwater invertebrates. *Chemosphere, 58*, 1255-1267.

Carpenter, S. R., Caraco, N. F., Correll, D. L., Howarth, R. W., Sharpley, A. N., & Smith, V. H. (1998). Nonpoint pollution of surface waters with phosphorus and nitrogen. *Ecological Applications, 8*, 559-568.

Commonwealth of Virginia (2005). Chesapeake Bay nutrient and sediment reduction strategy for the Shenandoah and Potomac River basins. http://www.dcr.virginia.gov/soil_and_water/documents/tsshenpoall032805.pdf

Daliparthy, J., Herbert, S. J., & Veneman, P. L. M. (1995). Excess dairy manure management: Impacts on water quality. In K. Steele (Ed.), *Animal waste and the land-water interface* (pp. 355-362). New York: Lewis Publishers.

Dodds, W. K., & Welch, E. B. (2000). Establishing nutrient criteria in streams. *Journal of the North American Benthological Society, 19*, 186-196.

Finlay-Moore, O., Hartel, P. G., & Cabrera, M. L. (2000). 17β-estradiol and testosterone in soil and runoff from grasslands amended with broiler litter. *Journal of Environmental Quality, 29*, 1604-1611.

Gore, J. A. (2006). Discharge measurements and streamflow analysis. In F. R. Hauer, & G. A. Lamberti (Eds.), *Methods in stream ecology, second edition* (pp. 51-78). New York: Elsevier.

Gould, M. C. (1995). Nitrate-nitrogen levels in well water in the little river/rooty creek agricultural non-point source hydrological unit area. In K. Steele (Ed.), *Animal waste and the land-water interface* (pp. 251-256). New York: Lews Publishers.

Guillette, L. J., & Edwards, T. M. (2005). Is nitrate an ecologically relevant endocrine disruptor in vertebrates? *Integrative and Comparative Biology, 45*, 19-27.

Hahlbeck, E., Griffiths, R., & Bengtsson, B.-E. (2004). The juvenile three-spined stickleback (*Gasterosteus aculeatus* L.) as a model organism for endocrine disruption I: Sexual differentiation. *Aquatic Toxicology, 70*, 287-310.

Hanselman, T. A., Graetz, D. A., & Wilkie, A. C. (2003). Manure-borne estrogens as potential environmental contaminants: A review. *Environmental Science and Technology, 37*, 5471-5478.

Herman, J. S., & Mills, A. I. (2003). Biological and hydrogeological interactions affect the persistence of 17β-estradiol in an agricultural watershed. *Geobiology, 1*, 141-151.

Homer, C., Dewitz, J., Fry, J., Coan, M., Hossain, N., Larson, C., et al., (2001). Completion of the 2001 National Land Cover Database for the conterminous United States. *Photogrammetric Engineering and Remote Sensing, 73*, 337-341.

Hutchins, S. R., White, M. V., Hudson, F. M., & Fine, D. D. (2007). Analysis of lagoon samples from different concentrated animal feeding operations for estrogens and estrogen conjugates. *Environmental Science and Technology, 41*, 738-744.

Hyer, K. E., Hornberger, G. M., & Herman, J. S. (2001). Processes controlling the episodic streamwater transport of atrazine and other agrochemicals in an agricultural watershed. *Journal of Hydrology, 254*, 47-66.

Iwanowicz, L. R., & Ottinger, C. A. (2009). Estrogens, estrogen receptors, and their role as immunoregulators in fish. In G. Zaccone, J. Meseguer, A. Garcia-Ayala, & B. G. Kapoor (Eds.), *Fish defenses, volume 1: Immunology* (pp. 277-322). Enfield, NH: Science Publishers.

Jenkins, M. B., Endale, D. M., Schomberg, H. H., & Sharpe, R. R. (2006). Fecal bacteria and sex hormones in soil and runoff from cropped watersheds amended with poultry litter. *Science of the Total Environment, 358*, 164-177.

Johnson, A. C., Williams, R. J., & Matthiessen, P. (2006). The potential steroid hormone contribution of farm animals to freshwaters, the United Kingdom as a case study. *Science of the Total Environment, 362*, 166-178.

Johnson, P. T. J., Townsend, A. R., Cleveland, C. C., Glibert, P. M., Howarth, R. W., McKenzie, V. J., et al., (2010). Linking environmental nutrient enrichment and disease emergence in humans and wildlife. *Ecological Applications, 20*, 16-29.

Karr, J. D., Showers, W. J., Gilliam, J. W., & Andres, A. S. (2001). Tracing nitrate transport and environmental impact from intensive swine farming using delta nitrogen-15. *Journal of Environmental Quality, 30*, 1163-1175.

Kellog, R. L., Lander, C. H., Moffitt, D. C., & Gollhon, N. (2000). Manure nutrients relative to the capacity of cropland and pastureland to assimilate nutrients: Spatial and temporal trends for the United States. USDA Natural Resources Conservation Service (Ed.). Fort Worth, TX. http://www.nrcs.usda.gov/technical/nri/pubs/manntr.pdf

Kjaer, J., Olsen, P., Bach, K., Barlebo, H. C., Ingerslev, F., Hansen, M., et al., (2007). Leaching of estrogenic hormones from manure-treated structured soils. *Environmental Science and Technology, 41*, 3911-3917.

Klotz, R. L. (1985). Factors controlling phosphorus limitation in stream sediments. *Limnology and Oceanography, 30*, 543-553.

Kolodziej, E. P., & Sedlak, D. L. (2007). Rangeland grazing as a source of steroid hormones to surface waters. *Environmental Science and Technology, 41*, 3514-3520.

Lagana, A., Bacaloni, A., De Leva, I., Faberi, A., Fago, G., & Marino, A. (2004). Analytical methodologies for determining the occurrence of endocrine disrupting chemicals in sewage treatment plants and natural waters. *Analytica Chemica Acta, 501*, 79-88.

Lange, A., Paull, G. C., Coe, T. S., Katsu, Y., Urushitani, H., Iguchi, T., et al., (2009). Sexual reprogramming and estrogenic sensitization in wild fish exposed to ethinylestradiol. *Environmental Science and Technology, 43*, 1219-1225.

Lindsey, B. D., Phillips, S. W., Donnelly, C. A., Speiran, G. K., Plummer, L. N., Bohlke, J.-K., et al., (2003). Residence times and nitrate transport in ground water discharging to streams in the Chesapeake Bay watershed. U.S. Geological Survey. Water Resources Investigations Report 03-4035.

Mallin, M. A., & Cahoon, L. B. (2003). Industrialized animal production - a major source of nutrient and microbial pollution to aquatic ecosystems. *Population and Environment, 24*, 369-385.

Marsh, L., Mullins, G., Habersack, M., & Collins, E. R. (2009). Land application of broiler and turkey litter for farming operations without a DEQ permit. *VCE publication 442-052*, http://pubs.ext.vt.edu/442/442-052/442-052.html.

Matthiessen, P., Arnold, D., Johnson, A. C., Pepper, T. J., Pottinger, T. G., & Pulman, K. G. T. (2006). Contamination of headwater streams in the United Kingdom by oestrogenic hormones from livestock farms. *Science of the Total Environment, 367*, 616-630.

Milliken, G. A., & Johnson, D. E. (1984). *Analysis of messy data. Volume 3: Analysis of covariance.* New York: van Nostrand Reinhold.

Miltner, R. J. (2010). A method and rationale for deriving nutrient criteria for small rivers and streams in Ohio. *Environmental Management, 45*, 842-855.

Mishra, A., Benham, B. L., & Mostaghimi, S. (2006). Sediment and nutrient losses from field-scale cropland plots treated with animal manure and inorganic fertilizer. *Water Air and Soil Pollution, 175*, 61-76.

Montgomery DC, Peck EA, Vining GG. Introduction to Linear Regression Analysis, Fourth Edition. Hoboken, NJ: John Wiley and Sons, 2006.

Mueller, D. K., & Spahr, N. E. (2006). Nutrients in streams and rivers across the nation - 1992-2001. U.S. Geological Survey Scientific Investigations Report 2006-5107, 51 p.

Muller, M., Rabenoelina, F., Balaguer, P., Patureau, D., Lemenach, K., Budzinski, H., et al., (2008). Chemical and biological analysis of endocrine-disrupting hormones and estrogenic activity in an advanced sewage treatment plant. *Environmental Toxicology and Chemistry, 27*(1649-1658).

Mullholland, P. J., Helton, A. M., Poole, G. C., Hall, R. O., Hamilton, S. K., Peterson, B. J., et al., (2008). Stream denitrification across biomes and its response to anthropogenic nitrate loading. *Nature, 452*, 202-206.

Nichols, D. J., Daniel, T. C., Moore, P. A., Edwards, D. R., & Pote, D. H. (1997). Runoff of estrogen hormone 17β-estradiol from poultry litter applied to pasture. *Journal of Environmental Quality, 26*, 1002-1006.

Palmer-Felgate, E. J., Jarvie, H. P., Withers, P. J. A., Mortimer, R. J. G., & Krom, M. D. (2009). Stream-bed phosphorus in paired catchments with different agricultural land use intensity. *Agriculture, Ecosystems, and Environment, 134*, 53-66.

Peterson, E. W., Davis, R. K., & Orndorff, H. A. (2000). 17β-estradiol as an indicator of animal waste contamination in mantled karst aquifers. *Journal of Environmental Quality, 29*, 826-834.

Petrovic, M., Sole, M., Lopez de Alda, M., & Barcelo, D. (2002). Endocrine disruptors in sewage treatment plants, receiving river waters, and sediments: Integration of chemical analysis and biological effects on feral carp. *Environmental Toxicology and Chemistry, 2146-2156*.

Raman, D. R., Williams, E. L., Layton, A. C., Burns, R. T., Easter, J. P., Daugherty, A. S., et al., (2004). Estrogen content of dairy and swine wastes. *Environmental Science and Technology, 38*, 3567-3573.

Rice, S. L., & Hale, R. C. (2009). Parameters for ultraperformance liquid chromatography/tandem mass spectrometric analysis of selected androgens versus estrogens in aqueous matrices. *Analytical Chemistry, 81*(16), 6716-6724.

Ripley, J., Iwanowicz, L. R., Blazer, V. S., & Foran, C. (2008). Utilization of protein expression profiles as indicators of environmental impairment of smallmouth bass (*Micropterus dolmieu*) from the Shenandoah River, Virginia, USA. *Environmental Toxicology and Chemistry, 27*, 1756-1767.

Robertson, L. S., Iwanowicz, L. R., & Marranca, J. M. (2009). Identification of centrarchid hepcidins and evidence that 17β-estradiol disrupts constitutive expression of hepcidin-1 and inducible expression of hepcidin-2 in largemouth bass (*Micropterus salmoides*). *Fish and Shellfish Immunology, 26*, 898-907.

Routledge, E. J., & Sumpter, J. P. (1996). Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environmental Toxicology and Chemistry, 15*, 241-248.

Salste, L., Leskinen, P., Virta, M., & Kronberg, L. (2007). Determination of estrogens and estrogenic activity in wastewater effluent by chemical analysis and the bioluminescent yeast assay. *Science of the Total Environment, 378*, 343-351.

Sanseverino, J., Gupta, R. K., Layton, A. C., Patterson, S. S., Ripp, S. A., Saidak, L., et al., (2005). Use of *Saccharomyces cerevisiae* BLYES expressing bacterial bioluminescence for rapid, sensitive detection of estrogenic compounds. *Applied and Environmental Microbiology, 71*, 4455-4460.

Shore, L. S., Correll, D. L., & Chakraborty, P. K. (1995). Fertilization of fields with chicken manure as a source of estrogens in small streams. In K. Steele (Ed.), *Animal waste and the land-water interface* (pp. 155-162). Boca Raton, FL: Lewis Publishers.

Sistani, K. R., Bolster, C. H., Way, T. R., Tobert, H. A., Pote, D. H., & Watts, D. B. (2010). Influence of poultry litter application methods on the longevity of nutrient and *E. coli* in runoff from tall fescue pasture. *Water Air and Soil Pollution, 206*, 3-12.

Soupir, M. L., Mostaghimi, S., & Yagow, E. R. (2006). Nutrient transport from manure applied to pastureland using phosphorus-based management strategies. *Journal of Environmental Quality, 35*, 1269-1278.

Steiner, L. D., Bidwell, V. J., Di, H. J., Cameron, K. C., & Northcott, G. L. (2010). Transport and modeling of estrogenic hormones in dairy farm effluent through undisturbed soil lysimeters. *Environmental Science and Technology, 44*, 2341-2347.

Swartz, C. H., Reddy, S., Benotti, M. J., Yin, H., Barber, L. B., Brownawell, B. J., et al., (2006). Steroid estrogens, nonylphenol ethoxylate metabolites, and other wastewater contaminants in groundwater affected by residential septic system on Cape Cod, MA. *Environmental Science and Technology, 40*, 4894-4902.

Thompson, M. L., Casey, F. X. M., Khan, E., Hakk, H., Larsen, G. L., & DeSutter, T. (2009). Occurrence and pathways of manure-borne 17β-estradiol in vadose zone water. *Chemosphere, 76*, 472-479.

U.S. Department of Agriculture (USDA) (1992). Chapter 3: Agricultural wastes and water, air, and animal resources. In USDA National Resources Conservation Service (Ed.), *Agricultural waste management handbook*.

Virginia Department of Conservation and Recreation (VADCR) (2010). Animal feeding operations database. http://webdat.dcr.virginia.gov/cfprog/dswc/afoprm.cfm

Virginia Department of Environmental Quality (VADEQ) (2006). Final 2006 305(b)/303(d) water quality assessment integrated report. http://www.deq.state.va.us/wqa/ir2006.htm

Vermeirssen, E. L. M., Burkj, R., Joris, C., Peter, A., Segner, H., Suter, M. J.-F., et al., (2005). Characterization of the estrogenicity of Swiss midland rivers using a recombinant yeast bioassay and plasma vitellogenin concentrations in feral male brown trout. *Environmental Toxicology and Chemistry, 24*, 2226-2233.

Weldon, M. B., & Hornbuckle, K. C. (2006). Concentrated animal feeding operations, row crops, and their relationships to nitrate in eastern iowa rivers. *Environmental Science and Technology, 40*, 3168-3173.

Withers, P. J. A., & Jarvie, H. P. (2008). Delivery and cycling of phosphorus in rivers: A review. *Science of the Total Environment, 400*, 379-395.

Wright, W. G. (1990). Ground-water hydrology and quality in the Valley and Ridge and Blue Ridge physiographic provinces of Clarke County, Virginia. U.S. Geological Survey Water-Resources Investigations Report 90-4134.

Yager, R. M., Southworth, S., & Voss, C. I. (2008). Simulation of ground-water flow in the Shenandoah Valley, Virginia and West Virginia, using variable-direction anisotropy in hydraulic conductivity to represent bedrock structure. U.S. Geological Survey Scientific Investigations Report 2008-5002

Ying, G.-G., Kookana, R. S., & Kumar, A. (2008). Fate of estrogens and xenoestrogens in four sewage treatment plants with different technologies. *Environmental Toxicology and Chemistry, 27*, 87-94.

Yonkos, L. T., Fisher, D. J., Van Veld, P. A., Kane, A. S., McGee, B. L., & Staver, K. W. (2010). Poultry-litter induced endocrine disruption in fathead minnow, sheepshead minnow, and mummichog laboratory exposures. *Environmental Toxicology and Chemistry, 29*, 2328-2340.

Young, W. F., Whitehouse, P., Johnson, I., & Sorokin, N. (2004). Proposed predited-no-effect concentrations (PNECs) for natural and synthetic steroid estrogens in surface waters. Environment Agency Technical Report P2-T04/1

http://publications.environment-agency.gov.uk/pdf/SP2-T04-TR1-e-p.pdf?lang=_e

Table 4- 1. Characteristics of each sampling site. These characteristics include: watershed percentages of land cover classes, watershed densities (#/1000 Acres) of animal feeding operations (AFOs), and total upstream permitted wastewater treatment plant (WWTP) effluent discharge in millions of gallons per day (MGD). Tributaries with upstream (US) and downstream (DS) sampling sites are indicated, as well as the total number of Shenandoah River subwatersheds located upstream of DS sites.

	Land cover %[1]			AFO Density				WWTP
	Past./ Hay	Crop	Devel	Poul.	Beef	Dairy	Total	MGD
Briery Br.	13	1.9	2.4	0.60	0.32	0.35	1.3	0
Cedar Cr. (DS; 5)	16	0.5	4.1	0	0.012	0	0.012	0.003*
Cedar Cr. (US)	5.0	0.2	1.9	0	0	0	0	0
Hawksbill (DS; 2)	29	2.5	12	0.72	0.32	0	1.0	1.8
Hawksbill (US)	29	2.8	9.1	0.98	0.43	0	1.4	0.2
Jennings Br.	17	1.2	8.2	0.13	0.22	0.044	0.40	0
Linville Cr.	64	4.2	9.8	1.3	0.47	0.71	2.5	0.03
Long Meadow R.	69	5.1	6.5	2.4	1.0	0.51	4.0	0
Mill Cr.	39	2.2	6.3	0.61	0.24	0.13	0.97	0
Mill Cr.	66	7.1	11	0.54	0.54	0.76	1.8	0
Meadow R.	61	4.3	19	0.082	0.33	0.49	0.90	0.015
Muddy Cr.	48	7.3	7.7	1.3	0.85	2.1	4.2	0.005
Naked Cr.	9.9	0.6	4.9	0.25	0	0	0.25	0
Passage Cr.	6.0	0.2	3.4	0.039	0	0	0.039	0.01*
Smith Cr. (DS; 4)	40	3.4	7.7	0.50	0.44	0.25	1.2	0.091
Smith Cr. (US)	35	2.7	2.9	0.51	0.44	0.37	1.3	0.03
Stony Cr. (DS; 3)	23	2.0	7.0	0.22	0.14	0.041	0.40	0.814
Stony Cr. (US)	8.0	0.3	6.5	0.17	0	0	0.17	0.639

[1]Percentage of forested land cover not shown; equal to 100- sum of other categories
* WWTPs for summer camps; only operating during August sampling periods

Table 4- 2. Range of analyte concentrations and loadings, as well as stream discharge, on each sampling date.

	Concentrations			Loadings			
	E2Eq [a] ng/L	DIN µg/L	PO_4-P [b] µg/L	E2Eq $mg/km^2/d$	DIN $g/km^2/d$	PO_4-P [b] $g/km^2/d$	Discharge [c] m^3/s
May 2008	<QL – 3.2	31 - 6,005	1.5 - 53.6				
Aug 2008	<QL – 1.1	43 - 7,588	2.3 – 169				
Mar 2009	<QL – 5.9	5.0 – 6,064	1.5 - 66.8	0.03- 1.8	0.8 - 604	0.2 – 26.3	0.03 – 0.80
May 2009	<QL – 3.9	44 – 6,145	2.8 - 55.9	0.07 - 1.4	27 - 5,841	1.4 – 60.6	0.07 – 3.3
Aug 2009	<QL – 7.2	9.7 – 7,140	4.0 – 108	0.01 - 0.43	0.6 - 1,201	0.4 – 28.6	0.02 – 0.70

[a]The quantitation limit (QL) for E2Eq (estrogenic activity as 17β-estradiol equivalents) was 0.31 ng/L.
[b]Excludes outlying concentrations and loadings of PO_4-P measured at Muddy Creek.
[c]Discharge data were collected and loadings estimated during 2009 sampling periods only.

Table 4- 3. Proportions of variance (R^2) explained by linear regressions between analytes (y; top=concentrations, bottom=loadings) and watershed densities of AFOs (x) and proportion of streamflow as WWTP effluent (x). The proportion of streamflow as WWTP effluent only had significant effects on PO_4-P concentrations, relationships with both estrogenic activity (E_2Eq) and DIN were not statistically significant.

y variable	E2Eq	DIN	PO_4-P	PO_4-P	PO_4-P
x variable	AFOs	AFOs	AFOs	WWTP	AFOs+WWTP
May 2008	$0.75^{\dagger\dagger}$	$0.81^{\dagger\dagger}$	0.27^{*}	NS	NS
Aug 2008	0.39^{**}	$0.60^{\dagger\dagger}$	0.39^{**}	NS	$0.57^{\dagger}$
Mar 2009	0.45^{**}	$0.59^{\dagger}$	NS	0.44^{**}	$0.59^{\dagger}$
May 2009	$0.63^{\dagger\dagger}$	$0.78^{\dagger\dagger}$	$0.57^{\dagger}$	NS	$0.66^{\dagger}$
Aug 2009	$0.58^{\dagger}$	$0.56^{\dagger}$	0.44^{**}	NS	NS
Mar 2009	0.23^{*}	0.42^{**}	NS	0.42^{**}	NS
May 2009	$0.51^{\dagger}$	$0.66^{\dagger\dagger}$	0.31^{*}	NS	0.41^{**}
Aug 2009	$0.70^{\dagger\dagger}$	$0.50^{\dagger}$	0.44^{**}	NS	NS

* $p<0.05$, ** $p\leq0.01$, † $p\leq0.001$, †† $p\leq0.0001$, "NS" = Not statistically significant ($p>0.05$) for univariate regression or multiple regression did not increase significance

Table 4- 4. **Proportions of variance (R^2) explained by linear regressions between analyte concentrations (y), land cover (x), and the agricultural index (AI; x).**

y variable	E2Eq	E2Eq	E2Eq	DIN	DIN	DIN	PO_4-P	PO_4-P	PO_4-P
x variable	PHay	Crop	AI	PHay	Crop	AI	PHay	Crops	AI
May 2008	$0.49^{\dagger}$	0.42^{**}	$0.67^{\dagger\dagger}$	$0.71^{\dagger\dagger}$	$0.66^{\dagger\dagger}$	$0.76^{\dagger\dagger}$	0.34^{**}	0.23^{*}	0.29^{*}
Aug 2008	NS	NS	0.28^{*}	0.38^{**}	0.34^{**}	$0.45^{\dagger}$	0.36^{**}	0.40^{**}	0.36^{**}
Mar 2009	NS	0.26^{*}	0.31^{*}	$0.50^{\dagger}$	0.44^{**}	$0.50^{\dagger}$	NS	NS	NS
May 2009	$0.52^{\dagger}$	$0.49^{\dagger}$	$0.66^{\dagger\dagger}$	$0.62^{\dagger\dagger}$	$0.56^{\dagger}$	$0.71^{\dagger\dagger}$	0.42^{**}	$0.50^{\dagger}$	$0.52^{\dagger}$
Aug 2009	0.48^{**}	0.42^{**}	$0.58^{\dagger}$	$0.48^{\dagger}$	0.44^{**}	$0.52^{\dagger}$	0.41^{**}	0.45^{**}	$0.50^{\dagger}$

* $p<0.05$, ** $p\leq0.01$, $^{\dagger}p\leq0.001$, $^{\dagger\dagger}p\leq0.0001$, "NS" = Not statistically significant ($p>0.05$)

.

Table 4- 5. **Mean analyte concentrations and loadings during each sampling period ± standard error, adjusted for relationships with watershed densities of AFOs (least-squares adjusted means). Letters indicate statistically different concentrations or loadings between sampling periods.**

	Concentrations			Loadings[1]		
	E2Eq ng/L	DIN µg/L	PO_4-P µg/L	E2Eq $mg/km^2/d$	DIN $g/km^2/d$	PO_4-P $g/km^2/d$
May 2008	0.48 ± 0.07^{abc}	794 ± 185	9.3 ± 2.2	-	-	-
Aug 2008	0.39 ± 0.06^{a}	933 ± 217	15.8 ± 3.6	-	-	-
Mar 2009	0.71 ± 0.10^{b}	490 ± 115	NS[2]	0.14 ± 0.03^{a}	96 ± 30^{a}	NS[2]
May 2009	0.44 ± 0.07^{ac}	741 ± 172	11.7 ± 2.7	0.31 ± 0.06^{b}	507 ± 159^{b}	7.9 ± 1.7^{a}
Aug 2009	0.66 ± 0.10^{bc}	708 ± 164	12.3 ± 2.8	0.09 ± 0.02^{a}	94 ± 29^{a}	1.5 ± 0.33^{b}

[1]Loadings were not calculated in 2008
[2]NS = Relationship with AFOs was not statistically significant

Figure 4- 1. Locations of the 18 study sites within the Shenandoah River watershed. The enlargement shows the 78 12-digit hydrologic unit code (HUC) subwatersheds of the Shenandoah River (indicated in grey) and the counties included in the entire watershed, relative to the state of Virginia.

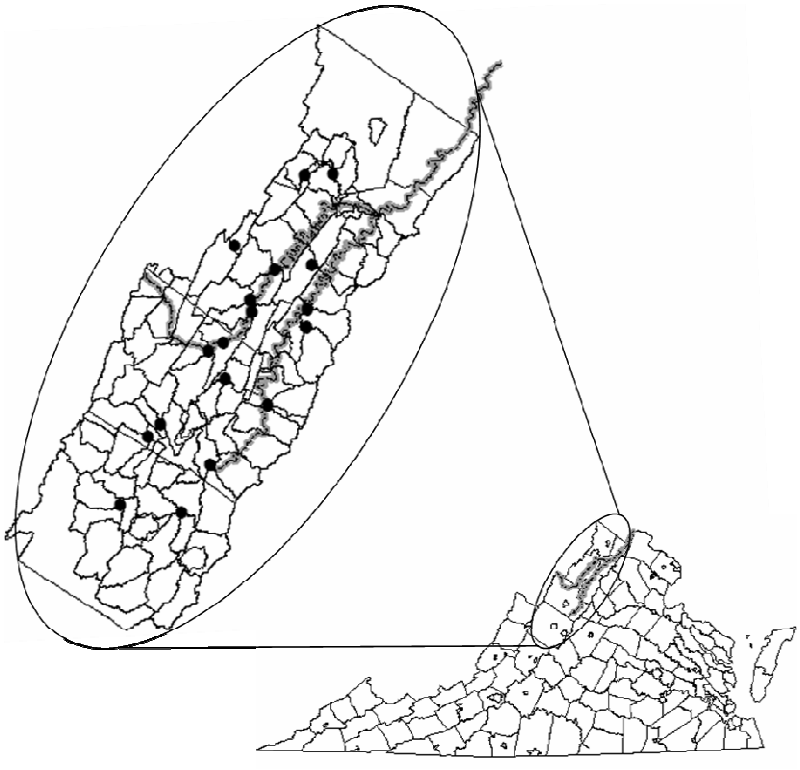

Figure 4- 2. Example of the bioluminescent yeast estrogen screen (BLYES) luminescent response to standards of 17β-estradiol (E2). Insets show derivation of standard curves within the low range (log_{10}[E2] vs. log_{10}Luminescence; Inset A) and high range (log_{10}[E2] vs. Luminescence; Inset B) of concentrations.

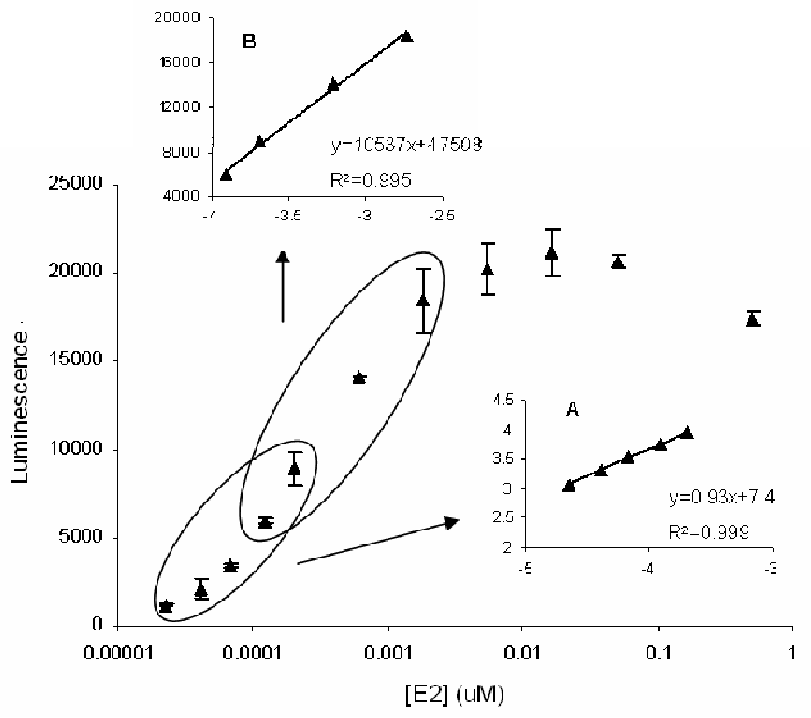

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Figure 4- 3. Relationship between watershed density of AFOs and DIN concentrations. The control line represents 1,000 µg/L. Symbol shape indicates sampling period with slopes for each sampling period in parentheses. Filled symbols indicate sites with no upstream WWTPs and open symbols represent sites with at least one upstream WWTP. There were no significant effects of either sampling period or WWTP presence/absence on DIN concentrations (ANCOVA, $p \geq 0.40$), so the overall regression is presented.

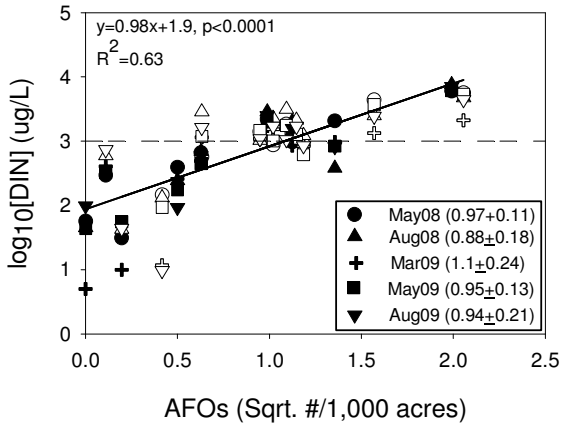


Figure 4- 4. Relationship between watershed density of AFOs and concentrations of E2Eq. Symbol shapes represent sampling period. A) Effects of sampling period on E2Eq concentrations. The control line represents 1ng/L. Slopes of regressions for each sampling period are indicated in the legend and were not significantly different (ANCOVA, p=0.56). There were significant differences in adjusted mean E2Eq concentrations between sampling periods (indicated by superscript letters). B) Effects of WWTP presence/absence on E2Eq concentrations. Filled symbols represent sites without upstream WWTPs and open symbols represent sites with WWTPs. Slopes were significantly different (ANCOVA, p=0.03), so a three-point evaluation was conducted and adjusted means were only significantly different at the lowest density of AFOs (x=0.10; p=0.047).

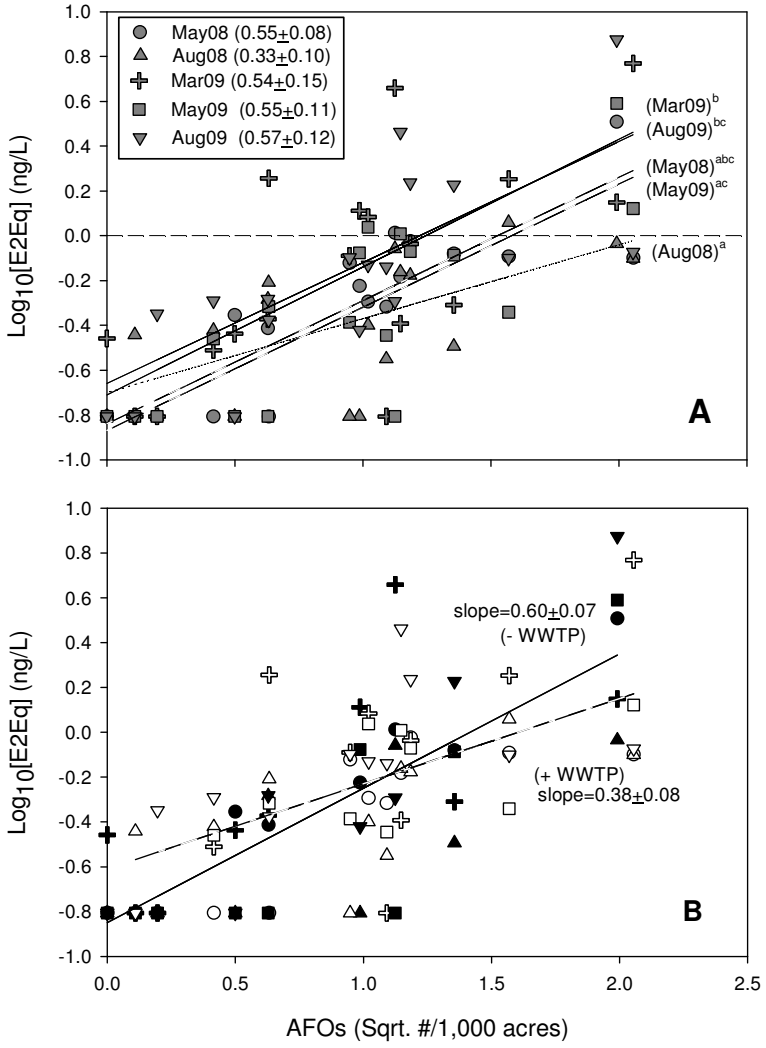
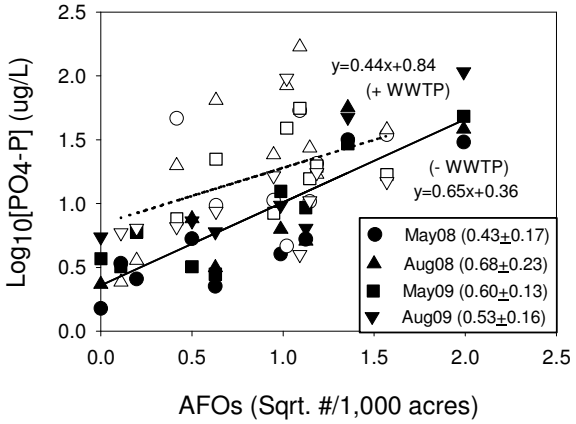


Figure 4- 5. Relationship between watershed densities of AFOs and PO_4-P concentrations. One site (Muddy Creek) was not included because of outlying concentrations. Symbol shape indicates sampling period (excluding March 2009), with slopes for each indicated in parentheses. There was no effect of sampling period on PO_4-P concentrations (ANCOVA, p=0.42). Filled symbols represent sites with no upstream WWTPs and open symbols represent sites with at least one upstream WWTP. There was a significant effect of WWTP presence/absence (ANCOVA, p=0.0006), indicated by the regression lines (+ or – WWTP). Slopes of regressions of the two categories of sites were not statistically different (p=0.26), and the adjusted mean PO_4-P concentration was significantly higher for sites with at least one upstream WWTP compared to sites with no upstream WWTPs (p=0.0008).



CHAPTER 5

Evaluation of spatial and temporal variability in population sex ratios of pleurocerid snails and their relationships with estrogenic compounds and other environmental variables

Serena Ciparis

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Abstract

When present in aquatic environments, steroidal estrogens and synthetic estrogenic compounds can interact with the endocrine system of aquatic organisms. Disruption of endocrine processes involved in sexual differentiation can ultimately lead to female-biased sex ratios in exposed populations. For gonochoristic freshwater snails in the family Pleuroceridae, little is known about the spatial and temporal variation in population sex ratios or potential relationships between sex ratios and exposure to estrogenic compounds. The variability in population sex ratios of *Leptoxis spp*, was evaluated within the Shenandoah River watershed (Virginia, USA), where agricultural operations are a source of estrogenic compounds to streams, and in other rivers in Virginia. Proportions of females varied amongst streams within the Shenandoah River watershed, ranging from 0.46-0.87. There was little within-site variation across generations of snails or when the same generation was examined in two different seasons. Proportions of females were not directly related to in-stream estrogenic activity or landscape sources of estrogenic compounds, but were negatively related to mean summer temperature at the sampling sites. Population sex ratios of *Leptoxis spp*. were female-biased at two of six sites in the Shenandoah River and one of five sites outside the basin. At the river sampling sites, proportions of females were positively related to specific conductivity. Overall, results suggest that site-specific factors can affect population sex ratios of pleurocerid snails. However, until more is known regarding mechanisms of sex determination and sexual differentiation in gastropods, population sex ratios should not be used as indicators of potential biological effects of estrogenic compounds.

Introduction

The presence of steroidal estrogens and synthetic estrogenic compounds in aquatic environments has raised concern regarding their effects on wildlife. The endocrine-disrupting effects of estrogenic compounds on male fish are well studied, and include inappropriate production of egg-yolk precursor proteins (vitellogenin) and the presence of oocytes in the testes (intersex). These effects have been observed in wild fish living downstream of wastewater treatment plant (WWTP) effluent discharges (e.g. Jobling et al., 1998). They have also been induced by laboratory exposures of fish to natural and synthetic estrogen compounds (Fenske et al., 2005; Hahlbeck et al., 2004; Lange et al., 2009), to WWTP effluents (Jobling et al., 2003; Tyler et al., 2005), and to extracts of manure from animal feeding operations (AFOs; Yonkos et al., 2010). Several laboratory exposures of fish to estrogens and estrogenic

compounds during early life stages resulted in complete feminization of genetic males and female-biased sex ratios (Fenske et al., 2005; Hahlbeck et al., 2004; Lange et al., 2009; Yonkos et al., 2010). In wild fish populations, changes from balanced to female-biased sex ratios have been observed along gradients of influence from WWTP effluent discharges (Duffy et al., 2009; Woodling et al., 2000; Vajda et al., 2008) and gradients of watershed agricultural intensity (Jeffries et al., 2008). Thus, anthropogenic introduction of estrogens and estrogenic compounds to aquatic environments may affect fish populations through disruption of sexual differentiation.

Similar to fish, critical periods of sexual development in mollusks appear to be susceptible to alteration by estrogens and estrogenic compounds. Exposures of freshwater and marine mussels to estrogens and estrogenic WWTP effluents during early stages of gametogenesis increased production of vitellogenin- and vitellin-like proteins (Gagne et al., 2000; Quinn et al., 2004) and expression of vitellogenin mRNA (Ciocan et al., 2010) in males and females. Exposure of undifferentiated adult infaunal estuarine clams to sediment spiked with a mixture of estrogens and alkylphenols induced significantly higher rates of intersex males relative to unexposed clams (Langston et al., 2007). Aqueous exposures of undifferentiated oyster larvae to nonylphenol induced hermaphroditism and female-biased sex ratios in the adult population, and these population characteristics were not present in unexposed organisms (Nice et al., 2003). Unlike bivalves, studies of the effects of estrogens and estrogenic compounds on gastropods have not involved exposures during periods of gametogenesis and sexual differentiation. However, exposures of adult snails to WWTP effluent and estrogenic compounds have resulted in significant reproductive system effects relative to unexposed snails, including stimulated embryo production (Duft et al., 2003; Jobling et al., 2003) and a significant increase in the size of accessory sex glands in females (Castro et al., 2007a; Oehlmann et al., 2000).

Studies of potential effects of estrogens and estrogenic compounds on mollusk populations are limited, particularly in streams and rivers, which are subject to acute effects of WWTPs and AFOs on water quality. In one study, sex ratios of wild freshwater mussel populations from a Canadian river changed relative to locations of WWTP discharges; sex ratios were male-biased upstream of the discharges and female-biased downstream (Gagne et al., 2011). Thus, population sex ratios of freshwater mollusks may be viable population-level indicators of exposure to estrogens or estrogenic compounds. Sex ratio is relevant to overall population health, since a substantial bias towards one sex could result in reduced reproductive success. Advantages of monitoring population-level effects in freshwater mollusks compared to fish include the limited mobility of mollusks, allowing assessment of

direct relationships between site and population characteristics, and the ability to repeatedly collect large numbers of organisms from stable populations. However, one disadvantage of utilizing mollusk population sex ratios as potential indicators of exposure to estrogenic compounds is that unlike fish, the mechanism for mollusks' apparent susceptibility to endocrine disruption requires further study. The presence of estrogens and enzymes required for their synthesis are well-documented in mollusks (reviewed by Janer and Porte, 2007 and Lafont and Mathieu, 2007), but the functional role of these compounds remain unclear (Ketata et al., 2008; Matthiessen, 2008). In addition, estrogen receptors have been isolated in several gastropod species and an octopus, but they do not appear to bind estrogen (Bannister et al., 2007; Kajiwara et al., 2006; Keay et al., 2006; Thornton et al., 2003). A second disadvantage of utilizing freshwater mollusk population sex ratios as potential indicators of exposure to estrogenic compounds is that variability of this basic population characteristic of many species is unknown, particularly those of freshwater gastropods (Lysne et al., 2008).

Population characteristics of freshwater snails in the family Pleuroceridae (superorder Caenogastropoda: order Sorbeoconcha: superfamily Cerithioidea) are generally not well studied, and several pleurocerid species are critically imperiled (Brown et al., 2008). *Leptoxis carinata* is an exception; this species is not endangered and basic life history and population characteristics have been previously described (Aldridge, 1982, Hendrix, 1986). Like all pleurocerid snails, *L. carinata* is gonochoristic with sexual reproduction, and sexes of individuals do not change during their lifetime. Limited studies of other freshwater caenogastropods suggest that sex is genetically determined (reviewed by Dillon, 2000 and Yusa, 2007). In gonochoristic species with genetic sex determination, expected population sex ratios are balanced (i.e. 1:1 or proportions of males or females = 0.5) (Fisher, 1930; Bull, 1983). Sex ratios of 1:1 have been documented in one population of *L. carinata* (Hendrix, 1986) and one population of the closely related species *Leptoxis dilatata* (Miller-Way and Way, 1989). Aldridge (1982) observed sex ratios ranging from 1:1 – 6.6:1 females: males in *L. carinata* populations from three different rivers, but potential causative agents were not discussed and the ratios may have been influenced by small sample sizes (12-23 snails). Population sex ratios ranging from balanced to female-biased have been observed for other pleurocerid snails (reviewed by Dillon, 2000), but samples were generally not collected over multiple seasons and generations or at multiple sites. Measures of spatial and temporal variability of population sex ratios are necessary in order to discern potential effects of environmental conditions, or contaminants like estrogens and estrogenic compounds, on this population characteristic.

The Shenandoah River (Virginia, USA) and its tributaries support large populations of *L. carinata*, with population densities as high as 3,000 snails/m^2 (Orth et al., 2009). Studies of smallmouth bass in the Shenandoah River have indicated a high proportion of intersex males relative to other basins, suggesting a possible biological effect of exposure to estrogens and estrogenic compounds (Blazer et al., 2007). Suspected sources of estrogens and estrogenic compounds in the Shenandoah River watershed include manure from over 1200 AFOs and the discharge of effluent from 87 municipal WWTPs. A recent study demonstrated significant relationships between watershed densities of AFOs and measured estrogenic activity in Shenandoah River tributaries; the influence of these AFOs on estrogenic activity outweighed any effect of WWTPs, likely due to the relatively low effluent volume generated by these facilities (Chapter 4). Thus, streams within the Shenandoah River watershed support large, stable populations of *L. carinata* and have documented gradients of measured estrogenic activity. This created the opportunity to assess the spatial and seasonal variability of population sex ratios of a gonochoristic gastropod and relationships between these population sex ratios and concentrations or sources of estrogens and estrogenic compounds.

The specific objectives of this study were to: 1) document variation in sex ratios of *L. carinata* in Shenandoah River tributaries, 2) compare these sex ratios with those of *Leptoxis spp*. in the Shenandoah River and other rivers in Virginia, and 3) assess relationships between population sex ratios and land use, environmental variables, and other population characteristics. The primary working hypothesis was that sex ratios in *Leptoxis spp.* populations would vary from balanced to female-biased, and that proportions of females in these populations would be related to sources and measured concentrations of estrogens and estrogenic compounds (as total estrogenicity). However, because of limited knowledge of the mechanisms of sex determination and differentiation in pleurocerid snails, relationships between sex ratios and other land use and environmental variables were also assessed. Other hypothesized causes for sex ratio variations were: variation in food resources (periphyton), variation in temperature, or inputs of other contaminants or stressors from general human activity, as these environmental variables can potentially affect both sex determination and sexual differentiation (Cook, 2002; Conover, 2004). Effects of snail population density and degree of parasitic infection on sex ratios were also evaluated.

Methods

Study sites

Study sites in the Shenandoah River watershed were selected to represent a gradient of influence from AFOs and municipal WWTPs, as described in detail in Chapter 2. Briefly, locations of AFOs (poultry, dairy, and beef) and locations of WWTPs and their total permitted effluent discharge were obtained from Virginia state agencies. For individual Shenandoah River tributaries, total densities of AFOs, WWTP presence/absence, and WWTP permitted effluent flow were quantified within delineated 12-digit, 6th level Hydrologic Unit Code (HUC 6) subwatersheds (40-160 km^2 in size). Twenty-five sites, located near outlets of the delineated HUC 6 subwatersheds, were sampled during a preliminary study in 2007. Fifteen sites in 12 Shenandoah River tributaries had relatively large populations of pleurocerid snails in stable riffles and were selected for the current study (Figure 5-1). Three of the tributaries drained multiple subwatersheds, and an upstream (US) sampling site was located in the primary subwatershed as well as a downstream (DS) site draining multiple subwatersheds. This created three sets of upstream-downstream pairs. In addition to watershed densities of AFOs and WWTP presence and total permitted effluent flow, land cover percentages were also quantified upstream of each sampling site. Land cover data (30 m resolution) were obtained from the 2001 National Land Cover Database (Homer et al., 2007), and reclassification and areal tabulation were used to quantify the watershed percentages of forest, developed land, pasture/hay, and cultivated crops upstream of each sampling site. Across the 15 sampling sites, ranges in watershed percentages of the four land cover types were: 19-92% forest, 5.0-69% pasture/hay, 0.22-7.3% cropland, and 1.9-12% developed land. These four land cover classes were also quantified as "local" percentages within a 300 m wide riparian buffer extending 1,000 m upstream of the site (Chapter 2). However, these local percentages did not have significant effects on variables of interest in this study and results are not presented or discussed. All land use characteristics were quantified using ArcGis 9.3 (ESRI, Redlands, CA).

Sampling sites in Virginia rivers were selected as part of a larger study of geographic variation in fish health (Orth et al., 2009). Five locations outside of the Shenandoah River watershed were selected: the Rappahannock River near Richardsville (RAPP), the Cowpasture River (James River basin; JA-1), the James River upstream of Buchanan (JA-2), the New River near Draper (NEW), and the North Fork Holston River upstream of Saltville (HOL) (Figure 5-1). Four sites were located in the North Fork of the Shenandoah River, with an upstream site located near Cootes Store (NF-1), followed by downstream sites near Mount Jackson (NF-2), Woodstock (NF-

3), and Strasburg (NF-4) (Figure 5-1). Two sites were located in the South Fork of the Shenandoah River, an upstream site in the North River above its confluence with the South River (SF-1) and a downstream site in the South Fork Shenandoah River near Luray (SF-2) (Figure 5-1). Land use characteristics were not quantified upstream of river sites due to the large watershed sizes and the resulting mixture of land uses present at the watershed scale.

Snail sampling

Leptoxis carinata was the only species of pleurocerid snail encountered at all Shenandoah River and James River basin sites and *Leptoxis dilatata* was the only species encountered at the New River site. At the Rappahannock River site, *Elimia virginica* and *Leptoxis carinata* were present, but only *L. carinata* was sampled for sex ratios due to their abundances in riffle areas. At the Holston River site, *Pleurocera uncialis* and *Leptoxis praerosa* were present, but only *L. praerosa* was sampled for sex ratios due to dominance in riffle areas. All species identifications were verified against a reference collection at the Smithsonian Institution National Museum of Natural History (Washington, DC).

Previous studies of *L. carinata* and *L. dilatata* have shown that these species are semelparous biennials; 2-year old snails lay eggs between late spring and mid-summer and die by late summer (Aldrige, 1982; Hendrix, 1986; Miller-Way and Way, 1989). Therefore, snails were not collected during the summer in order to avoid potential fluctuations in adult populations. Only adult (>1 yr old, >7 mm shell length) snails were included in sex ratio calculations because sexes are not morphologically distinct until the second summer of life (Aldrige, 1982; Hendrix, 1986; Miller-Way and Way, 1989, verified in Chapter 7). At Shenandoah River tributary sites, population sex ratios were determined for three generations of *L. carinata.* The 2006 generation was sampled 14-24 May 2008, the 2007 generation was sampled 5-15 October 2008 and 21 May-19 June 2009, and the 2008 generation was sampled 2-23 October 2009. The 2007 generation was sampled twice to assess sex ratio variability, either due to sampling or seasonal changes. To further assess seasonal variability, additional sampling of adult snails in 2008 and 2009 generations was conducted over 16 months (12 sampling dates) at two Shenandoah tributary sites: one with equal sex ratios (Briery Branch) and one with female-biased sex ratios (Long Meadow Run). Population sex ratios were determined for only two generations of *Leptoxis spp.* at river sites. The 2006 generation was sampled 2-16 June 2008 and the 2007 generation was sampled 19-27 October 2008.

At each study site, quadrat sampling at randomly selected locations in one riffle was performed. A Surber sampler (0.0929 m^2) was used at tributary sites, and a portable invertebrate box sampler (PIBS; 0.1 m^2) was used at river sites. During 2008, four replicates were collected at both tributary and river sites. During 2009 eight replicates were collected at tributary sites in order to increase the sample size. Locations for these replicates were selected based on preliminary current velocity measurements (Marsh McBirney Model 2000 Flow-Mate, Marsh McBirney, Inc., Frederick, MD) and represented gradients of faster (4 quadrats) and slower (4 quadrats) velocities measured at each site. After collection, juvenile and adult snails in each replicate sample were counted, and adults were retained for further analysis. For determination of population sex ratios, the collection target was a minimum of 120 adult snails. In spring 2008, if fewer than 120 adults were collected in the quadrat samples, adult snails were hand collected from random locations throughout the riffle area until the required number was achieved. For the fall 2008 collection, additional snails were collected using the Surber or PIBS sampler. For 2009 collections, additional snails were not required beyond the eight replicates. For the study of seasonal variation in sex ratios at two tributary sites, a specific number of adult snails was not targeted; adult snails collected within four quadrats (Surber) were used to determine sex ratios. After collection, all adults were transported to the laboratory in site water.

Adult snails were narcotized in 1% $MgCl_2$ in site water for 8-12 hours. In 2008, snails were not measured but in 2009, all snails from each replicate were measured with a digital caliper and placed into 1-mm size classes. Size classes were pooled for determination of sex ratios, except during the intensive sampling of Briery Branch and Long Meadow Run. For these sites, sex ratios for each size class were calculated separately during each collection period. After relaxation, the snails' shells were cracked with vise-grip pliers. Snails were sexed using a 2-point confirmation. Females were determined by presence of an egg-laying groove on the right side of the foot and developing oocytes in the ovary. Male pleurocerid snails do not have a penis; males were determined by absence of the egg-laying groove, the presence of a fleshy pad in its place, and examination of the testes. Snails infected with cercariae of digenetic trematodes were counted for determination of parasitic infection rates, but only uninfected snails were used to determine sex ratios since infection precludes gonad development and full development of the egg-laying groove. Only five apparent hermaphrodites (egg-laying groove and testes) were observed during the study and were not included in sex ratio calculations.

Prior to sampling, it was determined that a minimum of 96 sexed snails were required to allow 95% confidence in the accuracy of the estimated proportion of females within $\pm$ 0.1. This was determined using the

formula: $n = ((z_{\alpha/2})^2 * \pi(1-\pi))/E^2$, where n is the required sample size, z is 1.96 for 95% confidence, π is the expected proportion (0.5 for 1:1 sex ratio), and E is the desired accuracy (Ott and Longnecker, 2001). The desired number of 96 snails was almost always achieved, with a few exceptions, during individual sampling events due to higher than expected parasitic infection rates (Tables 2 and 3).

In addition to sampling for sex ratios, *L. carinata* were collected from tributary sites for analysis of bioaccumulative trace elements in 2008 (Chapter 3). Approximately 50 adult snails were hand-collected from the riffle, held in site water on ice for 24 hr, blotted dry, and immediately frozen. Soft tissue was removed from the shell, pooled within sites, freeze-dried, homogenized, and analyzed for As, Hg, Cd, Cr, Se, and Pb as described in Chapter 2. Only As, Hg, and Cd were above reporting limits at the majority of sampling sites (Table 1) and relationships between concentrations of these trace elements (µg/g dry weight) and population sex ratios were evaluated.

Environmental measurements

Environmental data were collected during spring sampling periods, during mid-summer (tributaries: 8-14 Aug. 2008 and 10-11 Aug. 2009, rivers: 6-24 Aug. 2008), and during late winter (10-15 March 2009; tributary sites only). Mid-summer was selected instead of fall in order to ensure that data could be collected during near-baseflow conditions and relatively stable water temperatures. Temperature (^oC) and specific conductivity (µS/cm) were measured at each site using a calibrated YSI 30 Salinity/Conductivity/Temperature meter (YSI Incorporated, Yellow Springs, OH). Water samples were filtered, preserved, and analyzed for nutrient concentrations (µg/L), including total dissolved inorganic N (DIN) and PO_4-P, as indicated in Chapter 4. Water samples were preserved, filtered, solid-phase extracted, and analyzed for estrogenic activity using a bioluminescent yeast estrogen screen (BLYES) as indicated in Chapter 4. Estrogenic activity was quantified as 17β-estradiol equivalents (ng/L). Mean seasonal temperatures and specific conductivity were used in data analysis; different years were pooled (tributary sites) due to the potentially high variability of point measurements, but spring and summer were separated due to large seasonal differences in these parameters. Mean concentrations of nutrients and estrogenic activity across both seasons and years (tributary sites) were used in data analysis because season/flow conditions did not have a significant effect on concentrations of these analytes at either tributary sites (Chapter 4) or river sites (paired t-tests, $p \geq 0.073$). Periphyton was collected during mid-summer from four rocks per riffle using a wire brush, bar-clamp sampler (9.62

cm^2), and deionized water. Following methods in Steinman et al., (2006), samples were filtered, chlorophyll a was extracted and quantified with a spectrophotometer, and ash-free dry mass (AFDM) was determined. Mean chlorophyll a ($\mu g/cm^2$) and AFDM (mg/cm^2) from the four replicates were calculated and pooled across years for tributary sites. The two measurements were linearly related, so only chlorophyll a was used in data analysis. Summary statistics for all environmental measurements are shown in Table 5-1.

Data analysis

The total numbers of males and females collected in all replicates were used to calculate sex ratios for each site during each sampling period. Sex ratios are presented as proportions of females (number of females/total number of sexed snails). Deviations from expected proportions (0.5 for each sex) and differences in sex ratios between seasons and generations at each sampling site were assessed using G-tests for goodness-of-fit and independence, respectively (Sokal and Rolf, 1995). Variables were tested for normality using the Shapiro-Wilk W test. Non-normal data were transformed when necessary (log_{10}, square root, or arcsine square root). Pearson correlation (Pearson's r) matrices and associated scatter plots were used to assess linearity in relationships between the proportion of females at each site and land use, environmental variables, and snail population characteristics, as well as correlations among land use and environmental variables. Statistical analyses were performed using SAS 9.2 (SAS Institute, Cary, NC), with a significance level of α=0.05.

Although proportions of females at the sampling sites were normally distributed without transformation, logistic regression was used to model relationships between this response and independent land use and environmental variables in order to avoid errors associated with the use of linear regression for a binomial response (Sokal and Rolf, 1995; Wilson and Hardy, 2001). Logistic regression (proc logistic, events/trials notation) was used to test the primary hypothesis of the study and the validity of linear relationships indicated by Pearson correlation analysis. Stepwise logistic regression (model entry criterion: p= 0.15) was used for exploratory analysis of effects of combinations of land use and environmental variables (independent) on the measured proportion of females (dependent). For tributary sites, variables that were highly correlated with watershed percentage of forest ($r \geq 0.85$, $p<0.0001$) and each other ($r \geq 0.75$, $p<0.0001$), including watershed percentage of pasture/hay and crops, DIN concentrations, and specific conductivity, were not included as candidates for selection. For logistic regression models, Wald χ^2 statistics were used to assess significance of parameters, the concordance index (c) was used to

assess predictive capability, Pearson residuals were used to assess model fit, and apparent overdispersion was assessed using Pearson's χ^2/degrees of freedom (SAS Institute, 1995; Wilson and Hardy, 2001). If values for Pearson's χ^2/degrees of freedom exceeded two, and the model met criteria for overdispersion as outlined in SAS Institute (1995), overdispersion was corrected using the Williams method. This method was selected due to the different numbers of snails utilized to determine population sex ratios (SAS Institute, 2010). The potential influence of outliers in the dataset was assessed using plots of Pearson χ^2 deletion differences and DfBetas (SAS Institute, 1995); none were influential and all were retained. The Hosmer-Lemeshow χ^2 test was used to assess overall predictive capability of logistic regression models, where $p<0.05$ represents a lack of predictive capability, but this test may be overly conservative (SAS Institute, 1995).

Results

Tributary sites

A total of 14,887 snails from Shenandoah River tributaries were included in sex ratio calculations (Table 5-2). The proportion of females at each site during each sampling period ranged from 0.36-0.94, and the mean proportion of females at each site ranged from 0.46-0.87 (Table 5-2). Sex ratios at nine sites were consistently female-biased; proportions of females were significantly higher than 0.5 (G-test, $p \leq 0.020$) during all four sampling periods (Table 5-2). One site (Linville Creek) had significantly female-biased sex ratios during only the spring 2008 sampling period (G-test, $p=0.0002$, Table 5-2). Four sites did not show any significant female bias during the four sampling periods, and two of these sites (Naked Creek and Cedar Creek DS) had significantly male-biased sex ratios during the spring 2008 sampling period (G-test, $p \leq 0.025$, Table 5-2). Along Cedar Creek, the sex ratio shifted from consistently female-biased at Cedar Creek US to balanced (or male-biased) at Cedar Creek DS, indicating a longitudinal shift within the same tributary (Table 5-2). The opposite pattern was observed between the US and DS sites on Hawksbill Creek (Table 5-2).

All but four sites had statistically similar proportions of females across the four sampling periods (G-test of independence, Table 5-2). Significantly different proportions of females during one or more sampling periods at Stony Creek US, Muddy Creek, and Jennings Branch may have been due to sampling error caused by habitat alteration at the sampling site, a gradual change in the population, and high parasitic infection rates, respectively.

However, these changes did not cause deviation from the overall trend of female-biased sex ratios at these sites during all sampling periods (Table 5-2). Hawksbill Creek US was the only site with fluctuating sex ratios. This site had a significantly male-biased population sex ratio during one sampling period (2007 generation, spring 2009) and a significantly female-biased population sex ratio during the next sampling period (2008 generation, fall 2009). There was also a large increase in the snail population density at this site during the last sampling period, which may reflect population instability. Overall, there was no pattern of different sex ratios between generations or seasons (2007 generation). Evaluation of sex ratios at two sampling sites for 16 months further indicated no seasonal changes in proportions of females, as the variation in proportions of females between months was minimal (Figure 5-2).

All calculated proportions of females at each site were included in analyses of relationships between sex ratios and land use, environmental variables, and other snail population characteristics. Contrary to the primary hypothesis, there were no linear relationships between proportions of females at tributary sites and mean concentrations of 17β-estradiol equivalents, watershed density of AFOs, or WWTPs, as indicated by correlation analysis ($p \geq 0.095$) and logistic regression analysis with the independent variables tested individually ($p \geq 0.21$) or in combination ($p \geq 0.41$). The relationship between proportions of females at tributary sites and mean concentrations of 17β-estradiol equivalents was nonlinear, the shape of the curve (apparent third order polynomial), suggests that the relationship was likely associative rather than predictive (Figure 5-3a). There was a similar nonlinear relationship between proportions of females at tributary sites and watershed densities of AFOs (Figure 5-3b). There were no other apparent nonlinear relationships between the proportions of females at tributary sites and land use or environmental variables. Although the presence and cumulative effluent flow of WWTPs did not have landscape-scale significant effects on proportions of females in *L. carinata* populations at tributary sites, the only major facility (discharge > 1 MGD) included in the study design was located on Hawksbill Creek, between the US and DS sites, and sex ratios did shift from balanced to female-biased between these two sites (Table 5-2).

The only variable with a significant linear relationship to the proportions of females at tributary sites was mean summer temperature ($r = -0.61$, $p<0.0001$). This relationship remained significant when modeled with logistic regression (coefficient= -0.227 ± 0.036, Wald $\chi^2=39.66$, $p<0.0001$, $c=0.576$). For this model (Figure 5-4), the Pearson residuals were small ($< \pm 2$) and showed no pattern, but the Hosmer-Lemeshow goodness-of-fit test indicated low predictive capability ($\chi^2=210.9$, $p<0.0001$). Inclusion of the only two variables weakly correlated with mean summer

temperature, arsenic in snail tissue (r = -0.53, p=0.041) and summer specific conductivity (r = -0.52, p=0.049), did not improve model fit, as coefficients for both variables were not significant ($p \geq 0.30$). Stepwise variable selection did not result in the inclusion of any variables other than mean summer temperature in the logistic regression model.

For *L. carinata* populations at tributary sites, densities of adults ranged from 75-2,332 snails/m^2, densities of juveniles ranged from 8-2,332 snails/m^2, and rates of parasitic infection were between 2% and 77%. When compared to other snail population characteristics, there were no apparent nonlinear relationships or significant linear relationships between the proportions of females at tributary sites and adult densities (p=0.41) or the proportions of snails infected with trematodes (p=0.93). There was no evidence that juvenile densities soon after hatching (2008 generation collected October 2008) affected eventual proportions of mature adult females (2008 generation collected October 2009) in *L. carinata* populations (p=0.080). There was also no evidence that proportions of adult females in *L. carinata* populations (2006 generation collected May 2008 and 2007 generation collected May 2009) affected the densities of juveniles soon after hatching (2008 generation collected October 2008 and 2009 generation collected October 2009) (p=0.10 and p=0.092, respectively).

There were no significant relationships between proportions of female *L. carinata* at tributary sites and proportions of individuals in any of the shell length classes (1 mm intervals for 7-12 mm and >12 mm) ($p \geq 0.26$) or proportions of individuals in small (7-9 mm; p=0.49), medium (9-11 mm, p=0.53), or large (>11 mm; p=0.74) size categories. For the two tributaries evaluated over a 16-month period, sex ratios were calculated for each individual size class, and the total proportions of females within each size class further illustrated the lack of a relationship between snail size and sex ratio (Figure 5-5).

River sites

A total of 4,018 snails within the genus *Leptoxis* were included in calculations of sex ratios at river sites (Table 5-3). Proportions of females ranged from 0.37-0.82 during the two sampling periods, and mean proportions of females ranged from 0.40-0.82 (Table 5-3). Sex ratios at three sites were consistently female-biased; proportions of females at the James River site (JA-2) and the two mid-river sites on the North Fork of the Shenandoah River (NF-3, NF-3) were significantly different from the expected proportion of 0.5 (G-test, $p \leq 0.0008$) during both sampling periods (Table 5-3). Proportions of females in populations of *Leptoxis spp*. were less than 0.5 at the Holston River, New River, and Rappahannock River sites, but sex ratios were significantly male-biased (G-test,

p=0.0001) at only the New River site during fall 2008 (Table 5-3). For all sites, the proportions of females were statistically similar between the two sampling periods (G-test of independence, G-values <1, $p \geq 0.34$)

Measured proportions of females from both sampling periods were included together in analyses of relationships between sex ratios and environmental variables and other snail population characteristics at river sites. Similar to tributary sites, the relationship between proportions of females and mean concentrations of 17β-estradiol equivalents at river sites was not significant (p=0.31). The only environmental variable with a significant linear relationship to proportions of females at river sites was summer specific conductivity ($r = 0.76$, $p<0.0001$). This relationship remained significant when modeled with logistic regression (coefficient= 0.0030 ± 0.0006, Wald $\chi^2=25.76$, $p<0.0001$, $c=0.631$). The predictive capability and fit of the model were not improved through addition of variables through stepwise selection, or addition of the variables correlated with summer specific conductivity (mean DIN concentration: $r =0.62$, $p=0.042$, and spring specific conductivity: $r = 0.75$, $p=0.0081$). For the final model (Figure 5-6), the Pearson residuals were small ($< \pm 2$) and showed no pattern, but the Hosmer-Lemeshow goodness-of-fit test indicated low predictive capability ($\chi^2=93.6$, $p<0.0001$).

For *Leptoxis spp*. populations at river sites, densities of adults ranged from 60-1,992 snails/m^2, densities of juveniles ranged from 5-1,045 snails/m^2, and rates of parasitic infection were between 0% and 79% .When compared to other snail population characteristics at the river sites, there were no significant linear relationships between the proportions of females and densities of adults (p=0.10) or the proportions of snails infected with trematodes (p=0.50). Proportions of females in *Leptoxis spp.* populations at river sites in May 2008 were not related to the density of juveniles soon after hatching (October 2008, p=0.83).

Discussion

The variation in proportions of females in *L. carinata* populations between sampling sites in Shenandoah River tributaries suggests that sex ratios were influenced by site-specific factors. Site-specific effects were further indicated by longitudinal changes in sex ratios between upstream and downstream site pairs in two Shenandoah River tributaries (Cedar Creek and Hawksbill Creek), and at sites in both the James River basin and North Fork of the Shenandoah River. With the exception of one site in the James River basin (JA-2), snail population sex ratios at all sampling sites outside of the Shenandoah River basin were not female-biased, suggesting that balanced sex ratios

are "normal" for *Leptoxis spp.* Previous studies of populations of *Leptoxis spp*. and other pleurocerid snails also found sex ratios ranging from balanced to female-biased (Aldridge, 1982; Hendrix, 1986; Miller-Way and Way, 1989; reviewed by Dillon 2000), but sampling was generally not conducted over multiple seasons and snail generations at multiple sites. The low within-site variation in proportions of females in *Leptoxis spp*. populations between seasons and snail generations indicates consistency in site-specific factors responsible for variation in population sex ratios between sites. These site-specific effects did not appear to be related to food availability, population densities, or infection with trematode parasites. However, the significant relationships between proportions of females and abiotic environmental variables, mean summer temperature and summer specific conductivity, support the idea that environmental conditions can affect either sex determination or sexual differentiation of pleurocerid snails.

Potential effects of environmental conditions on sexual differentiation

Exposure of individuals to contaminants during critical windows of development can result in disruption of normal sexual differentiation, phenotypic sex expression that differs from genotypic sex, and ultimately, biased population sex ratios. The primary hypothesis of this study was based on the idea that secondary sex ratios of *Leptoxis spp*. could be altered as a result of disruption of sexual differentiation by one type of contaminant (estrogens and estrogenic compounds). This is the suspected cause of sex ratio changes in wild fish populations (Duffy et al., 2009; Jeffries et al., 2008; Vajda et al., 2008) and freshwater mussel populations (Gagne et al., 2011) downstream of sources of estrogens and estrogenic compounds, relative to upstream populations. However, there were no significant relationships between proportions of females in *Leptoxis spp*. populations and measured estrogenic activity at tributary or river sites, or landscape sources of compounds contributing to estrogenic activity at tributary sites. One possible explanation is that estrogenic activity was only quantified during five sampling periods at tributary sites and two sampling periods at river sites. There were no consistent differences in estrogenicity between high and low flow conditions, but variability in concentrations of estrogens and estrogenic compounds over the time period corresponding to hatching through gametogenesis in *Leptoxis spp.* (i.e. August – March; Chapter 7) could be more important than general trends in concentrations in terms of potential site-specific effects on sexual differentiation and population sex ratios. Exposures of fish to natural and synthetic estrogens have shown that the

timing and duration of exposure is related to the degree of complete feminization within the exposed populations (Fenske et al., 2005; Hahlbeck et al., 2004; Lange et al., 2009).

Alternatively, sexual differentiation in *L. carinata* may not be susceptible to feminizing effects of estrogens. In a study of oysters, the weak xenoestrogen nonylphenol was an apparent cause of female-biased sex ratios in adult populations after exposure of undifferentiated larvae (Nice et al., 2003), but the actual role of steroidal estrogens in sexual differentiation of mollusks is unknown. Injections of oysters with 17β-estradiol during early stages of seasonal maturation induced sex reversal from males to females and resulted in female-biased sex ratios (Mori et al., 1969). In contrast, injections of undifferentiated juvenile scallops with 17β-estradiol stimulated morphological differentiation of gametes, but ultimately resulted in significantly more males than females relative to unexposed scallops (Wang and Croll, 2004). Aqueous exposures of undifferentiated mollusks to environmentally realistic estrogen concentrations, with subsequent evaluation of effects on sexual differentiation, have yet to be conducted.

Sexual differentiation of *L. carinata* could potentially be disrupted by non-estrogenic pathways. Retinoic acid signaling via the retinoid X receptor (RXR) has recently been implicated as a mechanism involved in development of male characteristics in female marine gastropods (imposex) after exposure to tributyltin (Castro et al., 2007b; Nishikawa et al., 2004). Sternberg et al. (2008) demonstrated that tributyltin stimulates development of male characteristics in females through inappropriate timing of RXR activation during the period of recrudescence in adult mudsnails. It appears possible that interference with retinoic acid signaling or direct suppression of RXR could suppress male development and stimulate development of female characteristics. Retinoid signaling is involved with sexual differentiation of rodents (summarized by Sternberg et al., 2008), but involvement of this signaling system in sexual differentiation of immature gastropods or other mollusks has yet to be studied.

Contaminants with the potential to suppress retinoid signaling systems were not specifically analyzed in the current study, but several have been documented in the Shenandoah River watershed. Arsenic can affect gene transcription dependent on ligand-activated retinoic acid receptor (RAR) forming a heterodimer with RXR; very low concentrations of arsenic stimulate gene transcription, while higher concentrations inhibit transcription (Davey et al., 2008). Relatively high concentrations of arsenic (1.2-10 μg/g dry wt.) have been measured in tissue from *L. carinata* and Asian clams from Shenandoah River tributaries, and several potential minor sources of arsenic are present in the Shenandoah River watershed (Chapter 3). The herbicide atrazine also appears to affect retinoid signaling systems,

although the mechanism of action is unclear (Lenkowski and McLaughlin, 2010; Solari et al., 2010). Elevated concentrations of agricultural pesticides, particularly atrazine, metolachlor, and dimethenamide, are suspected causes of reduced retinol concentrations in plasma and livers of bullfrogs in Canadian streams (Berube et al., 2005; Boily et al., 2005). Agricultural pesticides, including atrazine, simazine, prometon, and metolachlor, have been detected in groundwater and streams within the Shenandoah River watershed, with atrazine detected most frequently and in the highest concentrations (>0.1 µg/L) (Ator et al., 1998). In passive samplers deployed in the Shenandoah River and Cowpasture River (JA-1), concentrations of agricultural pesticides, including atrazine, simazine, metolachlor, and desethylatrazine, were highest at a North Fork Shenandoah River site (Alvarez et al., 2008). This site corresponds to the location of NF-2 in the current study, where female-biased sex ratios were observed. Outside of the Shenandoah River watershed, female-biased sex ratios were also observed at the James River site (JA-2). A tributary (Jackson River) upstream of JA-2 receives effluent from a large pulp and paper mill. The presence of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in bleach kraft mill effluent is well documented (USEPA 2007), and this compound interferes with retinoid signaling systems through several pathways (Nilsson and Hakansson, 2002).

The relationships between proportions of females in *Leptoxis spp.* populations at tributary and river sites and abiotic environmental variables may indirectly relate to the relative exposure of *Leptoxis spp*. to some or all of the aforementioned contaminants. Mean summer temperature was negatively related to proportions of females in *L. carinata* populations at tributary sties. Differences in summer temperatures between tributary sites may reflect the degree of exposure of *L. carinata* to groundwater. Groundwater is the primary contributor to summer baseflow in streams and sites close to areas of groundwater discharge likely have relatively low summer temperatures. Many streams in the Shenandoah River watershed are underlain by carbonate bedrock and spatial variation in groundwater contribution to streamflow is common in carbonate systems (Lindsey et al., 2003; Yager et al., 2008). Carbonate geology also creates the potential for rapid transport of contaminants from landscape sources to streams via groundwater (Ator et al., 1998). Thus, in addition to lower summer temperatures, *L. carinata* populations at sampling sites closer to groundwater discharges could also experience more consistent exposure to contaminants in groundwater or exposure to higher concentrations of these contaminants relative to populations at sampling sites further from groundwater discharges. The negative correlation between arsenic concentrations in snail tissue and mean summer temperature in Shenandoah River tributaries supports this idea, although arsenic concentrations in snail tissue were not directly related to proportions of females in *L. carinata* populations at tributary sites.

Summer specific conductivity was positively related to the proportions of females in *Leptoxis spp.* at river sites. Elevated conductivity in streams and rivers can occur as a result of runoff from agricultural and urban landscapes (Price and Lee, 2006; Walters et al., 2009) and discharge of effluent from municipal and industrial WWTPs (Gagne et al., 2011; Hall et al., 2009). In the North Fork of the Shenandoah River, major WWTP discharges and several tributaries draining areas of intensive agricultural production enter the river between NF-1 and NF-3, which corresponds to a shift in *L. carinata* population sex ratios from balanced to female-biased. As previously described, the tributary receiving bleached kraft mill effluent from a pulp and paper mill enters the James River between JA-1 and JA-2, which also corresponds to a shift in population sex ratios of *L. carinata*. In addition to ions, runoff and effluent discharges can introduce a number of contaminants to streams and rivers, including compounds with the potential to disrupt both estrogenic and retinoid signaling systems, which creates a potential mechanism for an indirect relationship between specific conductivity and proportions of females in *Leptoxis spp*. populations at river sites.

Potential effects of environmental conditions on sex determination

The significant negative relationship between mean summer temperature and proportions of females in *L. carinata* populations at tributary sampling sites was also evaluated as a potential indicator of an environmental influence on sex determination in *L. carinata*. Two mechanisms of genetic sex determination have been identified in gonochoristic gastropods: heterogametic (Avise et al., 2004; Barsiene et al., 2000; Vitturi et al., 1998), and oligogenic (small number of genes) (Yusa, 2007). The sex of an individual *Leptoxis spp*. is probably determined by one of these genetic mechanisms. If multiple genes are involved, the environmental influence on sex determination can increase, as can sex ratio variation across environmental conditions (Bull, 1983; Cook, 2002). Environmental sex determination (ESD) occurs when undifferentiated offspring have the potential to develop as either sex, depending on the conditions normally encountered during development (Conover, 2004). Temperature dependent sex determination (TSD) is a type of ESD that is well described in several species of fish and reptiles (Bull, 1983; Conover, 2004), but is not well studied in invertebrates. In populations of Atlantic silverside, *Menidia menidia*, offspring produced early in the breeding season when water temperatures are low primarily develop as females and grow larger than offspring produced later in the breeding season, which develop primarily as males (Conover, 2004). Growth in *Leptoxis spp*. is limited to the summer and fall after hatching and the spring and summer of the following

year (Aldrige, 1982; Miller-Way and Way, 1989). Enough biomass must be accumulated in order to overwinter and reproduce at 20-24 months of age, and females accumulate more biomass in terms of carbon and nitrogen than males (Aldridge, 1982). The relatively short period for growth, coupled with semelparity, suggests that earlier development of females relative to males could be advantageous for *Leptoxis spp*. For *L. carinata* populations in Shenandoah River tributaries, the observed period of egg-laying was April-July. If greater female production at lower water temperatures does indeed occur, it is a potential mechanism for production of more females in streams with lower water temperatures at the end of the egg-laying period, and the observed negative relationship between proportions of females in *L. carinata* populations and mean summer temperature in Shenandoah River tributaries.

For *L. carinata* populations in individual streams, shell length corresponds directly to age; individuals that hatch earlier attain larger sizes than individuals that hatch later in a given year. Thus, if early female development at lower water temperatures is an adaptive response, the proportions of females in larger size classes would be expected to be consistently high, and the proportions of females in smaller size classes would be expected to be low in streams with warm summer temperatures, and possibly higher in streams with cooler summer temperatures. However, there were no significant relationships between individual size classes or size class groupings (small, medium, large), and proportions of females in *L. carinata* populations in Shenandoah River tributaries. In addition, size-class specific sex ratios calculated from multiple collections of *L. carinata* from two Shenandoah River tributaries over a 16-month period indicated similar proportions of females in the dominant size classes (7-11mm), with a relative increase in the proportions of males in the largest size classes. These results were consistent for both tributaries, even though one had consistently female-biased sex ratios (Long Meadow Run) and the other had consistently balanced sex ratios (Briery Branch). Similar results were obtained from a single collection of another pleurocerid snail, *Elimia (Goniobasis) proxima*, from a population in a North Carolina river with a balanced sex ratio (Dillon 2000). It appears as if early development of females does not occur in *L. carinata* populations, and possibly in populations of other pleurocerid snails. Thus, water temperature is probably not directly related to sex determination in *L. carinata*.

Significance of female-biased sex ratios?

The significance of female-biased sex ratios within populations of pleurocerid snails is unknown. There were no significant relationships between proportions of females in *Leptoxis spp.* populations and densities of

juvenile snails. This suggests that greater proportions of females within populations do not result in greater production of surviving offspring. *Leptoxis carinata* females do produce fewer eggs in response to higher egg densities (Aldridge, 1982), and given the very low survival rate of eggs, there could be a cost to individual fitness. Similarly, individual fitness could be reduced in populations with strongly female-biased population sex ratios due to increased competition for mates. It is unclear whether populations with strongly biased sex ratios are "unhealthy". Reproductive failure during 2009 was observed at one site with female-biased sex ratios (Long Meadow Run; Chapter 7), but this was not observed at another site with similar proportions of females (Jennings Branch). Overproduction of one sex is adaptive in some species of birds and mammals, but characteristics of *L. carinata* do not meet the criteria for expected departure from equal allocation which include: low fecundity, high parental care, and intense sexual selection (Frank, 1990). It is possible that overproduction of females within *Leptoxis spp*. populations at some sampling sites is an adaptive response to stress, but a mechanism for evolution and maintenance of this pattern is unknown.

Conclusions

Results of this study provide some indirect support for the general hypothesis that sexual differentiation in pleurocerid snails can be affected by environmental contaminants. However, there was no direct evidence of an effect specific to compounds that can bind to the estrogen receptor, and it appears possible that sexual differentiation in these organisms can be disrupted through non-estrogenic pathways. Given the current interest in assessing effects of endocrine-disrupting compounds on aquatic organisms, further research on mechanisms of sex determination and sexual differentiation in mollusks appears to be of critical importance. For the notoriously understudied species of gonochoristic freshwater gastropods, knowledge of the significance of sex ratio variation could potentially increase their utility as bioindicators and benefit their conservation, particularly if female-biased sex ratios are an indication of contaminant exposure or stressed populations.

Literature cited

Aldridge, D. W. (1982). Reproductive tactics in relation to life-cycle bioenergetics in three natural populations of the freshwater snail, *Leptoxis carinata*. *Ecology, 63*(1), 196-208.

Alvarez, D., Cranor, W., Perkins, S., Schroeder, V., Werner, S., Furlong, E., et al., (2008). Reconnaissance of persistent and emerging contaminants in the Shenandoah and James River basins, Virginia, during spring of 2007. US Geological Survey. Open-file Report 2008-1231.

Ator, S. W., Blomquist, J. D., Brakebill, J. W., Denis, J. M., Ferrari, M. J., Miller, C. V., et al., (1998). Water quality in the Potomac river basin, Maryland, Pennsylvania, Virginia, West Virginia, and the District of Columbia, 1992-1996. U.S. Geological Survey. http://www.water.usgs.gov/pubs/circ1166

Avise, J. C., Power, A. J., & Walker, D. (2011). Genetic sex determination, gender identification, and pseudohermaphrotidism in the knobbed whelk, *Busycon carica* (Mollusca: Melongenidae). *Proceedings of the Royal Society of London, Series B: Biological Sciences, 271*, 641-646.

Bannister, R., Beresford, N., May, D., Routledge, E. J., Jobling, S., & Rand-Weaver, M. (2007). Novel estrogen receptor-related transcripts in *Marisa cornuarietis*; a freshwater snail with reported sensitivity to estrogenic chemicals. *Environmental Science & Technology, 41*, 2643-2650.

Barsiene, J., Ribi, F., & Barstye, D. (2000). Comparative karyological analysis of five species of *Viviparus* (Gastropoda: Prosobranchia). *Journal of Molluscan Studies, 66*, 259-271.

Berube, V. E., Boily, M. H., DeBlois, C., Dassylva, N., & Spear, P. A. (2005). Plasma retinoid profile in bullfrogs, *Rana castesbeiana*, in relation to agricultural intensity of sub-watersheds in the Yamaska River drainage basin, Quebec, Canada. *Aquatic Toxicology, 71*, 109-120.

Blazer, V. S., Iwanowicz, L. R., Iwanowicz, D. D., Smith, D. R., Young, J. A., Hedrick, J. D., et al., (2007). Intersex (testicular oocytes) in smallmouth bass from the Potomac River and selected nearby drainages. *Journal of Aquatic Animal Health, 19*, 242-253.

Boily, M. H., Berube, V. E., Spear, P. A., DeBlois, C., & Dassylva, N. (2005). Hepatic retinoids of bullfrogs in relation to agricultural pesticides. *Environmental Toxicology and Chemistry, 24*, 1099-1106.

Brown, K. M., Lang, B., & Perez, K. E. (2008). The conservation ecology of North American pleurocerid and hydrobiid gastropods. *Journal of the North American Benthological Society, 27*(2), 484-495.

Bull, J. J. (1983). *Evolution of sex determining mechanisms*. Menlo Park, CA: Benjamin/Cummings Publishing Company.

Castro, L. F. C., Melo, C., Guillot, R., Mendes, I., Queiros, S., Lima, D., et al., (2007a). The estrogen receptor of the gastropod *Nucella lapillus*: Modulation following exposure to estrogenic effluent? *Aquatic Toxicology, 84*, 465-468.

Castro, L. F. C., Lima, D., Machado, A., Melo, C., Hiromori, Y., Nishikawa, J. I., et al., (2007b). Imposex induction is mediated through the retinoid x receptor signalling pathway in the neogastropod *Nucella lapillus*. *Aquatic Toxicology, 85*, 57-66.

Ciocan, C. M., Cubero-Leon, E., Puinean, A. M., Hill, E. M., Minier, C., Osada, M., et al., (2010). Effects of estrogen exposure in mussels, *Mytilus edulis*, at different stages of gametogenesis. *Environmental Pollution, 158*, 2977-2984.

Conover, D. O. (2004). Temperature-dependent sex determination in fishes. In N. Valenzuela, & V. A. Lance (Eds.), *Temperature-dependent sex determination in vertebrates* (pp. 11-20). Washington, DC: Smithsonian Books.

Cook, J. M. (2002). Sex determination in invertebrates. In I. C. W. Hardy (Ed.), *Sex ratios: Concepts and research methods* (pp. 178-194). New York, NY: Cambridge University Press.

Davey, J. C., Nomikos, A. P., Wungjiranirun, M., Sherman, J. R., Ingram, L., Batki, C., et al., (2008). Arsenic as an endocrine disruptor: Arsenic disrupts retinoic acid receptor- and thyroid hormone receptor-mediated gene regulation and thyroid hormone-mediated amphibian tail metamorphosis. *Environmental Health Perspectives, 116*, 165-172.

Dillon, R. T., Jr. (2000). *The ecology of freshwater molluscs*. New York, NY: Cambridge University Press.

Duffy, T. A., McElroy, A. E., & Conover, D. O. (2009). Variable susceptibility and response to estrogenic chemicals in *Menidia menidia*. *Marine Ecology Progress Series, 380*, 245-254.

Duft, M., Schulte-Oehlmann, U., Tillmann, M., Weltje, L., & Oehlmann, J. (2003). Stimulated embryo production as a parameter of estrogenic exposure via sediments in the freshwater mudsnail *Potamopyrgus antipodarum*. *Aquatic Toxicology, 64*, 437-449.

Fenske, M., Maak, G., Schafers, C., & Segner, H. (2005). An environmentally relevant concentration of estrogen induces arrest of male gonad development in zebrafish, *Danio rerio*. *Environmental Toxicology and Chemistry, 24*(5), 1088-1098.

Fisher, R. A. (1930). *The genetical theory of natural selection*. Oxford, UK: Clarendon.

Frank, S. A. (1990). Sex allocation theory for birds and mammals. *Annual Review of Ecology and Systematics, 21*, 13-55.

Gagne, F., Blaise, C., Salazar, M., Salazar, S., & Hansen, P. D. (2001). Evaluation of estrogenic effects of municipal effluents to the freshwater mussel, *Elliptio complanata*. *Comparative Biochemistry and Physiology, Part C, 128*, 213-225.

Gagne, F., Bouchard, B., Andre, C., Farcy, E., & Fournier, M. (2011). Evidence of feminization in wild *Elliptio complanata* mussels in the receiving waters downstream of a municipal effluent. *Comparative Biochemistry and Physiology, Part C, 153*, 99-106.

Hahlbeck, E., Griffiths, R., & Bengtsson, B.-E. (2004). The juvenile three-spined stickleback (*Gasterosteus aculeatus* l.) as a model organism for endocrine disruption i: Sexual differentiation. *Aquatic Toxicology, 70*, 287-310.

Hall, T. J., Ragsdale, R. L., Arthurs, W. J., Ikoma, J., Borton, D. L., & Cook, D. L. (2009). A long-term, multitrophic level study to assess pulp and paper mill effluent effects on aquatic communities in four U.S. receiving waters: Characteristics of the study streams, sample sites, mills, and mill effluents. *Integrated Environmental Assessment and Management, 5*, 199-218.

Hendrix, S. S. (1986). Population biology of the pleurocerid snail, *Leptoxis carinata* (Brug.) in Marsh Creek, Adams County, PA. *American Malacological Union Bulletin, 4*(1), 119.

Homer, C., Dewitz, J., Fry, J., Coan, M., Hossain, N., Larson, C., et al., (2001). Completion of the 2001 National Land Cover Database for the conterminous United States. *Photogrammetric Engineering and Remote Sensing, 73*, 337-341.

Janer, G., & Porte, C. (2007). Sex steroids and potential mechanisms of non-genomic endocrine disruption in invertebrates. *Ecotoxicology, 16*, 145-160.

Jeffries, K. M., Nelson, E. R., Jackson, L. J., & Habibi, H. R. (2008). Basin-wide impacts of compounds with estrogen-like activity on longnose dace (*Rhinichthys cataractae*) in two prarie rivers of Alberta, Canada. *Environmental Toxicology and Chemistry, 27*, 2042-2052.

Jobling, S., Casey, D., Rodgers-Gray, T., Oehlmann, J., Schulte-Oehlmann, U., Pawlowski, S., et al., (2003). Comparative responses of molluscs and fish to environmental estrogens and estrogenic effluent. *Aquatic Toxicology, 65*, 205-220.

Jobling, S., Nolan, M., Tyler, C. R., Brighty, G., & Sumpter, J. P. (1998). Widespread sexual disruption in wild fish. *Environmental Science & Technology, 32*, 2498-2506.

Kajiwara, M., Kuraku, S., Kurokawa, T., Kato, K., Toda, S., Hirose, H., et al., (2006). Tissue preferential expression of estrogen receptor gene in the marine snail, *Thais clavigera*. *General and Comparative Endocrinology, 148*, 315-326.

Keay, J., Bridgham, J. T., & Thornton, J. W. (2006). The *Octopus vulgaris* estrogen receptor is a constitutive transcriptional activator: Evolutionary and functional implications. *Endocrinology, 147*, 3861-3869.

Ketata, I., Denier, X., Hamza-Chaffai, A., & Minier, C. (2008). Endocrine-related reproductive effects in molluscs. *Comparative Biochemistry and Physiology, Part C, 147*, 261-270.

Lafont, R., & Mathieu, M. (2007). Steroids in aquatic invertebrates. *Ecotoxicology, 16*, 109-130.

Lange, A., Paull, G. C., Coe, T. S., Yoshinao, K., Urushitani, H., Iguchi, T., et al., (2009). Sexual reprogramming and estrogenic sensitization in wild fish exposed to ethinylestradiol. *Environmental Science and Technology, 43*, 1219-1225.

Langston, W. J., Burt, G. R., & Chesman, B. S. (2007). Feminization of male clams *Scrobicularia plana* from estuaries in southwest UK and its induction by endocrine-disrupting chemicals. *Marine Ecology Progress Series, 333*, 173-184.

Lenkowski, J. R., & McLaughlin, K. A. (2010). Acute atrazine exposure disrupts matrix metalloproteinases and retinoid signaling during organ morphogenesis in *Xenopus laevis*. *Journal of Applied Toxicology, 30*, 582-589.

Lindsey, B. D., Phillips, S. W., Donnelly, C. A., Speiran, G. K., Plummer, L. N., Bohlke, J.-K., et al., (2003). Residence times and nitrate transport in ground water discharging to streams in the Chesapeake Bay watershed. U.S. Geological Survey. Water Resources Investigations Report 03-4035.

Lysne, S. J., Perez, K. E., Brown, K. M., Minton, R. L., & Sides, J. D. (2008). A review of freshwater gastropod conservation: Challenges and opportunities. *Journal of the North American Benthological Society, 27*, 463-470.

Matthiessen, P. (2008). An assessment of endocrine disruption in mollusks and the potential for developing internationally standardized mollusk life cycle test guidelines. *Integrated Environmental Assessment and Management, 4*, 274-284.

Miller-Way, C. A., & Way, C. M. (1989). The life history of *Leptoxis dilatata* (Conrad) (Prosobranchia: Pleuroceridae) from the Laurel Fork River, West Virginia. *American Midland Naturalist, 122*, 193-198.

Mori, K. (1969). Effect of steroid on oyster-iv. Acceleration of sexual maturation in female *Crassostrea gigas* by estradiol-17b. *Bulletin of the Japanese Society of Fisheries Science, 35*, 1077-1079.

Nice, H. E., Morritt, D., Crane, M., & Thorndyke, M. (2003). Long-term and transgenerational effects of nonylphenol exposure at a key stage in the development of *Crassostrea gigas*. Possible endocrine disruption? *Marine Ecology Progress Series, 256*, 293-300.

Nilsson, C. B., & Hakansson, H. (2002). The retinoid signaling system - a target in dioxin toxicity. *Critical Reviews in Toxicology, 32*, 211-232.

Nishikawa, J. I., Mamiya, F., Kanayama, T., Nishikawa, T., Shiraishi, F., & Horiguchi, T. (2004). Involvement of the retinoid x receptor in the development of imposex caused by organotins in gastropods. *Environmental Science & Technology, 38*, 6271-6276.

Oehlmann, J., Schulte-Oehlmann, U., Tillmann, M., & Markert, B. (2000). Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part i: Bisphenol a and octylphenol as xeno-estrogens. *Ecotoxicology, 9*, 383-397.

Orth, D. J., Alexander, K. A., Ciparis, S., Frimpong, E., Henley, W., & Voshell, J. R. (2009). Investigation into smallmouth bass mortality in Virginia's rivers. http://www.dgif.virginia.gov/fishing/fish-kill/Smallmout-Bass-Mortality-Final-Rpt-VT-2009.pdf

Price, K., & Leigh, D. S. (2006). Comparative water quality of lightly- and moderately-impacted streams in the southern Blue Ridge mountains, USA. *Environmental Monitoring and Assessment, 120*, 269-300.

Quinn, B., Gagne, F., Costello, M., McKenzie, C., Wilson, J., & Mothersill, C. (2004). The endocrine disrupting effect of municipal effluent on the zebra mussel (*Dreissena polymorpha*). *Aquatic Toxicology, 66*, 279-292.

SAS Institute, Inc. (1995). *Logsistic regression examples using the SAS system, version 6, first edition*. Cary, NC: SAS Institute, Inc.

SAS Institute, Inc. (2010). *SAS/STAT 9.22 user's guide*. Cary, NC: SAS Institute, Inc.

Sokal, R. R., & Rohlf, F. J. (1995). *Biometry: The principles and practice of statistics in biological research.* (3rd ed.). New York, NY: W.H. Freeman.

Solari, M., Paquin, J., Ducharme, P., & Boily, M. H. (2010). P19 neuronal differentiation and retinoic acid metabolism as criteria to investigate atrazine, nitrite, and nitrate developmental toxicity. *Toxicological Sciences, 113*, 116-126.

Steinman, A. D., Lamberti, G. A., & Leavitt, P. R. (2006). Biomass and pigments of benthic algae. In F. R. Hauer, & G. A. Lamberti (Eds.), *Methods in stream ecology* (pp. 357-380). New York: Elsevier.

Sternberg, R. M., Hotchkiss, A. K., & LeBlanc, G. A. (2008). Synchronized expression of retinoid x receptor mRNA with reproductive tract recrudescence in an imposex-susceptible mollusc. *Environmental Science & Technology, 42*, 1345-1351.

Thornton, J. W., Need, E., & Crews, D. (2003). Resurrecting the ancestral steroid receptor: Ancient origin of estrogen signaling. *Science, 301*, 1714-1717.

Tyler, C. R., Spary, C., Gibson, R., Santos, E. M., Shears, J., & Hill, E. M. (2005). Accounting for differences in estrogenic responses in rainbow trout (*Oncorhynchus mykiss*: Salmonidae) and roach (*Rutilus rutilus*: Cyprinidae) exposed to effluents from wastewater treatment works. *Environmental Science & Technology, 39*, 2599-2607.

United States Environmental Protection Agency (USEPA) (2007). Exposure and human health reassessment of 2,3,7,8-tetrachlorodibenzo-p-dioxin (tcdd) and related compounds, National Acedemy of Sciences (NAS) review draft. *Part 1, Volume 1, Chapter 8.* , http://www.epa.gov/ncea/pdfs/dioxin/nas-review/

Virginia Department of Environmental Quality (VADEQ) (2005). Fish tissue analysis for metals 2005. http://www.deq.virginia.gov/fishtissue/fishtissue.html.

Vajda, A. M., Barber, L. B., Gray, J. L., Lopez, E. M., Woodling, J. D., & Norris, D. O. (2008). Reproductive disruption in fish downstream from an estrogenic wastewater effluent. *Environmental Science & Technology, 42*, 3407-3414.

Vitturi, R., Colomba, M. S., Caputo, V., & Pandolfo, A. (1998). XY chromosome sex systems in the neogastropods *Fasciolaria lignaria* and *Pisania striata* (Mollusca: Prosobranchia). *Journal of Heredity, 89*, 538-543.

Walters, D. M., Roy, A. H., & Leigh, D. S. (2009). Environmental indicators of macroinvertebrate and fish assemblage integrity in urbanizing watersheds. *Ecological Indicators, 9*, 1222-1233.

Wang, C., & Croll, R. P. (2004). Effects of sex steroids on gonadal development and gender determination in the sea scallop, *Placopecten magellanicus*. *Aquaculture, 238*, 483-498.

Wilson, K., & Hardy, I. C. W. (2002). Statistical analysis of sex ratios: An introduction. In I. C. W. Hardy (Ed.), *Sex ratios: Concepts and research methods* (pp. 48-92). New York: Cambridge University Press.

Woodling, J. D., Lopez, E. M., Maldonado, T. A., Norris, D. O., & Vajda, A. M. (2006). Intersex and other reproductive disruption of fish in wastewater effluent dominated Colorado streams. *Comparative Biochemistry and Physiology, Part C, 144*, 10-15.

Yager, R. M., Southworth, S., & Voss, C. I. (2008). Simulation of ground-water flow in the Shenandoah Valley, Virginia and West Virginia, using variable-direction anisotropy in hydraulic conductivity to represent bedrock structure. U.S. Geological Survey. Scientific Investigations Report 2008-5002.

Yonkos, L. T., Fisher, D. J., Van Veld, P. A., Kane, A. S., McGee, B. L., & Staver, K. W. (2010). Poultry-litter induced endocrine disruption in fathead minnow, sheepshead minnow, and mummichog laboratory exposures. *Environmental Toxicology and Chemistry, 29*, 2328-2340.

Yusa, Y. (2007). Causes of variation in sex ratio and models of sex determination in the Mollusca - an overview. *American Malacological Bulletin, 23*, 89-98.

Table 5- 1. Ranges of land use and environmental characteristics for sampling sites in Shenandoah River tributaries and several rivers in Virginia.

	Tributary sites	River sites [a]
Spring temp. (^oC) [b]	13.0 – 19.9	15.8 – 30.1
Summer temp. (^oC) [b]	19.2 – 24.5	22.4 – 27.6
Spring sp. conductivity (µS/cm) [b]	83.8 – 625	171 – 618
Summer sp. conductivity (µS/cm) [b]	102 – 413	154 – 514
17β-estradiol equivalents (ng/L) [c]	0.19 – 3.4	0.16 – 1.2
PO_4-P (µg/L) [c]	2.91 – 471	2.31 – 95.9
DIN (µg/L) [c]	36.62 – 6,589	62.82 – 2,637
Chlorophyll a (µg/cm^2) [d]	2.0 – 28	0.35 – 15
As in snail tissue (µg/g dry wt.)	2.5 – 10	-
Cd in snail tissue (µg/g dry wt.)	<0.010 – 1.5	-
Hg in snail tissue (µg/g dry wt.)	<0.010 – 0.45	-

[a] For river sites, metals in snail tissue were not measured
[b] Measured in May and August; tributaries = mean of 2008 and 2009, rivers = 2008 only
[c] Tributaries = mean of five sampling periods (May and August 2008, March, May and August 2009); rivers = mean of two sampling periods (May and August 2008)
[d] Measured in August; tributaries = mean of 2008 and 2009, rivers = 2008 only

Table 5- 2. Proportions of females in *Leptoxis carinata* populations in Shenandoah River tributaries during seasonal sampling of three generations of snails. The total numbers of snails sampled are indicated in parentheses. Overall mean proportions of females ± one standard deviation (SD), and results of G-tests of independence are also shown.

#	Site Name	May 2008 (2006 gen.)	Oct. 2008 (2007 gen.)	May 2009 (2007 gen.)	Oct. 2009 (2008 gen.)	Mean ± SD	G-value
1	Cedar Cr. –US	0.65 (148)	0.63 (185)	0.75 (92)	0.65 (133)	0.67 ± 0.05	4.2
2	Cedar Cr. –DS	*0.40* (125)	0.52 (218)	0.56 (130)	0.52 (315)	0.50 ± 0.07	7.8
3	Stony Cr. -US	0.72 (167)	0.58 (173)	0.69 (223)	0.65 (720)	0.66 ± 0.06	8.5*
4	Stony Cr. -DS	0.71 (83)	0.74 (316)	0.72 (229)	0.65 (344)	0.70 ± 0.04	6.3
5	Passage Cr.	0.55 (134)	0.51 (163)	0.52 (123)	0.50 (325)	0.52 ± 0.02	1.0
6	Mill Cr.	0.68 (97)	0.61 (175)	0.62 (241)	0.61 (245)	0.63 ± 0.03	1.8
7	Smith Cr.	0.68 (170)	0.63 (209)	0.67 (513)	0.60 (533)	0.65 ± 0.03	6.4
8	Hawksbill. –US	0.56 (201)	0.52 (157)	*0.41* (190)	0.60 (875)	0.52 + 0.08	24**
9	Hawksbill –DS	0.67 (134)	0.61 (224)	0.60 (648)	0.59 (526)	0.62 ± 0.04	3.2
10	Long Meadow R.	0.89 (127)	0.85 (142)	0.82 (255)	0.79 (126)	0.84 ± 0.04	5.3
11	Linville Cr.	0.60 (135)	0.56 (139)	0.53 (94)	0.55 (110)	0.56 ± 0.03	1.3
12	Naked Cr.	*0.36* (83)	0.49 (143)	0.48 (103)	0.52 (148)	0.46 ± 0.07	5.7
13	Briery Br.	0.46 (124)	0.50 (280)	0.50 (545)	0.47 (460)	0.48 ± 0.02	1.3
14	Muddy Cr.	0.68 (148)	0.58 (543)	0.58 (411)	0.55 (693)	0.60 + 0.06	8.8*
15	Jennings Br.	0.79 (28)	0.92 (180)	0.83 (60)	0.94 (326)	0.87 ± 0.07	47**

Underlined proportions indicate significantly female-biased sex ratios (G-test, $p \leq 0.04$)
Italicized proportions indicate significantly male-biased sex ratios (G-test, $p \leq 0.025$)
G-values for G-test of independence: *$p<0.05$, **$p<0.0001$

Table 5- 3. Proportions of females in *Leptoxis spp.* populations in Virginia rivers during seasonal sampling of two generations of snails. The total numbers of snails sampled are indicated in parentheses and overall mean proportions of females ± one standard deviation (SD) are shown.

Code	River	*Leptoxis spp.*	May 2008 (2006 gen.)	Oct 2008 (2007 gen.)	Mean ± SD
HOL	Holston R.	*L. praerosa*	0.46 (153)	0.46 (186)	0.46 ± 0.00
NEW	New R.	*L. dilatata*	0.42 (130)	*0.37* (238)	0.40 ± 0.03
RAPP	Rappahannock	*L. carinata*	0.44 (94)	0.43 (183)	0.43 ± 0.00
JA-1	Cowpasture R.	*L. carinata*	0.57 (138)	0.52 (187)	0.55 ± 0.04
JA-2	James R.	*L. carinata*	0.82 (147)	0.81 (308)	0.82 ± 0.01
NF-1	NF Shenandoah	*L. carinata*	0.45 (42)	0.50 (160)	0.48 ± 0.03
NF-2	NF Shenandoah	*L. carinata*	0.64 (144)	0.67 (444)	0.65 ± 0.02
NF-3	NF Shenandoah	*L. carinata*	0.65 (167)	0.69 (177)	0.67 ± 0.03
NF-4	NF Shenandoah	*L. carinata*	0.50 (131)	0.50 (142)	0.50 ± 0.00
SF-1	North River	*L. carinata*	0.58 (117)	0.53 (226)	0.55 ± 0.04
SF-2	SF Shenandoah	*L. carinata*	0.50 (262)	0.52 (242)	0.51 ± 0.02

Underlined proportions indicate significantly female-biased sex ratios (G-test, $p \leq 0.0008$)
Italicized proportion indicates significantly male-biased sex ratio (G-test, $p < 0.0001$)

Figure 5- 1. Locations of sampling sites in rivers (letters) relative to counties in Virginia and locations of sampling sites in tributaries (numbers) within the Shenandoah River watershed (enlargement). Within the Shenandoah River watershed, the two forks of the river are indicated by the thin grey line and the 12-digit hydrologic unit code subwatersheds are outlined.

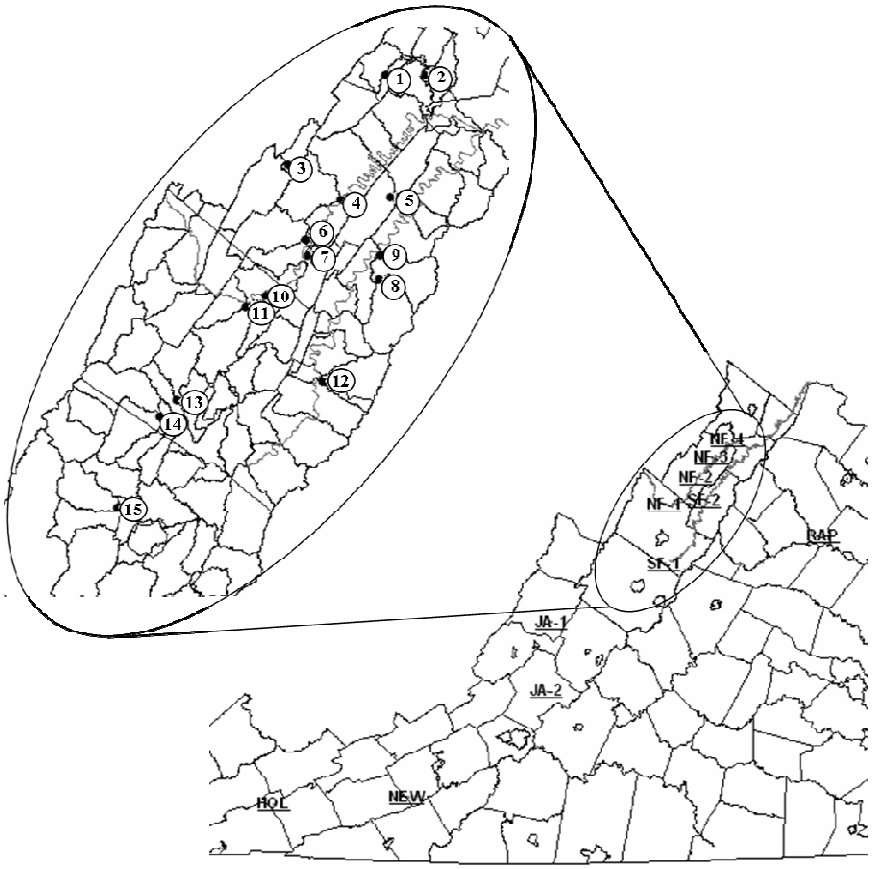


Figure 5- 2. Variation in the proportions of females at two Shenandoah River tributary sites over a 16-month period, Briery Branch (open) and Long Meadow Run (filled). Lines represent the overall mean proportion of females at each site.

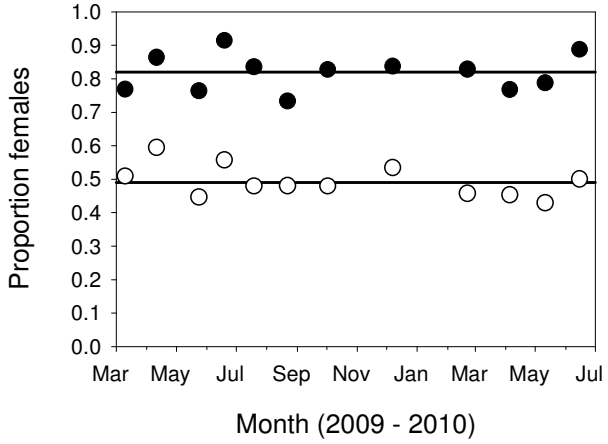


Figure 5- 3. Apparent nonlinear relationships between the proportions of females at Shenandoah River tributary sampling sites and estrogenic activity as 17β-estradiol equivalents (E2Eq; A) and watershed density of animal feeding operations (AFOs; B).

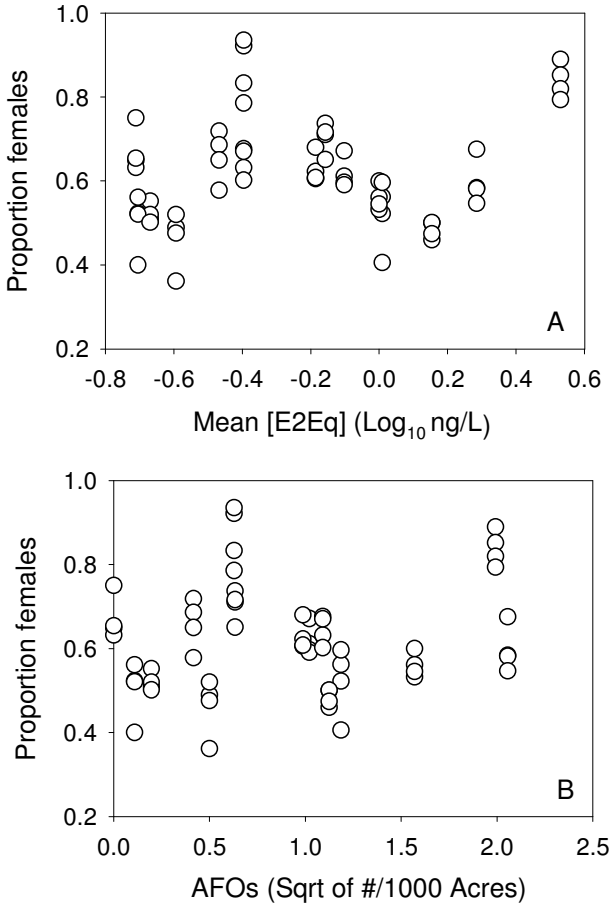


Figure 5- 4. Relationship between proportions of females at Shenandoah River tributary sampling sites relative to mean summer temperature. The solid line represents predicted values from logistic regression and dotted lines represent upper and lower 95% confidence intervals. The coefficient for mean summer temperature, intercept, and concordance index (c) for the logistic model are listed.

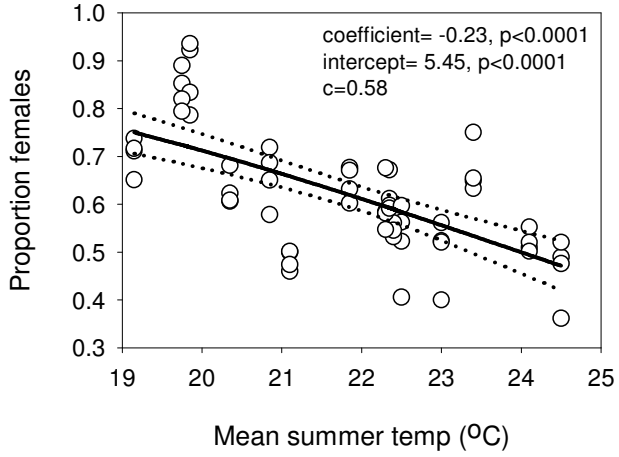


Figure 5- 5. Relationship between 1-mm size categories (shell length) of snails and total proportions of females observed over a 16-month period at two tributary sites, Briery Branch (hatched) and Long Meadow Run (filled). The total number of snails in each size category is listed above each bar.

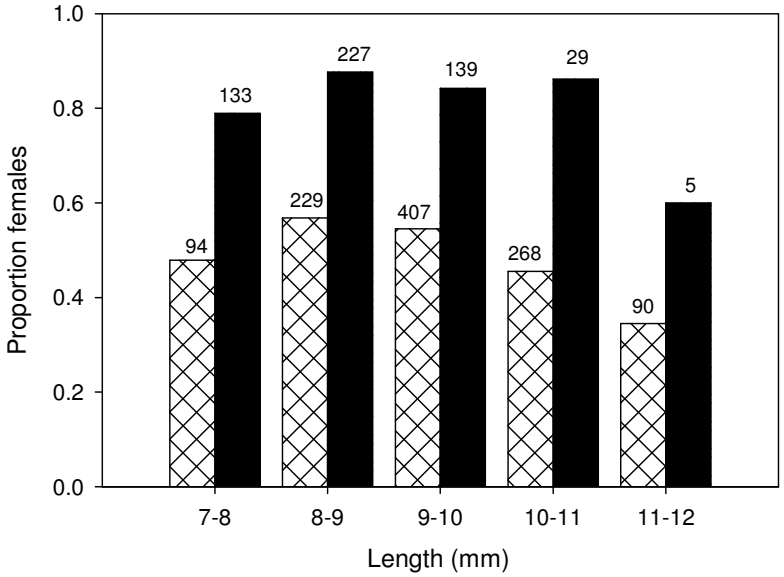
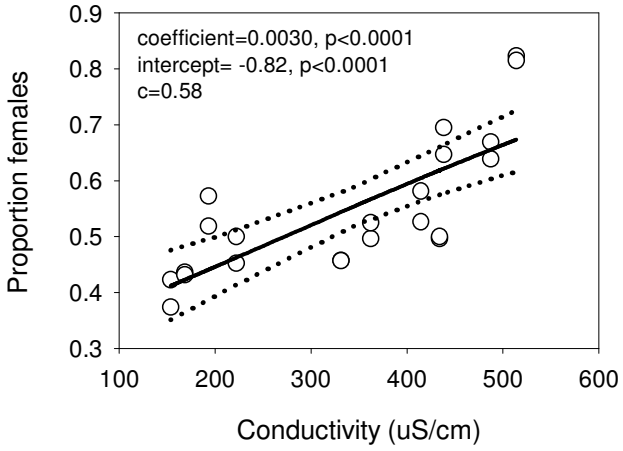


Figure 5- 6. Relationship between proportions of females at river sampling sites relative to summer specific conductivity measurements. The solid line represents predicted values from logistic regression and dotted lines represent upper and lower 95% confidence intervals. The coefficient for summer specific conductivity, intercept, and concordance index (c) for the logistic model are listed.



CHAPTER 6

Relationships between land use, nutrient enrichment in streams, and the densities and parasitic infection rates of populations of the pleurocerid snail *Leptoxis carinata*

Serena Ciparis

J. Reese Voshell, Jr.

Abstract

Nutrient enrichment is a widespread environmental problem in freshwater ecosystems. Eutrophic conditions caused by nutrient enrichment may result in higher infection prevalence of trematode parasites in host populations due to greater resource availability for molluscan first intermediate hosts. This study examined relationships between landscape sources of nutrients, in-stream environmental variables indicative of eutrophication, and the densities and degree of trematode infection of populations of the pleurocerid snail, *Leptoxis carinata*. Fifteen study sites were located in streams within the Shenandoah River watershed (Virginia, USA), where agricultural operations and other human activities have resulted in large-scale nutrient enrichment. Snail population densities were positively related to nutrient concentrations and landscape sources of nutrients, but increasing snail population densities were limited by an apparent threshold of watershed disturbance. Cercariae from five families of trematodes were identified in *L. carinata*, and infection rates were generally low (< 10 %). Neither total infection rates nor infection rates of individual trematode families showed a positive relationship with snail population densities or landscape features. However, there were significant relationships between infection prevalence of two trematode families (Notocotylidae and Opecoelidae) and physical and biological variables, including depth and benthic chlorophyll a concentrations, measured at both the site and sample scale. For trematodes within the family Opecoelidae, transmission between hosts may be inhibited by eutrophic conditions that support high snail population densities. *Leptoxis carinata* appears to be a useful species for monitoring biological effects of eutrophication and investigating trematode transmission dynamics in lotic systems.

Introduction

The presence of excess nutrients in aquatic ecosystems has become a widespread cause of environmental degradation in freshwater, estuarine, and coastal marine environments (Carpenter et al., 1998). Excess nutrients initiate the process of eutrophication, which begins with an increase in algal biomass, and can ultimately result in physical, biological, and chemical changes that cause losses of biodiversity and ecosystem services (Smith et al., 1999). There is increasing evidence that nutrient enrichment in aquatic environments can also lead to an increased risk of parasitic and infectious disease in humans and wildlife (McKenzie and Townsend, 2007). Nutrient enrichment may cause an increase in disease by increasing the fitness of pathogens (Bruno et al., 2003), or by

increasing the algal and detrital resources available to vectors and intermediate hosts of pathogens and parasites (Johnson et al., 2007; Reiskind et al., 2004; Zendt and Bergersen 2000).

Snails are common inhabitants of lentic and lotic freshwater environments and are first intermediate hosts for digenetic trematodes (phylum Platyhelminthes: class Trematoda: subclass Digenea). Trematodes require a molluscan host to complete their life cycle, and factors that affect snail populations may similarly affect trematode populations. Numerous experiments have shown that nutrient addition to freshwater environments results in an increase in snail growth, density, biomass, and reproductive output due to an increase in the biomass of attached algae (Chase and Knight, 2006; Johnson et al., 2007; Rosemond et al., 1993; Thomas and Daldorph, 1994; Wojdak, 2005). Eutrophic conditions that support large populations of snails may result in greater prevalence of parasitic infections due to increased probability of contact between snails and trematode eggs or miracidia (McKenzie and Townsend, 2007). Positive relationships between trematode infections in snails and second intermediate hosts (e.g. amphibians) and the density and species richness of snail populations have been documented in mesocosm experiments and field studies of lentic environments (Johnson et al., 2007; Rohr et al., 2008; Voutilainen et al., 2009). However, other studies have shown that the abundance of vertebrate definitive hosts (e.g. birds) and habitat suitability for these hosts are the most important factors regulating trematode prevalence in snail populations (Bass and Weis 2008; Byers et al., 2008; Hechinger and Lafferty, 2005; Skirnisson et al., 2004). Similar to intermediate hosts, greater species richness and abundance of definitive hosts may be supported by eutrophic water bodies due to greater abundance of food (Hoyer and Canfield, 1994; Soldanova et al., 2010).

Relationships between nutrients, snail populations, and trematode infections are not well studied in lotic environments. Snails in the family Pleuroceridae (superorder Caenogastropoda: order Sorbeoconcha: superfamily Cerithioidea) are often the most abundant invertebrate grazers in southeastern streams (Richardson et al., 1988; Rosemond et al., 1993). Population dynamics of pleurocerid snails and periphyton are tightly coupled (summarized by Brown et al., 2008), but landscape-scale ecological responses of pleurocerid snail populations to nutrient enrichment have yet to be studied. Furthermore, several studies have documented trematodes in pleurocerid snails from individual sites (e.g. Hendrix, 1978; Hoffman and Dunbar, 1963; Krist, 2000; Tolley-Jordan and Owen, 2008; Wetzel and Shreve, 2003), but few studies have investigated trematode species richness or infection prevalence over larger spatial scales, particularly in pleurocerid genera other than *Elimia*. Over large spatial scales, the effects of nutrient enrichment on lotic pleurocerid snail populations and trematode infection prevalence may be similar to

those observed in lentic environments. However, compared to lentic systems, lotic systems have much higher variability in physical habitat. These diverse microhabitats systems may be the most important factors structuring snail and trematode populations at smaller spatial scales (Johnson and Brown, 1997; Tolley-Jordan and Owen, 2008; Wetzel and Shreve, 2003), and could potentially overshadow larger scale effects of eutrophication.

The Shenandoah River (Virginia, USA) and its tributaries support large populations of the pleurocerid snail *Leptoxis carinata*, with densities as high as 3,000 snails/m^2 (Orth et al., 2009). Few studies on parasitism in this species have been conducted (Hendrix, 1978; Hoffman and Dunbar, 1963; Hoffman et al., 1985). Nutrient enrichment in the Shenandoah River is a recognized problem, with nutrient sources attributed to agricultural operations and outdated wastewater treatment plants (WWTPs) (Commonwealth of Virginia, 2005). A recent study indicated linear relationships between nutrient concentrations in Shenandoah River tributaries and watershed densities of animal feeding operations (AFOs) as well as upstream presence of WWTPs (Chapter 4). The large, stable populations of *L. carinata* in streams within the Shenandoah River watershed, coupled with gradients of nutrient concentrations and nutrient sources, created the opportunity to examine relationships between eutrophication and parasitism in lotic environments.

The first objective of this study was to evaluate relationships between population densities of *Leptoxis carinata* and measured concentrations of nutrients and periphyton, as well as landscape sources of nutrients in the Shenandoah River watershed. The second objective was to document the different types of digenetic trematode parasites utilizing *L. carinata* as an intermediate host. The third objective was to assess relationships between trematode infection rates in *L. carinata* and population densities, environmental variables, and landscape characteristics, to test the working hypothesis that nutrient enrichment leads to higher population densities of *L. carinata* and higher trematode infection rates within these populations. The fourth objective was to examine relationships between trematode infection rates and sample-scale physical habitat variables and snail population densities to assess the effects of microhabitats on trematode prevalence in lotic systems.

Methods

Study sites

Study sites in the Shenandoah River watershed were selected to represent a gradient of influence from AFOs and municipal WWTPs as described in detail in Chapter 2. Briefly, locations of AFOs (poultry, dairy, and

beef) and locations of WWTPs and their total permitted effluent discharge were obtained from Virginia state agencies. For individual Shenandoah River tributaries, total densities of AFOs, WWTP presence/absence, and WWTP permitted effluent flow were quantified within delineated 12-digit, 6th level Hydrologic Unit Code (HUC 6) subwatersheds (40-160 km^2 in size). Twenty-five sites, located near outlets of the delineated HUC 6 subwatersheds, were sampled during a preliminary study in 2007. Fifteen sites in 12 Shenandoah River tributaries had relatively large populations of pleurocerid snails in stable riffles and were selected for the current study (Figure 6-1; Table 6-1). Three of the tributaries drained multiple subwatersheds, and an upstream (US) sampling site was located in the primary subwatershed as well as a downstream (DS) site draining multiple subwatersheds (Table 6-1). Watershed size upstream of the 15 sampling sites ranged from 39.8-335 km^2.

In addition to AFOs and WWTPs, land cover percentages were quantified upstream of each sampling site. Land cover data (30 m resolution) were obtained from the 2001 National Land Cover Database (Homer et al., 2007), and reclassification and areal tabulation were used to quantify the watershed percentages of forest, developed land, pasture/hay, and cultivated crops upstream of each sampling site. Across the 15 sampling sites, ranges in watershed percentages of the four land cover types were: 19-92% forest, 5.0-69% pasture/hay, 0.22-7.3% cropland, and 1.9-12% developed land. These four land cover classes were also quantified as "local" percentages within a 300 m wide riparian buffer extending 1,000 m upstream of the site (Chapter 2). However, these local percentages did not have significant effects on variables of interest in this study and results are not presented or discussed. All land use characteristics were quantified using ArcGis 9.3 (ESRI, Redlands, CA).

Snail sampling

Leptoxis carinata was the only species of pleurocerid snail encountered at all sampling sites in Shenandoah River tributaries; the species identification was verified using the reference collection at the Smithsonian Institution National Museum of Natural History (Washington, DC). Other families of snails (Physidae and Planorbidae) were rarely encountered at the sampling sites and were not included in this study. Previous studies of *L. carinata* have shown that individuals are semelparous biennials; 2-year old snails lay eggs between late spring and mid-summer and die off by late summer (Aldrige, 1982; Hendrix, 1986). Therefore, snails were not collected during the summer in order to avoid potential fluctuations in adult populations. Only adult (>1 yr old, >7 mm shell length) snails were dissected for detection of infection by trematode cercariae. Infection rates were determined for three generations of

L. carinata. The 2006 generation was sampled 14-24 May 2008, the 2007 generation was sampled 5-15 October 2008 and 21 May -19 June 2009, and the 2008 generation was sampled 2-23 October 2009.

At each study site, quadrat sampling at random locations in one riffle was performed using a Surber sampler (0.0929 m^2). The riffles sampled at all study sites had rocky substrate of similar size (cobble through small boulder). During 2008, four replicate samples were collected. During 2009, eight replicate samples were collected in order to increase the sample size, and current velocity and depth were quantified at each quadrat location using a Marsh-McBirney Model 2000 Flow-mate meter (Marsh McBirney, Inc., Frederick, MD). Current velocity and depth measurements were utilized in sample-scale statistical analyses (2009 data), and all measurements from a site were averaged for use in site-scale analyses (all data).

After collection, juvenile and adult snails in each replicate sample were counted, and adults were returned to the laboratory in site water. In 2008, snails were not measured. In 2009, snails were measured with a digital caliper and grouped into 1-mm size classes. Densities of large (> 11 mm) and medium-sized (9-11 mm) snails were utilized in sample-scale analyses. Densities of small snails (7-9 mm) were not utilized because at some sites, snails less than 8 mm were not mature and were not dissected.

Trematode identification

Adult snails were narcotized in 1% $MgCl_2$ in site water for 8-12 hours. Shells were cracked with vise grip pliers, and the soft tissue was removed and examined under a dissecting microscope. Snails infected with trematodes were identified by the presence of cercariae in either the gonad/gut complex or the rectum. Cercariae or gut/gonad tissue were sampled using forceps, placed on a microscope slide, and examined under a compound microscope (10x-40x). When sporocysts containing undeveloped cercariae (no tails or identifiable features) were found, cercariae were classified as "immature". Mature cercariae were identified to type using keys in Schell (1985). Snails with different types of cercariae were placed in individual, sterile Petri dishes in deionized water and cercariae were allowed to emerge for 1-2 hours. Cercariae were then removed using transfer pipettes and placed in certified DNA-free microcentrifuge tubes in 95% ethanol. Five replicates of each identified cercarial type were subjected to genetic screening based on polymerase chain reaction (PCR) of 18S small subunit RNA.

Genomic DNA was extracted from preserved cercariae using the Qiagen DNeasy Tissue Kit (Valencia, CA), and stored at 4 °C prior to amplification. For PCR amplification, a PCR cocktail containing 1 μM of each

primer: TF (5'- GCT TGT CTC AGA GAT TAA GCC – 3') and TR (5' – ACG GAA ACC TTG TTA CGA C – 3') was added to GoTaq® Green Master Mix (Promega Corporation, Madison, WI). All primers were purchased from Integrated DNA Technologies (Coralville, IA) and designed as universal trematode primers. The primers were used with a PCR cycling profile consisting of 5 min denaturation step at 95 °C; 35 cycles of 30 sec at 95 °C, 30 sec at 56 °C, 1 min at 72 °C, and a 10 min extension at 72 °C. After amplification, 10 µL of the DNA sample was identified by electrophoresis at 90 V for two hours on a gel containing 1.2% I.D.NA® agarose (FMC Bioproducts, Rockland, ME).

The PCR products were cleaned using QIAquick PCR Purification Kit (Qiagen, Valencia, CA). Sequencing reactions were performed using Applied Biosystems Big Dye Cycle Sequencing Kit (Foster City, CA). Briefly, 2 µL of Big Dye, 1 µL of one primer (either TF or TR), and 2 µL of the template DNA were added to 5 µL of water. This was done for both the forward and reverse primer. The samples were then run on the following PCR cycling profile: 25 cycles of 30 sec at 96 ºC, 15 sec at 55 ºC, and 4 min at 60 ºC, and a 10 min extension at 72 °C. The reactions were cleaned with Agencourt CleanSEQ (Beckman Coulter Genomics, Beckman Coulter Inc., Brea, CA). Cleaned sequencing reactions were loaded onto an Applied Biosystems 3100 Genetic Analyzer (Foster City, CA). Samples were sequenced in both directions. Resulting sequences were analyzed with BioEdit software. Sequencing provided taxonomic identification using the most similar sequence identified in the National Center for Biotechnology Information (NCBI) GenBank catalog.

The lowest taxonomic levels of the best matches between sequences are presented below, but these results should be interpreted with caution. A species match requires that the species has been previously sequenced and entered in the GenBank database. In addition, reference adult specimens were not cultured or collected for morphological confirmation. Within the framework of this study, the greatest conservative confidence was in family level identifications, which is suitable identification of probable secondary hosts. Therefore, family and superfamily (see results) were used in analysis of relationships between different types of trematodes and land use, environmental variables, and snail population densities.

Environmental measurements

Environmental data were collected during spring sampling periods, during mid-summer (8-14 Aug. 2008 and 10-11 Aug. 2009), and during late winter (10-15 March 2009). Mid-summer was selected instead of fall in order

to ensure that data could be collected during near-baseflow conditions. Water samples were filtered, preserved, and analyzed for nutrient concentrations (µg/L), including total dissolved inorganic N (DIN) and PO_4-P, as indicated in Chapter 4. Mean concentrations of nutrients were used in statistical analysis because season/flow conditions did not have a significant effect on concentrations of these analytes (Chapter 4). None of the sampling sites had a closed canopy, so periphyton was collected during mid-summer sampling periods (2008 and 2009) from four rocks per riffle using a wire brush, bar-clamp sampler (9.62 cm^2), and deionized water. Following methods in Steinman et al., (2006), samples were filtered, chlorophyll a was extracted and quantified with a spectrophotometer, and ash-free dry mass (AFDM) was determined. Mean benthic chlorophyll a (µg/cm^2) and AFDM (mg/cm^2) from the four replicates were calculated and pooled across years. The two measurements were linearly related, so only benthic chlorophyll a was used in statistical analysis.

Data analysis

Pearson correlation (Pearson's r) matrices and associated scatter plots were used to assess relationships between and amongst land use variables, environmental variables, and snail population densities (total densities; adults and juveniles). The relationship between watershed size and snail population density was also assessed. Significant linear relationships and nonlinear relationships were modeled using linear or polynomial regression analysis, respectively. Effects of the presence of WWTPs on nutrient concentrations and snail population densities were also assessed using t-tests. Principal components analysis (centered and scaled data) was used to create reduced, orthogonal variables from correlated land uses at the watershed scale, including land cover, AFO density, and WWTP permitted effluent flow. Resulting principal component scores were used as independent variables in regression models.

Trematode infection rates are presented as percentages/proportions, where infection rate represents the number of infected snails divided by the total number of adult snails collected/examined. The total numbers of adult snails collected in all replicates were used to calculate infection rates for each site during each sampling period. Differences in infection rates between seasons at each sampling site were assessed using the G-test of independence (Sokal and Rolf, 1995). Pooled infection rates for all sampling periods were used to assess the relative contribution of individual trematode types to overall infection rates.

The infection rates (total and individual types of trematodes) for individual sampling periods were used in site-scale assessment of relationships with snail population densities (snails/total m^2 sampled), environmental variables, and land use variables. The relationship between watershed size and trematode infection rates was also assessed. These relationships were modeled using logistic regression (number of infected/total number examined in events/trials notation). Logistic regression was also used to model sample-scale relationships between trematode infection rates, calculated for each replicate, and corresponding depth, current velocity, and snail densities. For both site- and sample-scale analyses, relationships between trematode infection rates and snail densities were assessed for both total densities (adults and juveniles) and adult densities, since infection rates were only quantified in adults (individual cohort). For all logistic regression models, Wald χ^2 statistics were used to assess significance of parameters, the concordance index (c) was used to assess predictive capability, Pearson residuals were used to assess model fit, and apparent overdispersion was assessed using Pearson's χ^2/degrees of freedom (SAS Institute, 1995; Wilson and Hardy, 2001). If values for Pearson's χ^2/degrees of freedom exceeded two, and the model met criteria for overdispersion as outlined in SAS Institute (1995), overdispersion was corrected using the Williams method. This method was selected due to the different numbers of snails utilized to determine infection rates (SAS Institute, 2010). The Hosmer-Lemeshow χ^2 test was used to assess overall predictive capability of logistic regression models, where $p<0.05$ represents a lack of predictive capability, but this test may be overly conservative (SAS Institute, 1995). Although not appropriate for prediction, linear regressions of independent variables found to be significant in logistic regression models and logit transformed infection rates (modified as $\ln(1+(p/1-p))$ to account for incidences of no infection) were utilized to estimate the proportion of variance explained by the independent variables (R^2 value).

Prior to regression analyses, all variables were examined for potential outliers; critical values were defined as 1.5*interquartile range (Q3-Q1), added to Q3 (75^{th} percentile) and subtracted from Q1 (25^{th} percentile). Outliers were flagged and their effect on statistical analyses was tested, as described below. Variables were tested for normality using the Shapiro-Wilk W test. Non-normal variables were transformed when necessary ($\log_{10}$, square root, or arcsine square root). Statistical analyses were performed using SAS 9.2 (SAS Institute, Cary, NC), with a significance level of $\alpha=0.05$.

Results

Snail population densities

Mean population densities of *L. carinata* ranged from 364-1,775 snails/m^2 at the 15 sampling sites (Table 6-1). Mean DIN concentrations ranged from 36.67-6,589 µg/L and mean PO_4-P concentrations ranged from 2.91-470 µg/L. Snail population densities were positively related to both DIN ($p=0.004$, $R^2=0.14$) and PO_4-P ($p=0.001$, $R^2=0.17$, Figure 6-2). Mean concentrations of benthic chlorophyll a ranged from 2.0-28 ug/cm^2. The relationship between snail population densities and benthic chlorophyll a was nonlinear ($p<0.0001$, $R^2=0.29$, Figure 6-2). Benthic chlorophyll a was not directly related to concentrations of DIN ($p=0.15$) or PO_4-P ($p=0.93$, Figure 6-2).

As described previously (Chapter 4), watershed density of AFOs was positively related to both PO_4-P concentrations ($p<0.0009$, $R^2=0.59$), and DIN concentrations ($p<0.0001$, $R^2=0.73$), and mean concentrations of PO_4-P were significantly higher at sites with upstream WWTPs (t-test, $p=0.002$). Compared to watershed-scale land cover percentages, watershed density of AFOs was the best predictor of nutrient concentrations and snail population densities, but the relationship with snail population densities was nonlinear (2^{nd} order polynomial, $p=0.001$, $R^2=0.22$, Figure 6-3). Mean snail population densities were significantly higher at sites with upstream WWTPs (t-test, $p=0.02$). Snail population densities were positively related to the total upstream permitted WWTP effluent flow ($p=0.005$, $R^2=0.13$, Figure 6-3). When all watershed-scale land use variables were combined using principal components analysis, two principal components (PCs) had eigenvalues greater than one. The cumulative variance explained by PC 1 and PC 2 was 92%, with 63% and 29% explained by PC 1 and PC 2, respectively. Eigenvectors of PC 1 were: 0.54 for AFO density, 0.53 for cropland, 0.52 for pasture/hay, 0.39 for developed land, and 0.06 for permitted WWTP effluent flow. Eigenvectors of PC 2 were: 0.80 for permitted WWTP effluent flow, 0.54 for developed land, -0.17 for AFO density, -0.17 for cropland, and -0.15 for pasture/hay. Similar to watershed AFO density, the relationship between PC 1 and snail population densities was nonlinear (2^{nd} order polynomial, $p<0.0001$, Figure 6-3), but the proportion of variance explained ($R^2=0.28$) was greater than for watershed AFO density alone. The relationship between snail population densities and PC 2 was not statistically significant, either alone or when combined with PC 1. Relationships between snail population densities and local-scale land cover variables, as well as watershed size, were also not statistically significant.

There were weak relationships between snail population densities and physical variables at different spatial scales. Across sampling sites, snail population densities were positively related to mean current velocity where

samples were collected ($p=0.03$, $R^2=0.08$), but were not related to mean water depth. At the sample-scale, both total and adult snail densities were positively related to depth ($p=0.03$, $R^2=0.02$, and $p=0.02$, $R^2=0.03$, respectively). Adult snail densities were negatively related to current velocity ($p=0.01$, $R^2=0.03$), but the relationship between total snail densities and current velocity was not statistically significant.

Trematode prevalence

A total of 14,534 snails from Shenandoah River tributaries were examined for the presence of trematode cercariae and 1,523 snails were infected, for an overall infection rate of 10.5%. During individual sampling periods, infection rates ranged from 0-70 % (Table 6-1). Only five sites had significantly different infection rates between spring and fall sampling periods (Table 6-1), and for all of these sites, infection rates were greater in the spring. Several other sites had higher trematode infection rates in spring 2009 compared to fall 2008 (Table 6-1) which is notable because snails were from the same generation (2007). Due to very high stream flows and turbid conditions, lower numbers of adult snails were collected during spring 2008. This may have introduced some variability into the dataset due to the low numbers of snails examined, particularly at Cedar Creek US and DS, Passage Creek, and Linville Creek (Table 6-1). Overall variability in infection rates between sampling periods was generally low (Table 1). Mean infection rates were low ($\leq$ 10 %) at the majority of sites sampled. Three sites had mean infection rates between 11 and 15 %, two sites had mean infection rates between 15 and 20 %, and one site (Jennings Branch) had an exceptionally high mean infection rate of 49 % (Table 6-1). Outlying infection rates (70 %) were measured at Jennings Branch during both May sampling periods, and these two measurements were not included in statistical analysis of relationships between total infection rates and other variables.

Five types of cercariae were identified: cotylomicrocercous cercariae, monostome cercariae, longifurcate-pharyngeate cercariae of the vivax type, and xiphidiocercariae of the ubiquita and virgulate types (Schell, 1985). Cotylomicrocercous cercariae were located in the rectum of *L. carinata* and all other types were located in the gonad/digestive gland complex. Regardless of location, all trematode types precluded development or caused degeneration of the testes and ovaries. Information regarding identifications, potential secondary and intermediate hosts, and previous identifications in *L. carinata* is summarized in Table 6-2.

Cotylomicrocercous cercariae are produced by trematodes in the family Opecoelidae (Schell, 1985), and genetic screening provided confirmation of this family for all five replicates. The greatest percent match was with

Opecoelidae spp. (92 %, Accession # AY218105.1), which suggests that the species encountered in the current study has yet to be entered into the database. One species of Opecoelidae, *Plagioporus hypentelii*, was identified by Hendrix (1978) in *L. carinata* in tributaries within the Monocacy River drainage (Marsh Creek and Middle Creek, PA). This species encysts in aquatic insects and the definitive host is the northern hogsucker. Other genera within Opecoelidae encyst in amphipods or small fish, and definitive hosts include several species of fishes (Table 6-2). Vivax cercariae are produced by trematodes in the family Cyathocotylidae (Schell, 1985), and genetic screening provided confirmation of this family for all five replicates. The greatest percent match was with *Holostephanus dubinini* (96-97 %, Accession # AY245707.1). Cyathocotylid cercariae have been observed in *L. carinata* in a tributary of the Potomac River (Opequon River, WV) and were identified as *Neogogatea spp.* (Hoffman and Dunbar, 1963). Cercariae of both *Neogogatea* and *Holostephanus* encyst in muscle tissue of several freshwater fishes and definitive hosts are piscivorous birds (Table 6-2).

Monostome cercariae and xiphidiocercariae do not appear to have been previously described in *L. carinata*. Genetic screening indicated that monostome species collected in the current study were members of the family Notocotylidae. All five replicates were a 99 % match to *Notocotylus spp*. (Accession # AJ287547.1), which encyst on vegetation utilize waterfowl as definitive hosts (Table 6-2). Xiphidiocercariae of virgulate and ubiquita types are produced by several families of trematodes within the superfamily Microphalloidea (Schell, 1985). Genetic screening confirmed collection of both Microphallidae (produce ubiquita type cercariae) and Lecithodendriidae (produce virgulate type cercariae). The specimens within Microphallidae were a 96% match to both *Maritrema oocysta* (Accession # AJ287534.1) and *Microphallus primas* (Accession # AJ287541.1). *Microphallus spp.* and *Maritrema spp.* utilize amphipods, isopods, and crayfish as second intermediate hosts, and birds, mammals, fishes, and turtles as definitive hosts (Table 6-2). Some trematodes within *Microphallus* do not use a second intermediate host and encyst within the snails (Schell, 1985). The specimens within Lecithodendriidae were a 98% match to several unspecified Lecithodendriidae spp. (Accession #s EU199xx.x), which generally encyst in aquatic insects and utilize insectivorous birds, bats, or amphibians as definitive hosts (Table 6-2).

Genetic screening revealed that the xiphidiocercariae were not always typed correctly as either virgulate or ubiquita (i.e. Lecithodendriidae or Microphallidae). Sporocysts of these two types of xiphidiocercariae are similar in appearance and frequent collection of cercariae that were not fully mature may have contributed to errors in identification of distinguishing characteristics such as relative tail length and presence of a ventral sucker and the

virgula organ (Schell, 1985). Therefore, both types of xiphidiocercariae were combined and identified as Microphalloidea for statistical analysis.

Cercariae within the family Cyathocotylidae were found at 10 sites over the course of the study (Figure 6-4), but were only found at five sites more than once: Briery Branch, Muddy Creek, Jennings Branch, Smith Creek, and Cedar Creek DS. Because of the infrequent collection of this family, infection rates were not utilized in statistical analysis of relationships between individual types of cercariae and other variables. All other cercarial types were found at all sites during three out of four sampling periods, with the exception of Opecoelidae which was never found at Long Meadow Run (Figure 6-4). The dominant family of trematodes varied depending on the site, and one family was not dominant across all of the sites (Figure 6-4). Only four double infections were observed over the course of the study. A double infection of Opecoelidae and Microphalloidea was observed in one snail from Naked Creek in May 2008 and one snail from Stony Creek DS in May 2009. A double infection of Opecoelidae and immature cercariae was observed in one snail from Briery Branch in May 2009 and in one snail from Jennings Branch in October 2009. Outlying Opecoelidae infection rates (>20 %) were measured at Jennings Branch during all four sampling periods and were not included in statistical analyses of relationships between infections with this type of trematode and other variables.

Relationships between trematode infection rates and other variables

There were no statistically significant relationships between snail population densities (total or adults only) and either the total proportion of snails infected with trematodes (Figure 6-5) or proportions of snails infected with individual types of trematodes ($p \geq 0.57$). There were also no statistically significant relationships between total proportions of infected snails or proportions of snails infected with individual types of trematodes and any watershed-scale land use variables, including AFO density and the combined effect of land use variables as PC 1, or watershed size. There were no statistically significant relationships between any land use or environmental variable and proportions of snails infected with Microphalloidea.

Total proportions of infected snails were related to mean benthic chlorophyll a concentrations (polynomial) and the mean depth at each site ($p<0.0001$, Table 6-3). The overall predictive capability of the logistic model was low (Table 6-3), and the proportion of variance explained by these variables in a linear regression with logit transformed proportions of infected snails was $R^2=0.25$. Proportions of snails infected with Opecoelidae and

Notocotylidae were related to the variables that were related to the total proportion of infected snails (Table 6-3, Figure 6-5). Proportions of snails infected with Opecoelidae were related to mean benthic chlorophyll a (polynomial; $p<0.0001$, Table 3, Figure 5). The overall predictive capability of the logistic model was low (Table 6-3), and the proportion of variance explained by a linear regression with logit transformed proportions was $R^2=0.25$. Proportions of snails infected with Notocotylidae were positively related to the mean depth at each site (Table 6-3, Figure 6-5). The overall predictive capability of the logistic model was low (Table 6-3), and the proportion of variance explained by depth in a linear regression with logit transformed proportions was $R^2=0.24$. Two outlying proportions of snails infected with Notocotylidae were measured at Stony Creek DS in May 2008 and October 2009 (0.16 and 0.14, respectively). These proportions did not affect the statistical significance of the relationship between infection rates and depth ($p<0.0001$ if removed), but they are not shown in Figure 5 in order to better illustrate the overall relationship.

The only other variable with a statistically significant relationship to the proportions of infected snails was PO_4-P, and the negative relationship was specific to proportions of snails infected with Opecoelidae (Figure 6-5, coefficient= -0.98, Wald $\chi^2=9.30$, $p=0.002$, $c=0.62$). The overall predictive capability of the logistic model was low (Hosmer-Lemeshow $\chi^2=136.6$, $p<0.0001$), and the proportion of variance explained by PO_4-P concentrations in a linear regression with logit transformed proportions was $R^2=0.11$. Concentration of PO_4-P did not remain significant ($p=0.40$) when combined with other significant independent variables in a logistic regression model.

Sample-scale relationships between trematode infection rates and other variables

Sample-scale (within each quadrat) analyses of relationships between independent variables and trematode infection rates were only conducted for data collected in 2009 when eight replicate samples were collected at each site during each sampling period (n=240). Statistical analyses of these data did not include outlying infection rates measured in all eight replicates from a particular site because of the potential for a site-scale effect to overshadow a sample-scale effect. Outlying values that were excluded include: total proportions of infected snails (0.45-0.80) at Jennings Branch in May 2009, proportions of snails infected with Opecoelidae (0.31-0.56) at Jennings Branch in May 2009, and proportions of snails infected with Notocotylidae (0.11-0.18) at Stony Creek DS in October 2009.

There were no statistically significant relationships between trematode infection rates and current velocity or between proportions of snails infected with Microphalloidea and any sample-scale environmental or density

variable. The total proportions of snails infected with trematodes were positively related to depth and negatively related to densities of adult snails (p=0.0003, Table 6-4). The logistic model was predictive (p=0.07, Table 6-4), but the proportion of variance explained by these variables in a linear regression with logit transformed proportion of infected snails was R^2=0.03. Similar to results for site-scale analyses, proportions of snails infected with Opecoelidae and Notocotylidae were related to sample-scale variables that were related to the total proportions of infected snails. Proportions of snails infected with Opecoelidae were negatively related to the density of adult snails (p=0.0001, Table 6-4, Figure 6-6). The predictive capability of the logistic model was low (Table 6-4), and the proportion of variance explained by these variables in a linear regression with logit transformed proportions was also low (R^2=0.03). This variability appears to be due to the replicates that had Opecoelidae infection rates of zero, if these replicates were excluded, the relationship would have been stronger (Figure 6-6). Proportions of snails infected with Opecoelidae were not related to the total density of snails at the sample-scale (p=0.13), which suggests that the relationship with adult density is a cohort specific effect. Proportions of snails infected with Notocotylidae were positively related to depth (p<0.0001, Table 6-4), and the fit of the model was improved with the addition of the total densities of snails (p=0.03, Table 4), but not with the addition of adult snail densities (p=0.23). The predictive capability of the final model was moderate (Table 6-4), and the proportion of variance explained by both depth and total snail density in a linear regression with logit transformed proportions was R^2=0.10. Season (spring vs. fall) did not have a significant effect on any of the aforementioned sample-scale relationships.

Sample-scale densities of large snails (>11 mm) were positively related to total proportions of infected snails, proportions of snails infected with Opecoelidae, and proportions of snails infected with Notocotylidae (Table 6-4). Sample-scale densities of large snails were also positively related to proportions of snails infected with Microphalloidea (coefficient=0.37, Wald χ^2=12.9, p=0.0003, c=0.59). In logistic regression models, the predictive capability was greatest for proportions of snails infected with Notocotylidae (Table 6-4). The proportions of variance explained by sample-scale densities of large snails in linear regressions with logit transformed proportions of total infected snails and snails infected with individual trematode types were very low (R^2=0.02-0.08). Relationships between sample-scale densities of medium-sized snails (9-11 mm) and total proportions of infected snails or proportions of snails infected with individual trematode types were not statistically significant.

Discussion

Snail population densities

In streams, benthic chlorophyll a concentrations exceeding 150 mg/m^2 are considered to be "unnaturally high" (Biggs, 2000), and 200 mg/m^2 has been proposed as the boundary between mesotrophic and eutrophic conditions (Dodds et al., 1998). In the current study, mean benthic chlorophyll a exceeded 150 mg/m^2 at eight sites, and mean benthic chlorophyll a at five of these sites exceeded 200 mg/m^2. Interestingly, 150 mg/m^2 (15 µg/cm^2) was the inflection point of the relationship between benthic chlorophyll a concentrations and snail population densities. Below 150 mg/m^2, snail population densities showed a decreasing trend with increasing benthic chlorophyll a. This is primarily due to the four sites (Muddy Creek, Hawksbill DS, Briery Branch, and Stony Creek US) with very low benthic chlorophyll a concentrations and very high snail population densities, which is likely a result of low periphyton biomass maintained by intense grazing pressure. Three of these sites (Muddy Creek, Hawksbill DS, Stony Creek DS) contributed to the lack of a linear relationship between PO_4-P and benthic chlorophyll a concentrations; the relatively high PO_4-P concentrations may support high growth rates of periphyton, but this periphyton is rapidly grazed by snails, resulting in low periphyton biomass. Grazer-induced limitation of periphyton accrual under nutrient enriched conditions is well documented by field experiments (e.g. Biggs and Lowe, 1994).

Above 150 mg/m^2 benthic chlorophyll a, snail population densities increased with increasing chlorophyll a concentrations. There was no apparent effect of grazing, which could be due to the algal production outpacing snail population growth, perhaps because of an increase in unpalatable filamentous species (Marks and Lowe, 1989; Suren et al., 2003), or due to snails obtaining a greater proportion of nutrients from bacterial biomass (Morales and Ward, 2000). In addition, other stressors associated with eutrophic conditions (e.g. lower dissolved oxygen, higher suspended solids) may limit snail population growth at these sites. These stressors may have contributed to the variability in the relationship between snail population densities and high benthic chlorophyll a concentrations and also to variability in the response of benthic chlorophyll a concentrations to increasing nutrient concentrations (turbidity; Figueroa-Nieves et al., 2006).

The direct relationship between snail population densities and PO_4-P concentrations, as well as sources of PO_4-P such as WWTP effluent, illustrated the positive relationship between nutrient enrichment and population densities of pleurocerid snails. However, there was substantial variability in the linear relationship between snail population densities and PO_4-P concentrations. In addition, the relationship between snail population densities and

watershed density of AFOs, the dominant source of nutrients in streams within the Shenandoah River watershed (Chapter 4), was nonlinear. This further supports the idea that additional stressors associated with AFOs and/or resulting eutrophication limit snail population densities in streams with the highest watershed densities of these operations. Pleurocerid snails use gills for respiration ("prosobranchs"), which increases their sensitivity to environmental stressors such as suspended sediment and low dissolved oxygen concentrations relative to freshwater pulmonates (Brown, 2001). In agricultural streams, grazing animals (included in AFO calculations) are a source of suspended sediment and fine particulate organic matter which negatively affect sensitive aquatic biota (Braccia and Voshell, 2006; Niyogi et al., 2007), and runoff of manure from AFOs that is land-applied as fertilizer can introduce similar stressors (Mishra et al., 2006; Soupir et al., 2006). Watershed density of AFOs appeared to be the primary driver of the nonlinear relationship between snail population densities and watershed disturbance, but inclusion of other correlated land cover variables (pasture/hay, cropland, and developed land) through principal components analysis strengthened the relationship between watershed disturbance and snail population densities. Row crop agriculture and urbanization are well documented sources of nutrients, suspended solids, and other stressors to streams (Stone et al., 2005; Walters et al., 2009). Thus, there is an apparent direct relationship between population densities of pleurocerid snails and nutrient enrichment resulting from agricultural land use, but there is a threshold to the positive nature of this response. Beyond this threshold, population densities no longer increase, and appear to begin to decrease, likely due to the introduction of other environmental stressors.

Although land use and resulting nutrient enrichment appeared to be the main factors structuring snail population densities in the studied streams, there were some relationships between population densities and physical habitat variables. Sites with generally higher current velocities supported higher population densities of snails. This may reflect higher dissolved oxygen concentrations or less deposition of suspended solids on substrates colonized by snails, particularly at sites affected by high watershed densities of AFOs. However, at the sample scale, there was a negative relationship between densities of adult snails and current velocity. Johnson and Brown (1997) also found a negative correlation between densities of adult snails and current velocity, but proportions of juveniles were positively correlated with current velocity, which suggested that adults seek out flow refugia and juveniles do not. In the current study, contrasting relationships of adult densities and juvenile densities with current velocity would explain the observed lack of relationship between total snail densities and this physical habitat variable. Total snail densities and densities of adult snails were positively related to depth at the sample scale, which may reflect the

permanence of suitable habitat conditions at a particular sampling site. Although physical habitat variables did not explain a large proportion of variance in snail population densities, they likely contributed to variability in responses of snail population densities to land use and in-stream concentrations of nutrients and benthic chlorophyll a.

Trematode prevalence

The range in mean site-specific trematode infection rates of *L. carinata* populations (3.3-49 %) and the overall infection rate (10.5 %) were similar to those observed in other multi-site studies of snail populations (Byers et al., 2008; Soldanova et al., 2010; Tolley-Jordan and Owen, 2008; Urban, 2006). Esch et al. (2001) reported that 5-10 % infection rates are typical for natural snail populations. Only three sites in the current study had mean and overall infection rates greater than 15 %. Opecoelidae was the dominant trematode family at two of these sites (Jennings Branch and Naked Creek) and Notocotylidae was the dominant trematode family at the other site (Stony Creek DS). These sites may provide exceptional habitat for fishes (utilized by Opecoelidae) or waterfowl (utilized by Notocotylidae) relative to other sites, as habitat availability and site utilization by definitive hosts are often the primary factors structuring trematode infection rates in snail populations (Bass and Weis, 2008; Byers et al., 2008; Hechinger and Lafferty, 2005; Skirnisson et al., 2004). However, the exceptionally high trematode prevalence at Jennings Branch, particularly during spring sampling periods, was notable. Snail populations at this site also had exceptionally female-biased sex ratios (mean proportion of females=0.87; Chapter 4) and Asian clams from this site had the highest tissue concentrations of arsenic compared to other study sites in the Shenandoah River watershed (6 µg/g; Chapter 3). This led to suspicions that environmental stressors could be increasing snails' susceptibility to trematode infection at this site. However, attempts to develop an immune function assay for *L. carinata* to test this hypothesis were unsuccessful (Appendix A). For all sites with higher trematode infection rates in *L. carinata* populations, the trematode prevalence in second intermediate hosts is also expected to be relatively high, as the density of infected upstream hosts can determine infection rates in populations of the subsequent host (Thieltges, 2006).

The timing of infection of snail populations by trematodes is tightly coupled with the life history characteristics of the snail species (Kube et al., 2002). Trematodes begin to infect snails around the time of sexual maturation (Probst and Kube, 1999). *Leptoxis carinata* reproduce during spring through early summer of the second year of their lives and die off by the end of the summer (Aldridge, 1982; Hendrix, 1986). Gonad maturation begins

during the first spring after hatching and trematode infections appear to coincide with this time period; trematode infections in immature snails (< 5 mm) have been observed as early as February and March (Chapter 7). If trematodes cannot distinguish between developing snails and mature snails that will reproduce during that year, it provides a mechanism for the observed statistically significant differences in spring infection rates compared to fall infection rates as well as observed increases in infection rates within the same generation of snails (2007) between the fall and spring sampling periods.

The presence of Opecoelidae, Notocotylidae, and Microphalloidea at all of the sampling sites suggests general habitat suitability for secondary and definitive hosts. The only exception was the consistent lack of Opecoelidae in snails from Long Meadow Run. Relative to other studied streams, this stream had the smallest watershed (39.8 km^2), the second highest watershed density of AFOs, and the highest DIN concentrations. The small size or high level of disturbance may preclude the use of this stream by appropriate insect or fish hosts. Habitat suitability for fish hosts is also the most probable reason for the observed lack of uniform distribution of Cyathocotylidae across the studied streams. This study was intended as a large-scale preliminary assessment of the richness of trematodes utilizing *L. carinata* as a first intermediate host. Future in-depth morphological studies of cercariae, as well as investigations of likely second intermediate and definitive hosts and collection of adult trematodes, will allow further understanding of relationships between different species of trematodes and this one species of snail.

Relationships between trematode infection rates and other variables

Although the gradients of nutrient enrichment and landscape features that cause eutrophic conditions were directly related to population densities of *L. carinata*, higher snail population densities did not result in higher trematode infection rates within these populations. This was contrary to the working hypothesis of this study, which was based on the idea that higher snail population densities would increase the encounter rates between snails and trematode eggs or miracidia. The results reflect the importance of multi-host life cycles in determining trematode infection rates in first intermediate hosts. In addition, trematode infection rates in snail populations were not related to land use variables, which suggests that definitive host utilization of the study sites was not determined by landscape-scale factors. The importance of local factors in controlling the distribution and abundance of second intermediate and definitive hosts, and ultimately controlling trematode prevalence in first intermediate hosts, is well

documented in marine environments and lentic freshwater environments (Beyers et al., 2008; Hechinger and Lafferty, 2005; Skirnisson et al., 2004). Relationships between trematode infection rates in *L. carinata* and some of the measured in-stream environmental variables suggest that local factors are also important in determining trematode infection prevalence in lotic environments in the Shenandoah River watershed.

Eutrophic conditions in streams within the Shenandoah River watershed may affect the abundance of second intermediate hosts of trematodes in the family Opecoelidae. The nonlinear relationship between benthic chlorophyll a concentrations and Opecoelidae infection rates was similar to the relationship between benthic chlorophyll a and snail population densities, in that 150 mg/m^2 was the approximate inflection point. However, in contrast to snail population densities, Opecoelidae infection rates initially increased as benthic chlorophyll a increased and then decreased as conditions became more eutrophic. Aquatic insects are second intermediate hosts of *Plagioporus spp*. within Opecoelidae (Schell, 1985), which has been previously documented in *L. carinata* (Hendrix, 1978). With modest nutrient enrichment and resulting increases in benthic chlorophyll a concentrations, there is generally a positive linear relationship between densities of aquatic insects and benthic chlorophyll a (Lewis and McCutchen, 2010). However, as concentrations of nutrients and benthic chlorophyll a continue to increase, a gradual loss of sensitive aquatic insect taxa, such as Ephemeroptera, Plecoptera, and Trichoptera, can occur (Niyogi et al., 2007). Relative to responses observed for snail population densities, the inverse responses of Opecoelidae infection rates to both benthic chlorophyll a concentrations and PO_4-P concentrations, suggest that eutrophic conditions that support high snail population densities may result in a loss of second intermediate hosts for Opecoelidae, perhaps due to greater sensitivity to secondary effects of eutrophication. If the secondary intermediate hosts are grazers, snails may be better adapted to changes that occur in periphyton composition as a response to nutrient enrichment (Marks and Lowe, 1989; Suren et al., 2003). Competition between snails and second intermediate hosts of Opecoelidae for periphyton resources may further account for the low level of Opecoelidae infection at the lowest concentrations of benthic chlorophyll a, which as previously discussed, appeared to be related to intensive grazing by snails. Although the mechanism requires further study, eutrophication appears to affect Opecoelidae infection rates, but contrary to initial expectations, it does not result in increasing prevalence as a result of increasing snail population densities.

The prevalence of Notocotylidae infections in *L. carinata* populations was not related to indicators of eutrophication and was instead related to water depth, a physical habitat variable. In shallow lentic environments,

water depth affects trematode prevalence in snail populations, and the effect is directly related to habitat utilization by second intermediate and definitive hosts (Jokela and Lively, 1995; Sapp and Esch, 1994). In the current study, mean depth of sampling sites was likely related to the degree of utilization of upstream areas by waterfowl, the definitive hosts of Notocotylidae. In addition, variables that affect the transmission of Notocotylid trematodes between hosts, such as the rate of settling of eggs after waterfowl defecation or possibly the congregation of miracidia, may be positively related to the mean depth of sampling sites.

The observed lack of relationships between the prevalence of Microphalloidea infections in *L. carinata* populations and measured environmental variables may be due to stronger associations between definitive hosts of trematodes in this superfamily and the terrestrial environment, relative to trematodes within the families Opecoelidae and Notocotylidae. Terrestrial habitat variables, such as riparian vegetation, were not quantified for each study site and may have provided additional information regarding variability in Microphalloidea infection rates across sampling sites. Discrimination of site-specific infection rates of Microphallidae and Lecithodendriidae may have allowed greater inference regarding the potential interactions between second intermediate and definitive hosts and measured environmental variables at the sampling sites. However, there were no "unexplained relationships" between total rates of infection and environmental variables; variables that were related to the total rates of infection across sampling sites were related to infection rates of the trematode families Opecoelidae and Notocotylidae. This supports the idea that Microphalloidea infection rates in *L. carinata* populations in streams within the Shenandoah River watershed were related to variables that were not measured in the current study.

Sample-scale relationships between trematode infection rates and other variables

As expected, there were significant relationships between trematode infection rates in *L. carinata* and physical habitat variables at the sample scale. The observed lack of relationship between current velocity and trematode infection rates was unexpected, as a significant negative relationship between current velocity and trematode infection prevalence in pleurocerid snails has been previously documented (Tolley-Jordan and Owen, 2008; Wetzel and Shreve, 2003). However, these studies examined both pool and riffle habitats, in contrast to the current study, which focused only on riffles, likely resulting in less overall variation in current velocity. Similar to relationships observed in comparisons across study sites, sample-scale analyses demonstrated a significant positive relationship between water depth and overall trematode infection prevalence, and this relationship was specific to

trematodes in the family Notocotylidae. The sample-scale relationship between Notocotylidae infection rates and water depth is likely related to within-site variation in waterfowl utilization or variables that affect the transmission of Notocotylid trematodes between hosts. Regardless of scale, water depth appears to be a critical factor shaping prevalence of infection by Notocotylidae in *L. carinata* populations in streams within the Shenandoah River watershed. At the sample scale there was also a weak positive relationship between Notocotylidae infection rates and total snail densities, which may reflect density-dependence of transmission dynamics such as probability of contact between miracidia and *L. carinata*, or may be an artifact of the weak positive relationship between total snail densities and water depth at the sample scale.

Unlike results of analyses conducted across sampling sites, there was a significant relationship between snail population densities and trematode infection prevalence at the sample scale. However, the relationship was negative, which was the opposite of the expected trend, and was specific to trematodes in the family Opecoelidae. The negative sample-scale relationship between Opecoelidae infection rates and snail population densities may result from snails outnumbering the infective miracidia that hatch from Opecoelid eggs deposited in feces of definitive hosts. This dilution effect was suggested by Ewers (1964) after similar observation of a negative relationship between trematode infection rates and snail population densities. However, in contrast to the current study in which the negative relationship was due to densities of adult snails, the negative relationship observed by Ewers (1964) was due to densities of juvenile snails. As previously discussed for comparisons across sampling sites, conditions that promote high population densities of snails may negatively impact Opecoelid trematode transmission through effects on second intermediate hosts. In addition, environmental stressors related to eutrophication may vary across microhabitats and may negatively affect trematodes, particularly life stages that are free-living for a short period of time (eggs, miracidia, and cercariae). Reduced survival of cercariae and miracidia can result in low trematode prevalence in snail populations despite high densities of hosts (Lafferty, 1997). Effects of eutrophic conditions on transmission dynamics of trematodes in the family Opecoelidae require further study, but similar to analyses across study sites, sample-scale analyses failed to support the working hypothesis that nutrient enrichment results in increasing trematode infection prevalence as a result of increasing snail population densities.

The relationship between densities of large snails and proportions of snails infected with the three dominant types of trematodes was examined separately from other variables because the nature of the relationship is unclear. Snail size is frequently positively correlated with infection rates in snail populations (Byers et al., 2008; Jokela and

Lively, 1995; Soldanova et al., 2010), which is generally thought to be related to duration of exposure. Compared to pleurocerid snails, the effect of exposure duration is probably greater for long-lived marine snails and short-lived freshwater pulmonate snails, which reproduce quickly and have multiple generations of adults present at any one time. Alternatively, the positive relationship between large snails and proportions of snails infected with trematodes may be a direct result of infection. Increased growth rates as a result of trematode infection (gigantism) have been observed in another genus of pleurocerid snail (*Elimia)* which is iteroparous (Krist, 2000) and in other semelparous "prosobranchs" (Probst and Kube, 1999). Gigantism is thought to occur more often in semelparous species, like *L. carinata*, compared to iteroparous species (Sousa, 1983), because a greater proportion of resources that would be devoted to reproduction are diverted to the trematodes (due to effective castration of the snails). Given the life history characteristics of *L. carinata*, the positive relationship between densities of large snails and trematode infection rates is likely a result of infection rather than a result of increased exposure duration.

Conclusions

Population densities of *Leptoxis carinata* generally increased along a gradient of nutrient enrichment found in streams within the Shenandoah River watershed, although there was some variability in this relationship and an apparent threshold of watershed disturbance which limited growth of snail populations. Landscape-scale effects of eutrophication on snail population densities were significant, but similar effects on trematode infection rates within snail populations were not observed, which reflects the importance of local, in-stream physical and biological characteristics on transmission of trematodes between hosts. For trematodes within the family Opecoelidae, the site- and sample-scale effects of eutrophication appear to be opposite of those observed for snail population densities; conditions supporting high population densities of snails resulted in lower Opecoelidae infection rates within snail populations. This is one of the first studies of populations of *Leptoxis carinata* and trematode infection rates within these populations. Results suggest that *L. carinata* is a useful species for monitoring biological effects of eutrophication and investigating trematode transmission dynamics in lotic systems.

Literature cited

Aldridge, D. W. (1982). Reproductive tactics in relation to life-cycle bioenergetics in three natural populations of the freshwater snail, *Leptoxis carinata*. *Ecology, 63*(1), 196-208.

Bass, C. S., & Weis, J. S. (2008). Increased abundance of snails and trematode parasites of *Fundulus heteroclitus* (L.) in restored New Jersey wetlands. *Wetlands Ecology and Management, 16*, 173-182.

Biggs, B. J. F. (2000). Eutrophication of streams and rivers: Dissolved nutrient-chlorophyll relationships for benthic algae. *Journal of the North American Benthological Society, 19*, 17-31.

Biggs, B. J. F., & Lowe, R. L. (1994). Responses of two trophic levels to patch enrichment along a New Zealand stream continuum. *New Zealand Journal of Marine and Freshwater Research, 28*, 119-134.

Braccia, A., & Voshell, J. R. (2006). Environmental factors accounting for benthic macroinvertebrate assemblage structure at the sample scale in streams subjected to a gradient of cattle grazing. *Hydrobiologia, 573*, 55-73.

Brown, K. M. (2001). Mollusca: Gastropoda. In J. H. Thorp, & A. P. Covich (Eds.), *Ecology and classification of North American freshwater invertebrates, second edition* (pp. 297-325). New York: Academic Press.

Brown, K. M., Lang, B., & Perez, K. E. (2008). The conservation ecology of North American pleurocerid and hydrobiid gastropods. *Journal of the North American Benthological Society, 27*(2), 484-495.

Bruno, J. F., Petes, L. E., Harvell, C. D., & Hettinger, A. (2003). Nutrient enrichment can increase the severity of coral diseases. *Ecology Letters, 6*, 1056-1061.

Byers, J. E., Blakeslee, A. M. H., Linder, E., Cooper, A. B., & Maguire, T. J. (2008). Controls of spatial variation in the prevalence of trematode parasites infecting a marine snail. *Ecology, 89*, 439-451.

Carpenter, S. R., Caraco, N. F., Correll, D. L., Howarth, R. W., Sharpley, A. N., & Smith, V. H. (1998). Nonpoint pollution of surface waters with phosphorus and nitrogen. *Ecological Applications, 8*, 559-568.

Chase, J. M., & Knight, T. M. (2006). Effects of eutrophication and snails on Eurasian watermilfoil (*Myriophyllum spicatum*) invasion. *Biological Invasions, 8*, 1643-1649.

Commonwealth of Virginia (2005). Chesapeake Bay nutrient and sediment reduction strategy for the Shenandoah and Potomac river basins. http://www.dcr.virginia.gov/soil_and_water/documents/tsshenpoall032805.pdf.

Dodds, W. K., Jones, J. R., & Welch, E. B. (1998). Suggested classification of stream trophic state: Distributions of temperate stream types by chlorophyll, total nitrogen, and phosphorus. *Water Research, 32*, 1455-1462.

Esch, G. W., Curtis, L. A., & Barger, M. A. (2001). A perspective on the ecology of trematode communities in snails. *Parasitology, 123*, S57-S75.

Ewers, W. H. (1964). The influence of the density of snails on the incidence of larval trematodes. *Parasitology, 54*, 579-583.

Figueroa-Nieves, D., Royer, T. V., & David, M. B. (2006). Controls on chlorophyll-a in nutrient-rich agricultural streams in Illinois, USA. *Hydrobiologia, 568*, 287-298.

Hechinger, R. F., & Lafferty, K. D. (2005). Host diversity begets parasite diversity: Bird final hosts and trematodes in snail intermediate hosts. *Proceedings of the Royal Society of London, Series B: Biological Sciences, 272*, 1059-1066.

Hendrix, S. S. (1978). The life history and biology of *Plagioporus hypentelii* Hendrix 1973 (Trematoda: Opecoelidae). *Journal of Parasitology, 64*, 606-612.

Hendrix, S. S. (1986). Population biology of the pleurocerid snail, *Leptoxis carinata* (Brug.) in Marsh Creek, Adams County, PA. *American Malacological Union Bulletin, 4*(1), 119.

Hoffman, G. L., & Dunbar, C. E. (1963). Studies on *Neogogatea kentuckiensis* (cable, 1935) n. Comb. (Trematoda: Strigeoidea: Cyathocotylidae). *Journal of Parasitology, 49*, 737-744.

Hoffman, G. L., Fried, B., & Harvey, J. E. (1985). *Sanguinicola fontinalis* sp. Nov. (Digenea: Sanguinicolidae): A blood parasite of brook trout *Salvelinus fontinalis* (Mitchill), and longnose dace, *Rhinichthys cataractae* (Valenciennes). *Journal of Fish Diseases, 8*, 529-538.

Homer, C., Dewitz, J., Fry, J., Coan, M., Hossain, N., Larson, C., et al., (2001). Completion of the 2001 National Land Cover Database for the conterminous United States. *Photogrammetric Engineering and Remote Sensing, 73*, 337-341.

Hoyer, M. V., & Canfield, D. E. (1994). Bird abundance and species richness on Florida lakes: Influence of trophic status, lake morphology, and aquatic macrophytes. *Hydrobiologia, 279/280*, 107-119.

Johnson, P. D., & Brown, K. M. (1997). The role of current and light in explaining the habitat distribution of the lotic snail *Elimia semicarinata* (Say). *Journal of the North American Benthological Society, 16*, 545-561.

Johnson, P. T. J., Chase, J. M., Dosch, K. L., Hartson, R. B., Gross, J. A., Larson, D. J., et al., (2007). Aquatic eutrophication promotes pathogenic infection in amphibians. *Proceedings of the Academy of Natural Sciences, 104*, 15781-15786.

Johnson, P. T. J., Townsend, A. R., Cleveland, C. C., Glibert, P. M., Howarth, R. W., McKenzie, V. J., et al., (2010). Linking environmental nutrient enrichment and disease emergence in humans and wildlife. *Ecological Applications, 20*, 16-29.

Jokela, J., & Lively, C. M. (1995). Spatial variation in infection by digenetic trematodes in a population of freshwater snails (*Potamopyrgus antipodarum*). *Oecologia, 103*, 509-517.

Kube, S., Kube, J., & Bick, A. (2002). Component community of larval trematodes in the mudsnail *Hydrobia ventrosa*: Temporal variations in prevalence in relation to host life cycle. *Journal of Parasitology, 88*, 730-737.

Krist, A. C. (2000). Effect of the digenean parasite *Proteromera macrostoma* on host morphology in the freshwater snail *Elimia livescens*. *Journal of Parasitology, 86*, 262-267.

Lafferty, K. D. (1997). Environmental parasitology: What can parasites tell us about human impacts on the environment? *Parasitology Today, 13*, 251-255.

Lewis, W. M., & McCutchan, J. H. (2010). Ecological responses to nutrients in streams and rivers of the Colorado mountains and foothills. *Freshwater Biology, 55*, 1973-1983.

Marks, J. C., & Lowe, R. L. (1989). The independent and interactive effects of snail grazing and nutrient enrichment on structuring periphyton communities. *Hydrobiologia, 185*, 9-17.

McKenzie, V. J., & Townsend, A. R. (2007). Parasitic and infectious disease responses to changing global nutrient cycles. *EcoHealth, 4*, 384-396.

Mishra, A., Benham, B. L., & Mostaghimi, S. (2006). Sediment and nutrient losses from field-scale cropland plots treated with animal manure and inorganic fertilizer. *Water Air and Soil Pollution, 175*, 61-76.

Morales, J. B. T., & Ward, A. K. (2000). Differential incorporation of algae and bacteria by *Elimia clara* (Prosobranchia: Pleuroceridae) - A study using dual-labeled epilithon. *Journal of the North American Benthological Society, 19*, 289-297.

Niyogi, D. K., Koren, M., Arbuckle, C. J., & Townsend, A. R. (2007). Stream communities along a catchment land-use gradient: Subsidy-stress responses to pastoral development. *Environmental Management, 39*, 213-225.

Orth, D. J., Alexander, K. A., Ciparis, S., Frimpong, E., Henley, W., & Voshell, J. R. (2009). Investigation into smallmouth bass mortality in Virginia's rivers. http://www.dgif.virginia.gov/fishing/fish-kill/Smallmout-Bass-Mortality-Final-Rpt-VT-2009.pdf

Probst, S., & Kube, J. (1999). Histopathological effects of larval trematode infections in mudsnails and their impact on host growth: What causes gigantism in *Hydrobia ventrosa* (Gastropoda: Prosobranchia). *Journal of Experimental Marine Biology and Ecology, 238*, 49-68.

Reiskind, M. H., Walton, E. T., & Wilson, M. L. (2004). Nutrient-dependent reduced growth and survival of larval *Culex restuans* (Diptera: Culicidae): Laboratory and field experiments in Michigan. *Journal of Medical Entomology, 41*, 650-656.

Richardson, T. D., Scheiring, J. F., & Brown, K. M. (1988). Secondary production of two lotic snails (Pleuroceridae: Elimia). *Journal of the North American Benthological Society, 7*, 234-245.

Rohr, J. R., Schotthoefer, A. M., Raffel, T. R., Carrick, H. J., Halstead, N., Hoverman, J. T., et al., (2008). Agrochemicals increase trematode infections in a declining amphibian species. *Nature, 455*, 1235-1239.

Rosemond, A. D., Mulholland, P. J., & Elwood, J. W. (1993). Top-down and bottom-up control of stream periphyton: Effects of nutrients and herbivores. *Ecology, 7*, 1264-1280.

Sapp, K. K., & Esch, G. W. (1994). The effects of spatial and temporal heterogeneity as structuring forces for parasite communities in *Helisoma anceps* and *Physa gyrina*. *American Midland Naturalist, 132*, 91-103.

SAS Institute, Inc. (1995). *Logsistic regression examples using the SAS system, version 6, first edition*. Cary, NC: SAS Institute, Inc.

SAS Institute, Inc. (2010). *SAS/STAT 9.22 user's guide*. Cary, NC: SAS Institute, Inc.

Schell, S. C. (1985). *Handbook of the trematodes of North America north of Mexico*. Moscow, Idaho: University Press of Idaho.

Skirnisson, K., Galaktionov, K. V., & Kozminsky, E. V. (2004). Factors influencing the distribution of digenetic trematode infections in a mudsnail (*Hydrobia ventrosa*) population inhabiting salt marsh ponds in Iceland. *Journal of Parasitology, 90*, 50-59.

Smith, V. H., Tilman, G. D., & Nekola, J. C. (1999). Eutrophication: impacts of excess nutrient inputs on freshwater, marine, and terrestrial ecosystems. *Environmental Pollution, 100*, 179-196.

Soldanova, M., Selbach, C., Sures, B., Kostadinova, A., & Perez-del-Olmo, A. (2010). Larval trematode communities in *Radix auricularia* and *Lymnaea stagnalis* in a reservoir system of the Ruhr River. *Parasites and Vectors, 3*, 56.

Soupir, M. L., Mostaghimi, S., & Yagow, E. R. (2006). Nutrient transport from manure applied to pastureland using phosphorus-based management strategies. *Journal of Environmental Quality, 35*, 1269-1278.

Sousa, W. P. (1983). Host life history and the effect of parasitic castration on growth: A field study of *Cerithidea californica* Haldeman (Gastropoda: Prosobranchia) and its trematode parasites. *Journal of Experimental Marine Biology and Ecology, 73*, 273-296.

Stone, M. L., Whiles, M. R., Webber, J. A., Williard, K. W. J., & Reeve, J. D. (2005). Macroinvertebrate communities in agriculturally impacted southern Illinois streams: Patterns with riparian vegetation, water quality, and in-stream habitat quality. *Journal of Environmental Quality, 34*, 907-917.

Suren, A. M., Biggs, B. J. F., Kilroy, C., & Bergey, L. (2003). Benthic community dynamics during summer low-flows in two rivers of contrasting enrichment, 1. Periphyton. *New Zealand Journal of Marine and Freshwater Research, 37*, 53-70.

Thieltges, D. W. (2007). Habitat and transmission - effect of tidal level and upstream host density on metacercarial load in an intertidal bivalve. *Parasitology, 134*, 599-605.

Thomas, J. D., & Daldorph, P. W. G. (1994). The influence of nutrient and organic enrichment on a community dominated by macrophytes and gastropod molluscs in a eutrophic drainage channel: Relevance to snail control and conservation. *Journal of Applied Ecology, 31*, 571-588.

Tolley-Jordan, L. R., & Owen, J. M. (2008). Habitat influences snail community structure and trematode infection levels in a spring-fed river, Texas, USA. *Hydrobiologia, 600*, 29-40.

Urban, M. C. (2005). Road facilitation of trematode infections in snails of northern Alaska. *Conservation Biology, 20*, 1143-1149.

Voutilainen, A., van Ooik, T., Puurtinen, M., Kortet, R., & Taskinen, J. (2009). Relationship between prevalence of trematode parasite *Diplostomum* sp. and population density of its snail host *Lymnaea stagnalis* in lakes and ponds in Finland. *Aquatic Ecology, 43*, 351-357.

Walters, D. M., Roy, A. H., & Leigh, D. S. (2009). Environmental indicators of macroinvertebrate and fish assemblage integrity in urbanizing watersheds. *Ecological Indicators, 9*, 1222-1233.

Wetzel, E. J., & Shreve, E. W. (2003). The influence of habitat on the distribution and abundance of metacercariae of *Macravestibulum obtusicaudum* (Pronocephalidae) in a small Indiana stream. *Journal of Parasitology, 89*, 1088-1090.

Wilson, K., & Hardy, I. C. W. (2002). Statistical analysis of sex ratios: An introduction. In I. C. W. Hardy (Ed.), *Sex ratios: Concepts and research methods* (pp. 48-92). New York: Cambridge University Press.

Wojdak, J. M. (2005). Relative strength of top-down, bottom-up, and consumer species richness effects on pond ecosystems. *Ecological Monographs, 75*, 489-504.

Zendt, J. S., & Bergersen, E. P. (2000). Distribution and abundance of the aquatic oligochaete host *Tubifex tubifex* for the salmonid whirling disease parasite *Myxobolus cerebralis* in the upper Colorado river basin. *North American Journal of Fisheries Management, 20*, 502-512.

Table 6- 1. Information for the 15 study sites in streams within the Shenandoah River watershed sampled in 2008 and 2009.

Site #	Stream Name	AFOs / 1,000 acre [a]	WWTP (MGD)[b]	Benthic Chl. a [c] (µg/cm^2)	Snails / m^2 ± SE [d]	% Infected Snails [e]					Spring vs. Fall [f]
						Spr. 2008	Fall 2008	Spr. 2009	Fall 2009	Mean ± SE	
1	Cedar Crk.-US	0	0	10.5	364 ± 103	0**	11	12*	8.3	7.9 ± 2.7	0.97
2	Cedar Crk.-DS	0.01	0	19.5	517 ± 162	13**	5.2	9.7	4.0	8.0 ± 2.1	0.009
3	Stony Crk.-US	0.17	0.64	2.0	1,354 ± 256	1.9	4.3	5.1	4.5	3.9 ± 0.7	0.76
4	Stony Crk.-DS	0.40	0.81	23.8	1,446 ± 462	19**	16	13	18	17 ± 1.4	0.15
5	Passage Crk.	0.04	0	5.4	576 ± 164	14**	7.2	2.2*	6.3	7.5 ± 2.5	0.38
6	Mill Crk.	0.97	0	16.2	1,046 ± 184	20**	3.3	16	18	14 ± 3.8	0.19
7	Smith Crk.	1.2	0.09	28.3	1,686 ± 300	2.2**	5.4	6.6	4.3	4.6 ± 0.9	0.19
8	Hawksbill-US	1.4	0.20	23.5	728 ± 282	12*	13	12	8.9	11 ± 0.9	0.20
9	Hawksbill-DS	1.0	1.8	2.0	1,671 ± 206	3.2*	2.8	4.3	4.9	3.8 ± 0.5	0.87
10	Long Meadow	4.0	0	23.1	1,113 ± 333	1.8	3.2*	5.2	3.1	3.3 ± 0.7	0.48
11	Linville Crk.	2.5	0.03	16.6	520 ± 101	9.6*	6.8*	16	7.6	10 ± 2.1	0.033
12	Naked Crk.	0.25	0	8.0	480 ± 71	21**	16*	21	9.2	17 ± 2.8	0.008
13	Briery Br.	1.3	0	4.6	1,175 ± 225	12	5.1	10	12	10 ± 1.7	0.91
14	Muddy Crk.	4.2	0.01	4.6	1,324 ± 169	11	14	16	9.2	12 ± 1.5	0.017
15	Jennings Br.	0.40	0	22.4	1,775 ± 471	70**	28	70	26	49 ± 13	0.0001

[a] Watershed density of animal feeding operations (AFOs)

[b] Total upstream permitted effluent flow of wastewater treatment plants (WWTPs) in millions of gallons per day (MGD)

[c] Mean benthic chlorophyll a concentrations from rocks sampled during summer 2008 and summer 2009

[d] Mean densities of snails (adults and juveniles) collected during four sampling periods ± standard error (SE)

[e] Percentages of snails infected with trematode cercariae, calculated from all replicate samples collected within a sampling period (four in 2008 and eight in 2009), as well as the mean of all sampling periods ± standard error (SE)

[f] P-values from G-tests of independence on pooled proportions of infected snails in spring and fall sampling periods at each sampling site, underlined p-values were significant at α=0.05

* less than 100 adult snails collected/examined, ** less than 50 adult snails collected/examined

Table 6- 2. Types of trematode cercariae utilizing *Leptoxis carinata* as a first intermediate host. Cercarial type, family identification, probable secondary and definitive hosts, and previous observations in *L. carinata* are listed.

Type	Family	Secondary hosts [b]	Definitive hosts [b]	Previously observed?
Cotylomicrocercous	Opecoelidae	Aquatic insects or fish	Fish	Yes [c]
Vivax	Cyathocotylidae	Fish	Picscivorous birds	Yes [d]
Monostome	Notocotylidae	Encyst on plant material	Birds (waterfowl)	No
Ubiquita [a]	Microphallidae [a]	Amphipods, isopods, crayfish or none	Birds, mammals, fish, or turtles	No
Virgulate [a]	Lecithodendriidae [a]	Aquatic insects	Birds, bats, or amphibians	No

[a] Xiphidiocercariae; produced by trematodes within the superfamily Microphalloidea
[b] As described in Schell (1985)
[c] Hendrix (1978)
[d] Hoffman and Dunbar (1963)

Table 6- 3. Results of logistic regressions between site-scale independent variables and total proportions of infected snails, proportions of snails infected with Opecoelidae, and proportions of snails infected with Notocotylidae. For each significant variable (insignificant indicated by -), coefficients and Wald χ^2 values are shown. For full models (all preceding significant variables), Wald χ^2 values, the concordance index (c), and Hosmer and Lemeshow (H-L) goodness of fit χ^2 values are shown.

Site-scale variables	Total proportion infected				Proportion infected w/ Opecoelidae				Proportion infected w/ Notocotylidae			
	Coeff	χ^2	c	H-L	Coeff	χ^2	c	H-L	Coeff	χ^2	c	H-L
[Chlor a][a]	0.17	13.8 †			0.45	34.0 ††			-	-		
[Chlor a] ^2	-0.006	12.3 †			-0.018	35.0 ††			-	-		
Depth	1.76	10.3 †			-	-			3.94	23.5 ††		
Full model		21.8 ††	0.62	66.7 ††		35.1 ††	0.70	80.9 ††		23.5 ††	0.64	80.9 ††

[a] Concentration of benthic chlorophyll a ($\mu g/cm^2$)
[b] Local percentage of developed land (within 1,500 m^2 of the site)
** $p \leq 0.01$, $^{\dagger} p \leq 0.001$, $^{\dagger\dagger} p \leq 0.0001$

Table 6- 4. Results of logistic regressions between sample-scale independent variables and total proportions of infected snails, proportions of snails infected with Opecoelidae, and proportions of snails infected with Notocotylidae. For each significant variable (insignificant indicated by -), coefficients and Wald χ^2 values are shown. For full models (all preceding significant variables), Wald χ^2 values, the concordance index (c), and Hosmer and Lemeshow (H-L) goodness of fit χ^2 values are shown. The effect of snail size was assessed separately.

Sample-scale variables	Total proportion infected				Proportion infected w/ Opecoelidae				Proportion infected w/ Notocotylidae			
	Coeff	χ^2	c	H-L	Coeff	χ^2	c	H-L	Coeff	χ^2	c	H-L
Depth	1.06	11.3 ††			-	-			1.37	15.2 ††		
Adult dens.[a]	-0.50	7.67 **			-1.15	14.6 ††			-	-		
Total dens.[a]	-	-			-	-			0.50	4.65 *		
Full model		16.36 †	0.57	14.2		14.6 ††	0.67	49.1 ††		20.5 ††	0.58	18.9 *
Large snails[b]	0.36	22.2 ††	0.57	74.4 ††	0.57	13.4 †	0.61	89.5 ††	0.37	12.9 †	0.59	11.2

[a] Density (snails/m^2)
[b] Density of snails > 11 mm
$^{*}p<0.05$, $^{**}p\leq0.01$, $^{\dagger}p\leq0.001$, $^{\dagger\dagger}p\leq0.0001$

Figure 6- 1. Locations of sampling sites in streams (numbers) within the Shenandoah River watershed. The enlargement is placed in the context of counties in the state of Virginia. Within the Shenandoah River watershed, the two forks of the river are indicated by the thin grey line and the 12-digit hydrologic unit code subwatersheds are outlined.

Figure 6- 2. Relationships between snail population densities (four sampling periods) at study sites within Shenandoah River tributaries and environmental variables. These variables include: mean concentrations of PO_4-P (A) and benthic chlorophyll a (B), as well as the relationship between PO_4-P and benthic chlorophyll a at these study sites (C). Lines represent statistically significant linear (A) or polynomial (B) regressions, with relevant information listed above or below each line. The relationship between PO_4-P and benthic chlorophyll a (C) was not statistically significant.

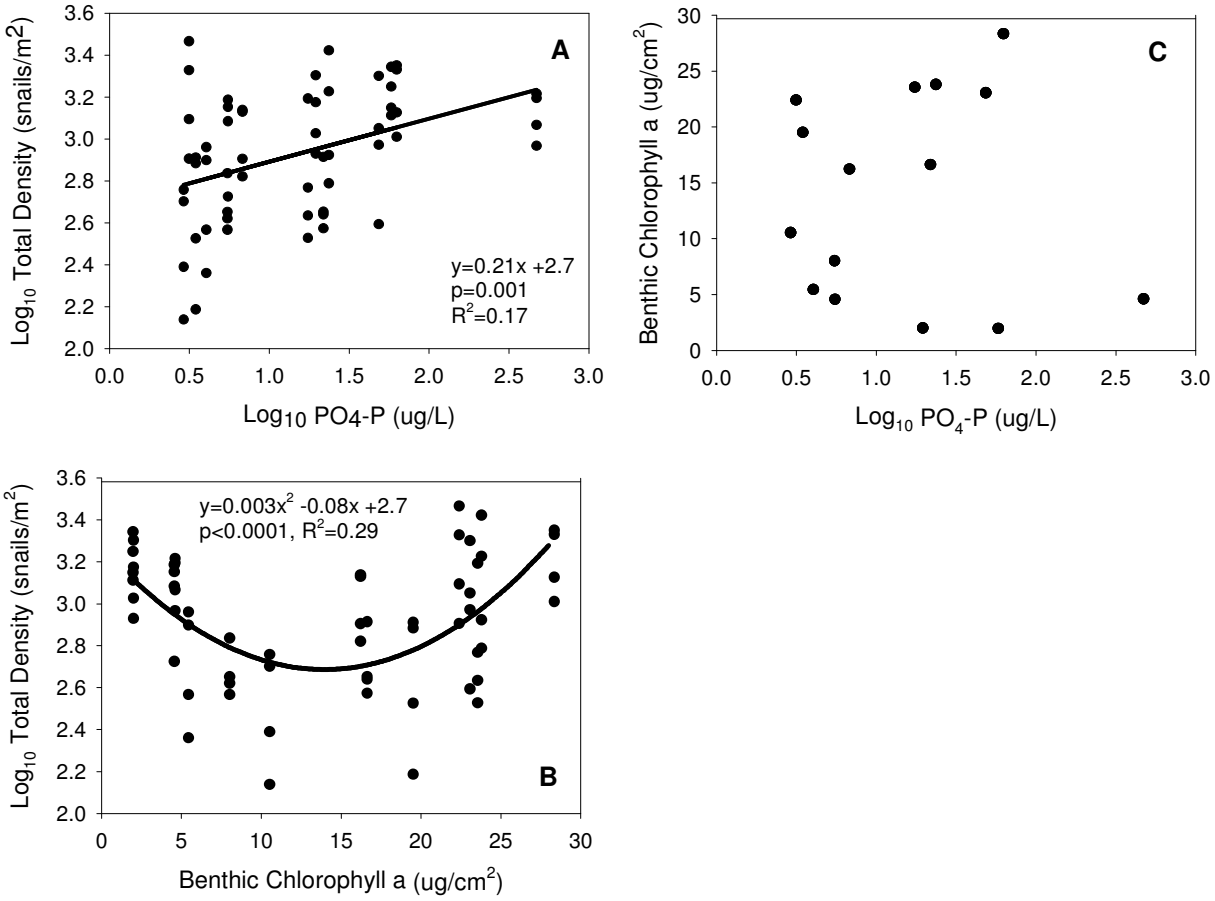


Figure 6- 3. Relationships between snail population densities (four sampling periods) at study sites within Shenandoah River tributaries and land use variables. These variables include: watershed densities of animal feeding operations (AFOs; A), total upstream permitted effluent flow of wastewater treatment plants (WWTPs) in millions of gallons per day (MGD; B), and principal component 1 (PC 1) from the combination of all watershed-scale land use variables (C). Lines represent statistically significant polynomial (A and C) or linear regressions (B), with relevant information listed below each line.

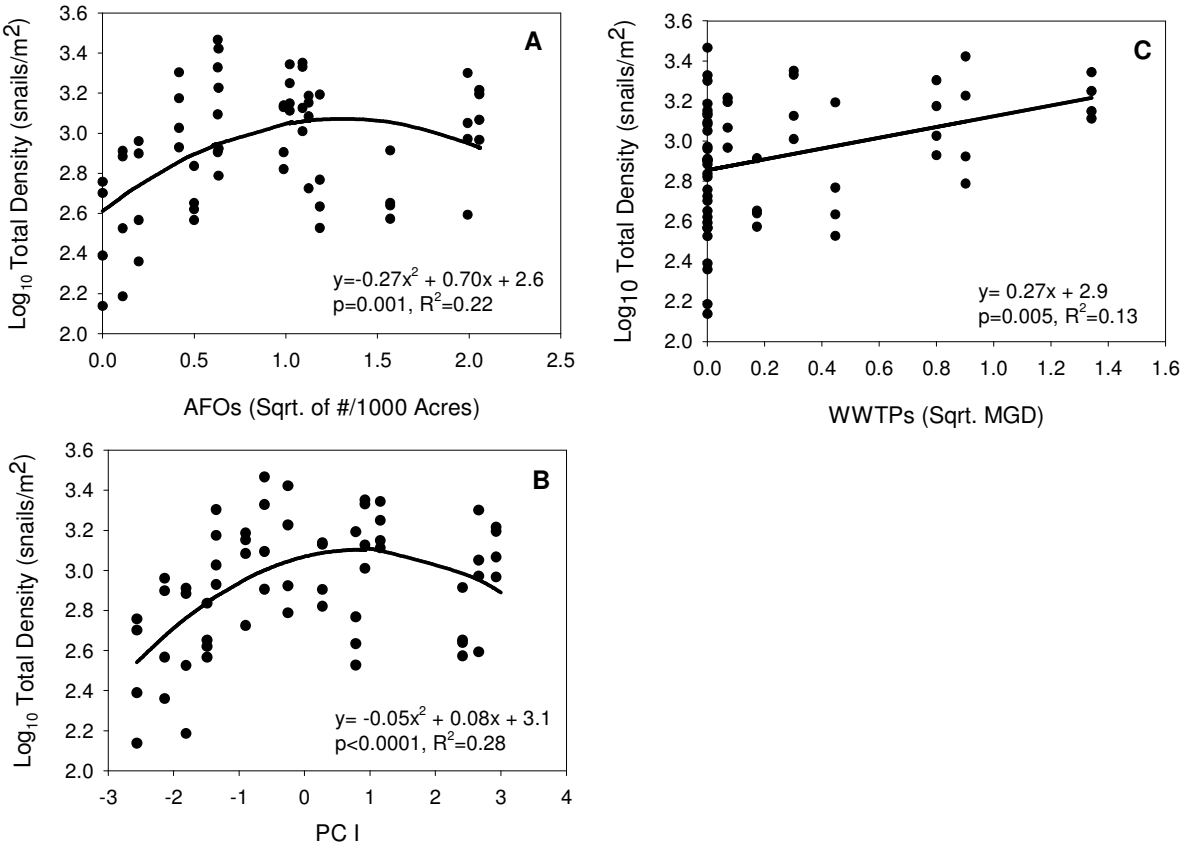


Figure 6- 4. **Total trematode infection rates in snail populations at each study site, including infection rates of individual types of trematodes. The number above each bar represents the total number of snails examined over the course of the study (four sampling periods).**

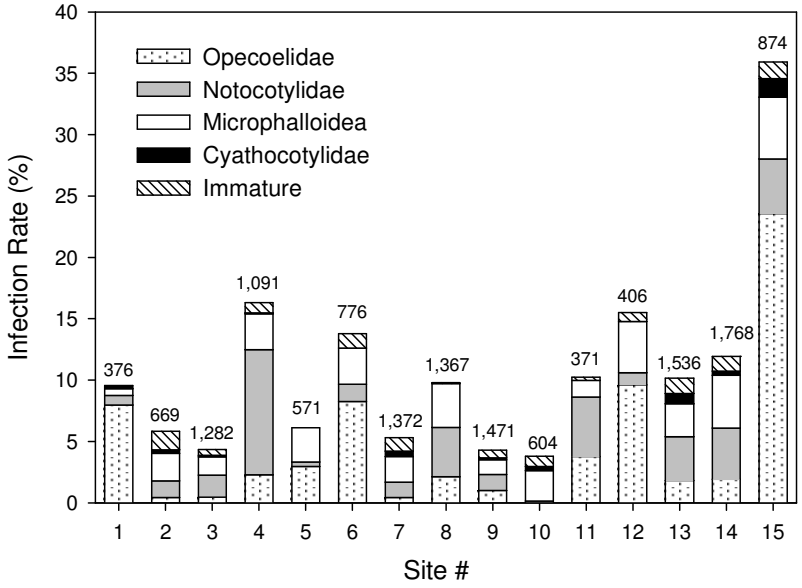


Figure 6- 5. **Relationships between proportions of snails infected with trematodes (four sampling periods) at study sites within Shenandoah River tributaries and other variables. These variables include: snail population densities (A), mean benthic chlorophyll a (B), mean depth at sampling sites (C), and mean concentrations of PO_4-P (D). Proportions of infected snails represent all observed types of trematodes (A), or individual families of trematodes (B, C, E). Lines represent statistically significant logistic regressions (B-D), with relevant information listed above each line. The relationship between population densities of snails and the proportion of infected snails (A) was not statistically significant.**

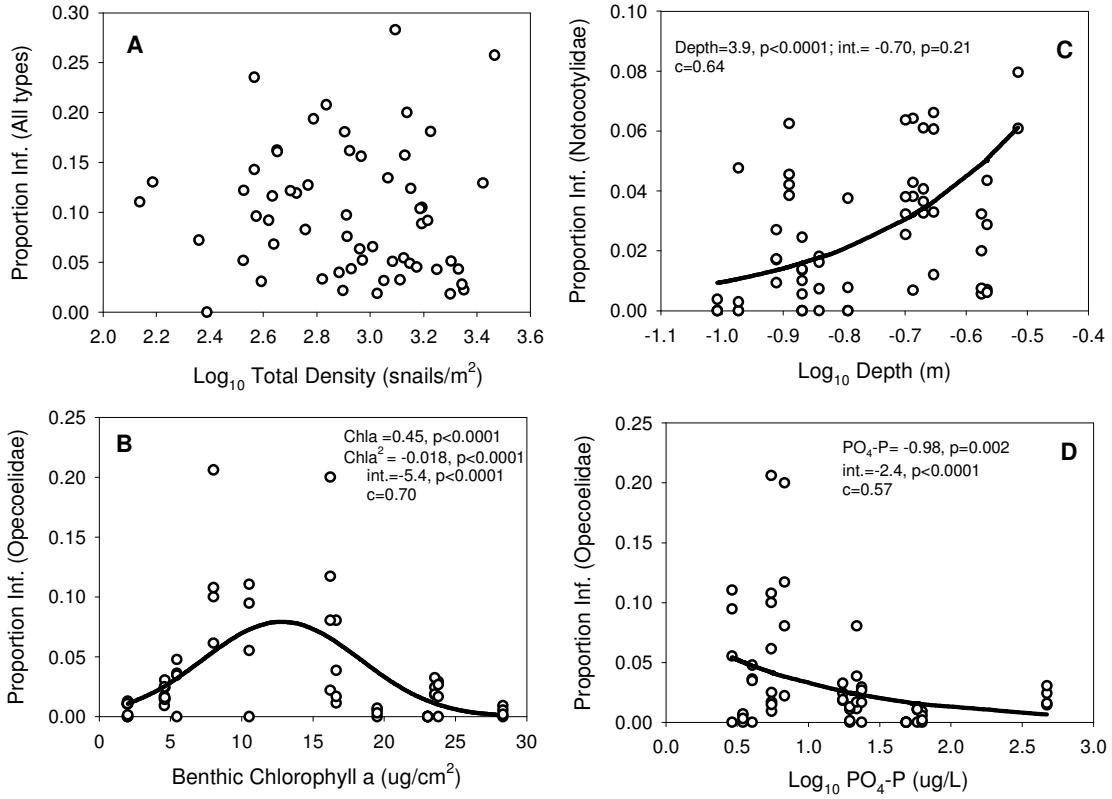
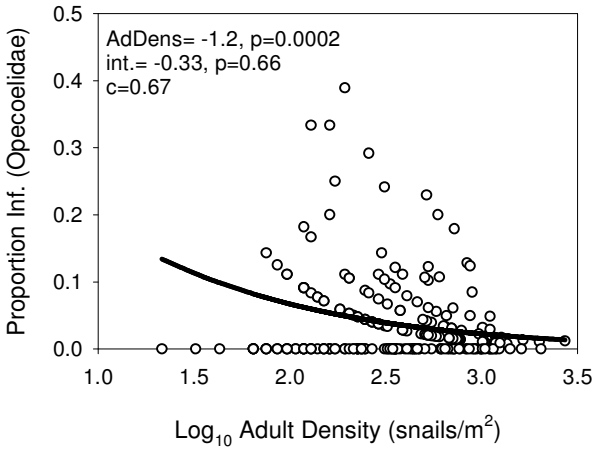


Figure 6- 6. Relationship between proportions of snails infected with trematodes in the family Opecoelidae and population densities of adult snails within individual replicate samples. Replicate samples (n=8) were collected over two sampling periods in 2009 from study sites within 15 Shenandoah River tributaries (n=240). The line represents a statistically significant logistic regression model, with relevant information listed above it.



CHAPTER 7

Description of gamete development in a pleurocerid snail, *Leptoxis carinata*, using morphological and histological techniques

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J. Reese Voshell, Jr.

Abstract

Little is known about the timing of sexual development in pleurocerid snails or the effect of environmental conditions on this process. The objectives of this study were to describe gamete development in *Leptoxis carinata* with respect to snail size and season, and to compare this process between two streams with different environmental conditions. Morphological and histological examinations of two generations of *L. carinata* were conducted for 16 months at two sites in the Shenandoah River watershed (Virginia, USA); one site represented reference conditions and the other was impacted by agricultural activities. Water temperatures were similar between sites during the sampling period (paired t-test, $p=0.84$). Population sex ratios were consistently female-biased at the impacted site (mean 81% females), compared to balanced sex ratios at the reference site (mean 49% females). Morphologically, sexes did not become fully distinct at the reference site until approximately 15 months after hatching, and there was an additional seven month delay in morphological development at the impacted site. Histological observations demonstrated that gamete production began earlier than indicated by external morphology; the majority of snails from both sites were producing gametes eight months after hatching. Histological comparisons of mature snails showed differences in gamete production between study sites, both male and female snails at the impacted site had significantly fewer acini (paired t-test, $p \leq 0.027$) and there were apparent differences in oocyte developmental stages and male acini compared to the reference site. The differences in sex ratios and gamete production between the two study sites suggest that environmental factors other than water temperature may affect pleurocerid snail sexual development.

Introduction

Environmental contaminants can disrupt sexual development of wildlife through several mechanisms. The compound p,p'-DDE, a degradation product of the insecticide DDT, can disrupt sexual differentiation in alligators through an anti-androgenic mechanism of action, causing female-biased sex ratios (Milnes et al., 2005). The anti-fouling compound tributyltin causes development of male accessory sex organs in female caenogastropods (imposex), through inappropriate activation of retinoic acid signaling via the retinoid X receptor (RXR; Castro et al.,

2007a; Nishikawa et al., 2004; Sternberg et al., 2008). Compounds that mimic the action of endogenous estrogens (estrogenic compounds) can disrupt sexual differentiation in fishes, causing development of oocytes in the testes of males and phenotypic expression of female secondary sex characteristics in genotypic males (Fenske et al., 2005; Hahlbeck et al., 2004; Jobling et al., 1998; Jobling et al., 2003). Estrogenic compounds may also affect sexual differentiation in bivalves. Female-biased sex ratios were observed in populations of adult oysters exposed to alkylphenols as undifferentiated larvae, compared to balanced sex ratios in unexposed oysters (Nice et al., 2003). Female-biased sex ratios have also been observed in populations of freshwater mussels living downstream of wastewater treatment plants (WWTPs), which are known sources of estrogenic compounds, compared to balanced sex ratios upstream of these operations (Gagne et al., 2011). In freshwater gastropods, exposure to estrogenic compounds appears to increase reproductive output (Duft et al., 2003; Jobling et al., 2003) and cause a significant increase in the size of accessory sex glands in adult females (Castro et al., 2007b; Oehlmann et al., 2000). However, few studies have examined the effects of estrogenic compounds, or other contaminants, on processes related to sexual differentiation of freshwater gastropods, despite their widespread distribution in aquatic environments and potential utility as sentinel organisms.

The freshwater snail *Leptoxis carinata* (superorder Caenogastropoda: order Sorbeoconcha: superfamily Cerithioidea: family Pleuroceridae), is present in high densities (up to 3,000/m^2) in the streams and rivers within the Shenandoah River watershed (Virginia, USA). A recent study of population sex ratios of *L. carinata* in this watershed showed large variation amongst 15 sampled streams, with proportions of females ranging from 0.46-0.87, and longitudinal variation in sex ratios along the length of the North Fork of the Shenandoah River (Chapter 5). The spatial variation in population sex ratios across sites was consistent, but there was little within-site variation in population sex ratios across different seasons and generations of snails. Taken together, these results suggest that site-specific environmental conditions may affect sexual differentiation in this species. However, the effects of specific contaminants or stressors on this process cannot be evaluated until more is known about the processes of sexual differentiation and gonad development in *L. carinata*, including an appropriate timeframe for controlled exposures. Studies of the effects of estrogenic compounds on populations of exposed fish have demonstrated that the timing and duration of exposure are critical in terms of the magnitude of observed effects (Fenske et al., 2005; Lange et al., 2009).

Previous studies have documented the basic life history characteristics of *Leptoxis carinata* and a closely related species, *Leptoxis dilatata*. (Aldridge, 1982; Hendrix, 1986; Miller-Way and Way, 1989). These two species of *Leptoxis spp*. are semelparous biennials. Only 2-year old snails participate in reproduction, egg-laying occurs in spring through early summer, and the 2-year old snails die off by the end of the summer. Eggs hatch in two to six weeks, and juvenile snails are sexually dimorphic by the following summer. Although the general reproductive pattern has been determined for *Leptoxis spp*., the timing of sexual differentiation and gonad maturation is unknown, and the details of these processes are not well studied in the entire family Pleuroceridae. The objectives of this study were 1) to use morphological and histological techniques to describe gonad maturation in *L. carinata,* and 2) to compare this process between two streams with different environmental conditions.

Methods

Snails were collected 12 times between March 2009 and June 2010 from two streams in the Shenandoah River watershed, Briery Branch (BRIR) and Long Meadow Run (LOMR). The two streams reflect differences in the degree of impact from agricultural operations, with BRIR representing reference conditions and LOMR representing impacted conditions (Table 7-1). In addition, the population sex ratios of *L. carinata,* initially determined during field sampling of several Shenandoah River tributaries in spring and fall of 2008, differed between these sites (Table 7-1). Collections were approximately monthly, with bimonthly collections in the late fall and winter/early spring. Snails were collected from within four randomly assigned quadrats (Surber sampler, 0.0929 m^2) on each sampling date and the presence or absence of eggs was recorded. Water temperature and specific conductivity were recorded with a YSI 30 Salinity/Conductivity/Temperature meter (YSI Incorporated, Yellow Springs, OH). Snails were held in site water in glass jars until they were returned to the laboratory.

Prior to dissection, snails were narcotized with a 1% solution of $MgCl_2$ in site water and the total shell length (SL) of all collected snails was determined with a digital caliper (0.01 mm precision). Snails were separated into 1-mm size categories of snails, counted, and removed from their shells. For each sampling date, percentages of snails in each size class were calculated. Ages for all snails were estimated based on hatching times between May and July, which corresponds to egg-laying periods observed by Aldridge (1982) and in the current study (see results). Aldridge (1982) also observed an 18 day lag between egg-laying and hatching of juvenile snails. Therefore,

the egg-laying period roughly corresponds to the hatching period, and in the current study, all SL categories collected within a given sampling period represent variation in snail ages (smaller snails are younger/hatched later).

Sexually mature female pleurocerid snails can be identified by an egg-laying groove on the right side of the foot, the presence of developing oocytes in the ovary, and the presence of a white capsule gland (Figure 7-1a). Males do not have a penis, sexually mature males have a fleshy pad on the right side of the foot, and sex confirmation is accomplished by examination of the testes (Figure 7-1b). In mature males, sperm is visible within the gonoduct and is readily released if a small piece of the testis is removed with forceps.

Using a dissecting microscope, external morphological characteristics of the gonad/digestive gland complex, including color and estimated percentage of the complex with apparent development of ovaries or testes, was described for each 1-mm size category collected on each sampling date. For maturing females, the presence and degree of development of the egg-laying groove and capsule gland was also described. Sexually mature individuals were classified as adults and immature individuals were classified as juveniles, with either immature or developing gonads. Individuals with obvious infections of digenetic trematodes were noted, but were not included in any morphological or histological analyses. All snails were fixed and preserved in 10% neutral buffered formalin (histological grade).

For histological analysis, six adult males, six adult females, and 11-13 snails from each of the dominant juvenile size categories collected on each sampling date were randomly subsampled using a gridded glass Petri dish in 70% ethanol. The gonad/digestive gland complex was dissected from the rest of the body and dehydrated with ascending concentrations of ethanol, cleared in xylene, and embedded in paraffin wax. The gonad/digestive gland was always oriented so that the right side would be evaluated (cut first). Thick sections (5 μm) within the first 50-100 μm of tissue were obtained using a microtome and stained with hematoxylin and eosin. Light microscopy was used to examine the thick sections (10x-40x).

In gonochoristic gastropods, gametes develop from primordial germ cells located in gonadal acini (hollow cavities) that develop within connective tissue (Awaji and Hamano, 2004; Voltzow, 1991). The acini develop first, but sexes are indistinguishable until gametogenesis. For individual *L. carinata* evaluated using histology, the entire thick section of the gonad was examined for the presence of acini. All acini within each section were counted. The only exception was for fully developed males; when more than 100 acini were present, 100 was recorded as the number of acini, due to difficulty in obtaining accurate counts within the field of view. Oocytes are easily identified

at the early stages of development compared to primary spermatocytes, so the presence of developing oocytes was used as the first indication of gametogenesis in collected snails. For all thick sections of the gonad of differentiated females (juveniles and mature), the numbers of acini containing oocytes were counted in addition to the total number of acini, and the proportion of acini with oocytes was calculated. For evaluation of gonad development in juvenile snails, the total number of acini counted for all snails within each size class collected on a particular date was divided by the number of snails evaluated to determine the mean number of acini per snail. For differentiated females, the total number of acini with oocytes was divided by the number of females evaluated to determine the mean number of acini with oocytes per female, and the proportions of acini with oocytes were calculated.

For histological comparisons of mature snails between Briery Branch (reference) and Long Meadow Run (impacted), the total number of females within each size class evaluated on each sample date, and the total numbers of acini and acini with oocytes counted within the thick sections, were used to calculate the total proportions of acini with oocytes and the mean number of acini with oocytes, per female. For mature males, the total number of evaluated males and the total number of counted acini were used to calculate the mean number of acini per male. All acini of mature males contained sperm. Histological endpoints and water temperatures were compared between sites using t-tests (α=0.05).

Results and Discussion

Population characteristics and environmental conditions

When the study began in March 2009, two generations of snails were present at both sampling sites; adults that hatched in 2007 (19-21 months old), and juveniles that hatched in the summer of 2008 (7-9 months old) (Table 7-2). The 2007 generation was reproductively competent and a combination of SL and observations of external morphology of the soft tissues were used to distinguish the two generations. In March 2009, all snails at BRIR and LOMR with SL < 7 mm were 2008 generation juveniles (8-10 months old) and all snails with SL > 8 mm were 2007 generation adults (20-22 months old). Overlap between the two generations occurred at SL 7-8 mm as indicated by observations of external morphology; half of the snails had fully developed gonads and half of the snails had only partial gonad development (not reproductively competent).

At BRIR (reference site), growth of 2008 generation snails was evident between May 2009 (10-12 months old) and October 2009 (15-17 months old), with a gradual shift of the dominant SL category (Table 7-3). Eggs, laid by 2007 generation snails (~24 months old), were observed between May and July in 2009. In July 2009, the 2009 generation of snails became collectable at SL 1-2 mm (1-2 months old). By August 2009, the 2007 generation snails had died off, consistent with the previously documented 2-year life cycle of *L. carinata* (Aldridge, 1982), and all adult snails were the 2008 generation (13-15 months old). Growth of 2009 generation juvenile snails was apparent through the December collection period ($\leq$ 7 months old) and appeared to cease until April/May of 2010 (9-12 months old) (Table 7-3), which is also consistent with findings of Aldridge (1982). Egg-laying by 2008 generation snails began in May 2010 (22-24 months old). There was no overlap in SL categories between the 2008 adults and 2009 juveniles in the spring of 2010 (Table 7-3).

At LOMR (impacted site), growth of 2008 generation snails was evident between March and May 2009, and to a lesser extent, between May and October 2009 (Table 7-3). Compared to BRIR (reference), snails were larger at LOMR on the first sampling date (8-10 months old), but were smaller at maturity (October, 15-17 months old, Table 7-3). This indicates lower growth rates for snails at LOMR during their second summer of life, which is a critical time period for energy assimilation so that snails have enough energy stores to overwinter and reproduce during the next spring (Aldridge 1982). Water temperatures at BRIR and LOMR were not significantly different (paired t-test, p=0.84; Figure 7-2) over the course of the study. Therefore, water temperature is not likely the cause of lower growth rates at LOMR relative to BRIR.

In 2009, no eggs were observed at LOMR, and a 2009 generation was not produced as evidenced by the lack of juvenile snails in collections from late summer and fall of 2009 (Table 7-3). The 2007 adults died off in July (24-26 months old) without successfully reproducing. The reasons for this are unclear. The sex ratio at LOMR was highly biased toward females on all sampling dates, compared to a balanced sex ratio at BRIR (Figure 7-3). It is possible that the sex ratio was too unbalanced to allow successful mating.

Morphological observations

As previously described, fully developed females had a well developed egg-laying groove and ovipositor, a white capsule gland (cg), and developing oocytes covering the majority of the right side of the gonad/digestive gland

complex (Figure 7-1a). Mature males had testes covering the entire right side of the gonad/digestive gland complex (Figure 7-1b) and were producing sperm. The sexes are morphologically distinct when females develop an egg-laying groove and males have a thin layer of testes "coating" the entire right side of the gonad/digestive gland. Prior to this, gonad development appears to be indicated by the appearance of "blue stripes" over the right side of the predominantly brown digestive gland (dg) (Figure 7-1c). In females, oocytes appear to develop within the stripes, which gradually begin to take up a greater proportion of the right side of the gonad/dg (Table 7-4). In contrast, a more uniform blue/yellow/green layer of testes appears to develop over the right side of the brown dg for males (Figure 7-1d, Table 7-4).

At BRIR (reference site), the largest size class of 2008 juveniles (6-7 mm) had distinguishable males and females as early as March 2009 (9-11 months old), but the majority of juveniles were indistinguishable (Table 7-4). The 2008 juveniles became morphologically distinct (adults) in August 2009 (13-15 months old), which is consistent with other observations of *Leptoxis spp*. (Hendrix, 1986; Miller-Way and Way, 1989). By October 2009 (15-17 months old) all females had developing oocytes, an egg-laying groove, and a cg, and almost all males were producing sperm (Table 7-4). The 2009 juveniles remained morphologically indistinct through May 2010 (10-12 months old).

Morphological analysis of snails from LOMR (impacted site) revealed development of 2008 juveniles similar to BRIR through July 2009 (12-14 months old). However, while snails from BRIR were all morphologically distinct by August and females were fully developed in October 2009, LOMR adults were not morphologically distinct or fully developed at this time (Table 7-4). This corresponds to the time period when *Leptoxis carinata* should be fully mature in order to overwinter and reproduce during the following spring (Aldridge 1982). At LOMR, females did not reach full development until May 2010 (22-24 months old), which corresponds to the typical egg-laying period for *L. carinata*. It is possible that delayed female development could negatively impact reproduction at this site.

Thus, morphological development of gonads of 2008 generation *L. carinata* at LOMR (impacted) was delayed relative to BRIR (reference). This is likely related to the observations of lower growth rates of *L. carinata* at LOMR relative to BRIR.

Histological observations – timing of gametogenesis

A total of 367 juvenile snails (249 from BRIR, 118 from LOMR) were included in histological evaluations using microscopy. For 2008 generation snails from BRIR (reference), 11 of 12 snails examined from the dominant size class (3-4 mm) collected during the first sampling period (March 2009; 8-10 months old) had undifferentiated acini (Figure 7-4a), but one tissue section from one snail had one acinus with a developing oocyte (example in Figure 7-4b). Within this collection period, the mean number of acini per snail and the mean number of acini with oocytes per female showed general increasing trends with increasing size (Figure 7-5). These trends were also evident in subsequent sampling periods (Figure 7-5). The proportion of differentiated females in the size classes with the majority of snails showed a general increasing trend between March 2009 (8-10 months old) and June 2009 (12-14 months old) (Table 7-5). Snails with undifferentiated acini were observed in size classes with the majority of snails until July 2009 (12-14 months old) (Table 7-5). Developing spermatocytes were first distinguishable in snails from the largest size class (6-7 mm) collected during March 2009 (8-10 months old), and were first distinguishable in snails from a smaller size class (5-6 mm) the following month (Figure 7-4c, Table 7-5). In July 2009, snails in the 2008 generation were easily distinguishable as males or females and were considered adults (12-14 months old).

For 2009 generation snails from BRIR (reference), 11 of 13 snails from the 4-5 mm size class collected in December 2009 (5-7 months old) had undifferentiated acini, and two histological sections of gonad tissue (two snails) had multiple acini with oocytes (Figure 7-5, Table 7-5). None of the snails from the smaller size class (3-4 mm) had differentiated acini (Figure 7-5, Table 7-5). A similar pattern was observed in February 2010 (7-9 months old). Compared to the 2008 generation, there were apparently fewer differentiated females observed in the 2009 generation during April and May 2010 (Table 7-5), these females had generally lower mean numbers of acini with oocytes and all snails had generally lower mean numbers of acini (Figure 7-5) . During June 2010, the proportion of differentiated females in the dominant size class of the 2009 generation (11-13 months old) increased and was similar to the proportion of differentiated females observed in June 2009 (Table 7-5). Spermatocytes also became distinguishable in June, but snails with undifferentiated acini were still observed (Table 7-5). The apparent difference in the rate of gametogenesis in snails at BRIR between 2009 and 2010 may have been due to differences in spring water temperatures between the two years (Figure 7-2). The rate of increase (slope) in temperature between March and June was higher during 2009 (4.47) compared to 2010 (2.88) (Figure 7-2). However, the slopes and the difference in adjusted mean temperatures during this period, were not statistically significant between years

(ANCOVA, p=0.25 and p=0.71, respectively). Alternatively, differences in the egg-laying period between 2008 and 2009 could have led to differences in acquired biomass (growth) prior to overwintering, as snail size (as indicated by SL) appears to be an important factor in the timing of gametogenesis in L. *carinata*. The timing of egg-laying by *L. carinata* is driven by water temperature (Aldridge 1982). Thus, gametogenesis in juvenile *L. carinata* may be affected by water temperatures during the either the egg-laying period or the following spring, or possibly, a combination of the two.

Although there were apparent differences in the timing of gametogenesis and number of oocytes observed in the gonads of developing females between years, the results of this study suggest that there is a delay in the onset of gametogenesis in *L. carinata* after hatching. This delay could potentially create a "window of opportunity" for contaminants to disrupt the process of sexual differentiation in these organisms, as observed in studies of the effects of estrogenic compounds on sexual differentiation in fishes (Fenske et al., 2005; Lange et al., 2009). The results of this study also demonstrate that sexual differentiation of *Leptoxis spp*. occurs earlier than the second summer of life, which was previously thought to be the period of sexual differentiation based on morphological observations alone (Hendrix, 1986; Miller-Way and Way, 1989).

Histological observations – comparisons between study sites

Similar to BRIR, oocytes were observed in both majority size classes of 2008 generation snails collected from LOMR (impacted) in March 2009 (Table 7-5). However, in both size classes the observed proportion of differentiated females was apparently greater than in the majority size classes collected from BRIR, and this pattern was present throughout the spring 2009 sampling period (Table 7-5). This may have been due to either the larger size of the snails relative to snails at BRIR or the apparently sustained female-biased sex ratios at this site. Similar to BRIR, the first distinguishable spermatocytes were observed in snails in the 6-7 mm size class collected from LOMR in March 2009. In July 2009, males and females were distinguishable using histology, and were considered adults (Table 7-5). Quantitative comparisons of histological sections of gonad tissue from juvenile snails at both sites showed that both the mean numbers of acini per snail and the mean numbers acini with of oocytes per female were generally lower at LOMR compared to BRIR (Table 7-6), however only differences in the mean numbers of acini per snail were statistically significant (paired t-test, p=0.038). The proportions of acini with oocytes in developing females was similar between LOMR and BRIR (Table 7-6, paired t-test, p=0.38).

A total of 197 adult snails (81 from LOMR, 116 from BRIR) were examined in order to assess differences in gamete production between study sites. When the mean numbers of acini containing oocytes per female were compared between identical size classes of adult snails collected from BRIR and LOMR, the numbers were significantly higher (paired t-test, $p=0.027$) at BRIR (Table 7-7). However, the proportion of acini with oocytes was statistically similar (paired t-test, $p=0.055$) when compared between sites (Table 7-7). The mean numbers of acini with sperm per male in identical size classes of adult snails were also significantly higher at BRIR (t-test, $p<0.0001$) compared to LOMR (Table 7-7). There were other differences between the histological sections of gonad tissue of adult snails collected from BRIR and LOMR during October and December 2009 that were observed but not quantified. The oocytes in females from LOMR appeared to be generally smaller than at BRIR, more digestive gland tissue was visible in females from LOMR, and the oocytes in females from LOMR appeared to be in a different developmental stage than in snails from BRIR (Figure 7-6). In males, the shape of the acini appeared more irregular at LOMR than at BRIR and the tails of the sperm appeared to differ in morphology (Figure 7-7).

Thus, although the general progression of gametogenesis was similar between BRIR and LOMR during 2009, snails at LOMR demonstrated reduced numbers of productive acini in males and females and apparent lower or slower production of vitellogenic oocytes compared to snails at BRIR. In addition, the morphology of male acini and sperm appeared different at LOMR compared to BRIR. There are numerous possible stressors that could impact developing snails in LOMR. Of 15 streams in the Shenandoah River watershed studied between 2008 and 2009, LOMR had the highest mean concentrations of dissolved inorganic nitrogen (6,600 µg/L), total suspended solids (174 mg/L), and estrogenic activity (3.4 ng/L) compared to other sites. The effects of estrogenic compounds on sexual development in fishes are well-documented (Fenske et al., 2005; Hahlbeck et al., 2004; Jobling et al., 1998), and there is some evidence that nitrate can also interact with the endocrine system of aquatic organisms (Guillette and Edwards, 2005). Snails at LOMR also had the highest measured tissue concentration of arsenic relative to other study sites in 2008 (10 µg/g; Chapter 3). Long Meadow Run is a spring-fed stream, with a small watershed (39.8 km^2) dominated by agricultural land use. Other stressors, such as agricultural pesticides, could also be present as their mobility in the karst topography of the Shenandoah River watershed has been previously documented (Ator et al., 1998). Both arsenic and agricultural herbicides may interact with the retinoic acid signaling system (Berube et al., 2005; Davey et al., 2008), which appears to play a role in regulation of sexual development in gastropods (Sternberg et al. 2008).

Conclusions

Although preliminary, results of this study suggest that *L. carinata* do not undergo sexual differentiation immediately after hatching, and the timing of the process appears to be dependent on size and possibly water temperature. Sexual differentiation does precede apparent morphological differences between the sexes. Although the exact timing of sexual differentiation will probably vary yearly and between geographic regions, confirmation of a delay in the process has important implications for potential effects of environmental conditions on the process and the design of laboratory studies to assess these effects.

Environmental conditions other than water temperature appear to have the potential to affect growth and gonad development in *L. carinata*, and may reduce the quantity of mature gametes produced. Although the mechanisms require further study, it appears plausible that environmental stressors, reduction in production of mature gametes, female-biased sex ratios, and reproductive failure are linked. These interrelationships are particularly relevant for pleurocerid snails, because many species are both understudied and critically imperiled.

Literature cited

Aldridge, D. W. (1982). Reproductive tactics in relation to life-cycle bioenergetics in three natural populations of the freshwater snail, *Leptoxis carinata*. *Ecology, 63*(1), 196-208.

Ator, S. W., Blomquist, J. D., Brakebill, J. W., Denis, J. M., Ferrari, M. J., Miller, C. V., et al., (1998). Water quality in the Potomac river basin, Maryland, Pennsylvania, Virginia, West Virginia, and the District of Columbia, 1992-1996. U.S. Geological Survey. http://www.water.usgs.gov/pubs/circ1166

Awaji, M. and K. Hamano. (2004). Gonad formation, sex differentiation and gonad maturation processes in artificially produced juveniles of the abalone, *Haliotis discus hannai*. *Aquaculture,* 239, 397-411.

Berube, V. E., Boily, M. H., DeBlois, C., Dassylva, N., & Spear, P. A. (2005). Plasma retinoid profile in bullfrogs, *Rana castesbeiana*, in relation to agricultural intensity of sub-watersheds in the Yamaska River drainage basin, Quebec, Canada. *Aquatic Toxicology, 71*, 109-120.

Castro, L. F. C., Lima, D., Machado, A., Melo, C., Hiromori, Y., Nishikawa, J. I., et al., (2007a). Imposex induction is mediated through the retinoid x receptor signalling pathway in the neogastropod *Nucella lapillus*. *Aquatic Toxicology, 85*, 57-66

Castro, L. F. C., Melo, C., Guillot, R., Mendes, I., Queiros, S., Lima, D., et al., (2007b). The estrogen receptor of the gastropod *Nucella lapillus*: Modulation following exposure to estrogenic effluent? *Aquatic Toxicology, 84*, 465-468..

Davey, J. C., Nomikos, A. P., Wungjiranirun, M., Sherman, J. R., Ingram, L., Batki, C., et al., (2008). Arsenic as an endocrine disruptor: Arsenic disrupts retinoic acid receptor- and thyroid hormone receptor-mediated gene regulation and thyroid hormone-mediated amphibian tail metamorphosis. *Environmental Health Perspectives, 116*, 165-172.

Duft, M., Schulte-Oehlmann, U., Tillmann, M., Weltje, L., & Oehlmann, J. (2003). Stimulated embryo production as a parameter of estrogenic exposure via sediments in the freshwater mudsnail *Potamopyrgus antipodarum*. *Aquatic Toxicology, 64*, 437-449.

Fenske, M., Maak, G., Schafers, C., & Segner, H. (2005). An environmentally relevant concentration of estrogen induces arrest of male gonad development in zebrafish, *Danio rerio*. *Environmental Toxicology and Chemistry, 24*(5), 1088-1098.

Gagne, F., Bouchard, B., Andre, C., Farcy, E., & Fournier, M. (2011). Evidence of feminization in wild *Elliptio complanata* mussels in the receiving waters downstream of a municipal effluent. *Comparative Biochemistry and Physiology, Part C, 153*, 99-106.

Guillette, L. J., & Edwards, T. M. (2005). Is nitrate an ecologically relevant endocrine disruptor in vertebrates? *Integrative and Comparative Biology, 45*, 19-27.

Hahlbeck, E., Griffiths, R., & Bengtsson, B.-E. (2004). The juvenile three-spined stickleback (*Gasterosteus aculeatus* l.) as a model organism for endocrine disruption i: Sexual differentiation. *Aquatic Toxicology, 70*, 287-310.

Hendrix, S. S. (1986). Population biology of the pleurocerid snail, *Leptoxis carinata* (Brug.) in Marsh Creek, Adams County, PA. *American Malacological Union Bulletin, 4*(1), 119.

Jobling, S., Casey, D., Rodgers-Gray, T., Oehlmann, J., Schulte-Oehlmann, U., Pawlowski, S., et al., (2003). Comparative responses of molluscs and fish to environmental estrogens and estrogenic effluent. *Aquatic Toxicology, 65*, 205-220.

Jobling, S., Nolan, M., Tyler, C. R., Brighty, G., & Sumpter, J. P. (1998). Widespread sexual disruption in wild fish. *Environmental Science & Technology, 32*, 2498-2506.

Lange, A., Paull, G. C., Coe, T. S., Yoshinao, K., Urushitani, H., Iguchi, T., et al., (2009). Sexual reprogramming and estrogenic sensitization in wild fish exposed to ethinylestradiol. *Environmental Science and Technology, 43*, 1219-1225.

Miller-Way, C. A., & Way, C. M. (1989). The life history of *Leptoxis dilatata* (Conrad) (Prosobranchia: Pleuroceridae) from the Laurel Fork River, West Virginia. *American Midland Naturalist, 122*, 193-198.

Milnes, M.R., Byran, J.A., Medina, J.G., Gunderson, M.P., & Guillette, L.J. (2005). Developmental alterations as a result of in ovo exposure to the pesticide *p,p'*-DDE in *Alligator mississippiensis*. *General and Comparative Endocrinology,* 144, 257-263

Nice, H. E., Morritt, D., Crane, M., & Thorndyke, M. (2003). Long-term and transgenerational effects of nonylphenol exposure at a key stage in the development of *Crassostrea gigas*. Possible endocrine disruption? *Marine Ecology Progress Series, 256*, 293-300.

Nishikawa, J. I., Mamiya, F., Kanayama, T., Nishikawa, T., Shiraishi, F., & Horiguchi, T. (2004). Involvement of the retinoid x receptor in the development of imposex caused by organotins in gastropods. *Environmental Science & Technology, 38*, 6271-6276.

Oehlmann, J., Schulte-Oehlmann, U., Tillmann, M., & Markert, B. (2000). Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part i: Bisphenol a and octylphenol as xeno-estrogens. *Ecotoxicology, 9*, 383-397.

Sternberg, R. M., Hotchkiss, A. K., & LeBlanc, G. A. (2008). Synchronized expression of retinoid x receptor mRNA with reproductive tract recrudescence in an imposex-susceptible mollusc. *Environmental Science & Technology, 42*, 1345-1351.

Voltzow, J. (1991). Gastropoda: Prosobranchia. In: Harrison, F. W. and A. J. Kohn, Eds. *Microscopic anatomy of invertebrates.* New York, Wiley-Liss. pp. 111-252.

Table 7- 1. **Watershed characteristics and observed population sex ratios of *L. carinata* (2008) at the two sampling sites.**

Name	Code	Area km^2	% Forest	% Pasture & Hay	% Crop	% Devel.	AFO/1,000 A*	% Female
Briery Branch	BRIR	128	82.5	13.4	1.9	2.4	1.26	48
Long Meadow Run	LOMR	39.8	19.3	69.1	5.1	6.5	4.17	87

*Density of animal feeding operations (AFOs), # per 1000 acres

Table 7- 2. Generations of snails present at each site over the course of the study (2009-2010). The life stages at which each generation was sampled are underlined.

Generation	Hatch	Juvenile Development	Reproduction/ Death
1	2007	2008	<u>2009</u>
2	2008	<u>2009</u>	<u>2010</u>
3	<u>2009</u>	<u>2010</u>	2011

Table 7- 3. Percentages of 2008 and 2009 generation snails in 1-mm size classes during select months at each site. Percentages of size classes for the 2007 generation (adults in spring 2009) are not shown.

	Briery Branch[1] (reference)					Long Meadow Run[2] (impacted)				
Size	Mar 2009	May 2009	Oct 2009	Dec. 2009	May 2010	Mar 2009	May 2009	Oct 2009	Dec 2009	May 2010
1-2 mm			*2.5*							
2-3 mm			*31.4*	*15.6*						
3-4 mm	48.8	12.1	*56.5*	*32.2*	*14.4*					
4-5 mm	8.3	15.0	*9.6*	*51.1*	*44.8*	4.0	1.2			
5-6 mm	16.8	36.3		*1.1*	*36.0*	33.8	7.7	1.9	4.3	
6-7 mm	17.5	19.2		2.4	*4.8*	52.6	49.3	11.2	19.4	
7-8 mm	8.6	17.4	9.4	4.1	4.9	9.6	41.2	54.0	61.3	7.8
8-9 mm			18.9	16.6	21.7			28.0	12.9	41.2
9-10 mm			31.3	36.7	34.3			4.3	2.2	45.1
10-11 mm			32.2	24.9	21.0			0.6		5.9
> 11 mm			8.1	15.4	18.2					
Total #	457	240	661	259	268	302	169	161	93	51

[1] Size classes of the 2009 generation are indicated in italics
[2] Snails failed to reproduce in 2009, only size classes of the 2008 generation are shown

Table 7- 4. Description of the external morphology of the gonad/digestive gland complex and accessory structures of 2008 generation snails in March 2009 (8-10 months old) and October 2009 (15-17 months old), and a description of 2009 generation snails (< 5 mm) from Briery Branch in October 2009 (3-5 months old).

Size	Briery Branch (BRIR; reference) Mar-09	Briery Branch (BRIR; reference) Oct-09	Long Meadow Run (LOMR; impacted) Mar-09	Long Meadow Run (LOMR; impacted) Oct-09
2-3 mm	-	90% brown dg. Few flecks.	-	-
3-4 mm	Predominantly brown dg, very thin blue layer developing over some parts (10%). No egg l. groove.	80-90% brown dg w/some blue and yellow flecks.	-	-
4-5 mm	Mostly brown dg. Some blue as "stripes" only. No egg l. groove.	Brown dg with 25% blue stripes or yellow flecks. No egg l. groove.	Almost all dg. Very thin blue stripes.	-
5-6 mm	Mostly brown dg with blue stripes or thin blue layer over dg. No egg l. groove.	-	Primarily dg, blue stripes just forming. No egg l. groove.	All immature. Gonad stripes 5-10% of dg.
6-7 mm	Partial devel. Trace of egg l. grove on F, blue stripes over dg. Poss. M with more uniform "coating".	-	Blue stripes just starting to form. Trace of egg l. groove in 2/60 snails	All immature. Stripes 10-25% compared to dg.
7-8 mm	½ partial devel. Thin blue layer over dg with no internal structure. Poss. F with stripes and trace of egg l. groove. Poss. M with blue coating over dg. ½ mature (2007 gen.)	Mostly mature. F: all have egg l. groove, developing oocytes, cover 50% of rt. side. Some M producing sperm, others with full layer/coating of testes on the right side of the dg.	½ mostly dg, blue stripes starting to form. Some F with trace of egg l. groove. ½ mature (2007 gen.)	Most undeveloped. No trace of egg l. groove on F? w/ 25% stripes vs. dg. Future M? with more of coating vs. stripes.
> 8 mm	All mature. (2007 generation)	All mature. F: egg l. groove, small ovipositor, thin white cg, developing oocytes 75-100% of right side. M: gonad 100% of right side, producing sperm.	All mature (2007 gen.) F: Oocytes defined, white cg, more dg visible than at Briery Br. M: Fully developed, more dg visible and testes "thinner" than at Briery Br.	F: Trace of egg groove, gonad 50% compared to dg. Developing oocytes in stripes, small cg. M: coating of gonad, sperm production in some. Coating either uniform or over 75% of right side.

dg=digestive gland
egg l. groove = egg laying groove (females)
cg = capsule gland (females)

Table 7- 5. Proportions of differentiated females in subsamples of snails from size classes collected during each sampling period in 2009 (March-July) and 2010 (Dec.-June). Differentiated females were determined from observations of oocytes in acini from histological sections of gonad tissue. The proportion of snails within each size class is indicated in parentheses.

	BRIR (reference)				LOMR (impacted)	
Month (age)	3-4 mm	4-6 mm	6-8 mm	>8 mm	4-6 mm	6-8 mm
Mar. (8-10 mo.)	**0.08**[u] **(49%)**	0.48[u] (25%)	0.38[s] (26%)		0.67[u] (38%)	**0.58**[u,s] **(62%)**
Apr. (9-11 mo.)	**0.17**[u] **(43%)**	0.6[u, s] (20%)	0.50[s] (37%)		0.50[u] (32%)	**0.75**[u,s] **(68%)**
May (10-12 mo.)		**0.30**[u, s] **(51%)**	0.90 (37%)			**0.78**[u,s] **(91%)**
June (11-13 mo.)		**0.40**[u] **(44%)**	0.60[u,s] (33%)			**0.88**[u,s] **(90%)**
July (12-14 mo.)			**0.60**[s] **(37%)**	0.50[s] (28%)		**0.78**[s] **(68%)**
Dec. (5-7 mo.)	**0**[u] **(32%)**[1]	0.15[u] (52%)				
Feb. (7-9 mo.)	**0**[u] **(69%)**	0.08[u] (31%)				
Apr. (9-11 mo.)	**0.08**[u] **(65%)**	0[u] (33%)				
May (10-11 mo.)		**0.17**[u] **(81%)**				
June (11-13 mo.)		**0.55**[u,s] **(65%)**	0.33[u,s] (35%)			

[u]Undifferentiated acini
[s] Spermatocytes
[1]15% of collected snails were <3 mm; winter collection may have led to discrepancy between dominant size classes in Dec. and Feb. 2010

Table 7- 6. Mean numbers of acini per snail, acini with oocytes per female snail, and proportions of acini with oocytes observed in thick sections of gonad tissue of 2008 generation snails from Briery Branch (BRIR; reference) and Long Meadow Run (LOMR; impacted) during each sampling period (all size classes).

	# of acini per snail		# of acini with oocytes per F		Proportion of acini w/oocytes	
Month (age)	BRIR	LOMR*	BRIR	LOMR	BRIR	LOMR
Mar (8-10 mo.)	13	6.5	5.2	4.3	0.72	0.71
Apr (9-11 mo.)	13	6.0	6.4	3.5	0.73	0.62
May (10-12 mo.)	14	9.8	6.2	6.0	0.61	0.68
Jun (11-13 mo.)	17	5.6	5.7	4.6	0.56	0.86
July (12-14 mo.)	30	5.9	9.5	4.8	0.83	0.91

*Significantly different from BRIR (paired t-test, p=0.038)

Table 7- 7. Mean proportions of acini (female) with oocytes, numbers of acini containing oocytes per female, and numbers of acini containing sperm per male in 5 µm sections of gonad tissue from different size classes of adult snails (2008 generation; >13 months old) collected from Briery Branch (reference) and Long Meadow Run (impacted). Numbers of acini were significantly higher at Briery Branch (paired t-test, $p \leq 0.027$) when compared to matching size classes at Long Meadow Run. The proportion of acini with oocytes was not significantly different (paired t-test, p=0.055) when compared between matching size classes at each site.

		Briery Branch (BRIR)			Long Meadow Run (LOMR)		
Month	Size	Proportion acini with oocytes	# acini with oocytes/ F	# acini with sperm/ M	Proportion acini with oocytes	# acini with oocytes/ F	# acini with sperm/ M
Aug.	6-7 mm	0.91	8.0	66	0.88	5.7	7.5
Aug.	7-8 mm	0.99	14	67	0.95	6.3	13
Aug.	8-9 mm	1.0	18	86			
Aug.	>9 mm	1.0	9.2	97			
Oct.	6-7 mm				0.88	8.4	6.7
Oct.	7-8 mm	0.98	10.3	82	0.97	6.1	22
Oct.	8-9 mm	1.0	20	89	0.99	8.3	-
Oct.	>9 mm	1.0	18	85			
Dec.	> 6 mm	1.0	22	96	1.0	6.5	18
Mean		0.99	15	84	0.95	7.0	14

Figure 7- 1. Morphological characteristics of gonad development in *Leptoxis carinata*. A) Fully developed females have a well developed egg-laying groove and ovipositor (ov) on the right side of the foot, a white capsule gland (cg), and oocytes (oo) on the right side of the gonad/digestive gland. B) Males have a fleshy pad (fp) on the right side of the foot and testes (te) fully covering the right side of the gonad/digestive gland. C) Gonad development begins as blue stripes (arrow) and yellow flecks over a brown digestive gland. In females, the oocytes develop within the stripes and gradually expand to cover the digestive gland. D) In males, a thin "coating" of testes develops over the right side of the digestive gland.

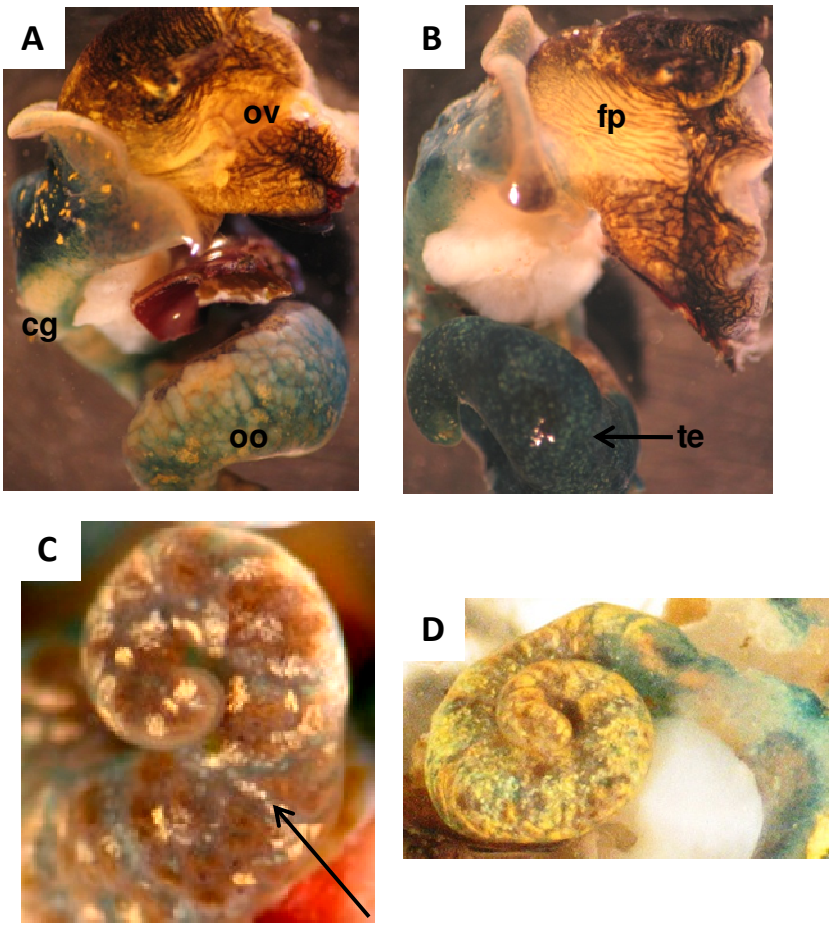


Figure 7- 2. Water temperature measured on each sampling date. Water temperature was not significantly different between the two sites (paired t-test, p=0.84).

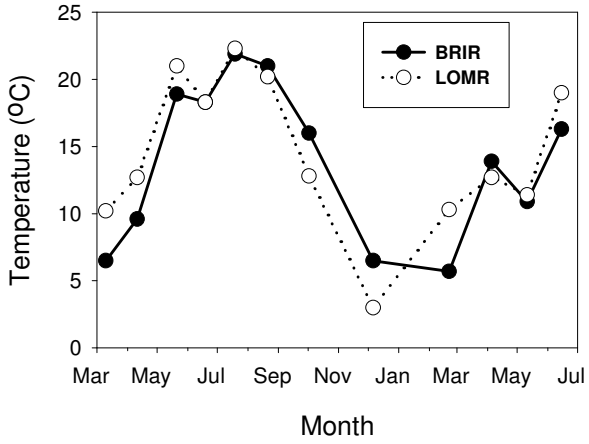


Figure 7- 3. Mean proportions of female snails at each site during the sampling period (n=11). Error bars represent standard deviation.

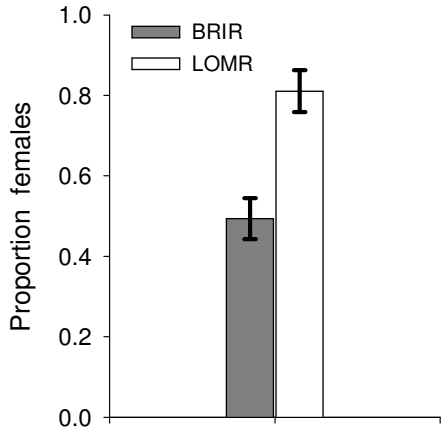


Figure 7- 4. Histological examination of gametogenesis in 2008 generation snails from Briery Branch (reference site). Scale bar represents 100 µm (20x). A) Undifferentiated acini (arrow) developing in connective tissue (stripes observed in external morphology) in a snail in the 3-4 mm size class collected in March 2009 (~8 months old). B) Developing oocytes (arrow) in acini within connective tissue in a snail in the 4-5 mm size class collected in April 2009 (~9 months old). C) Developing spermatocytes (arrow) in acini within connective tissue in a snail in the 5-6 mm size class collected in April 2009 (~9-10 months old).

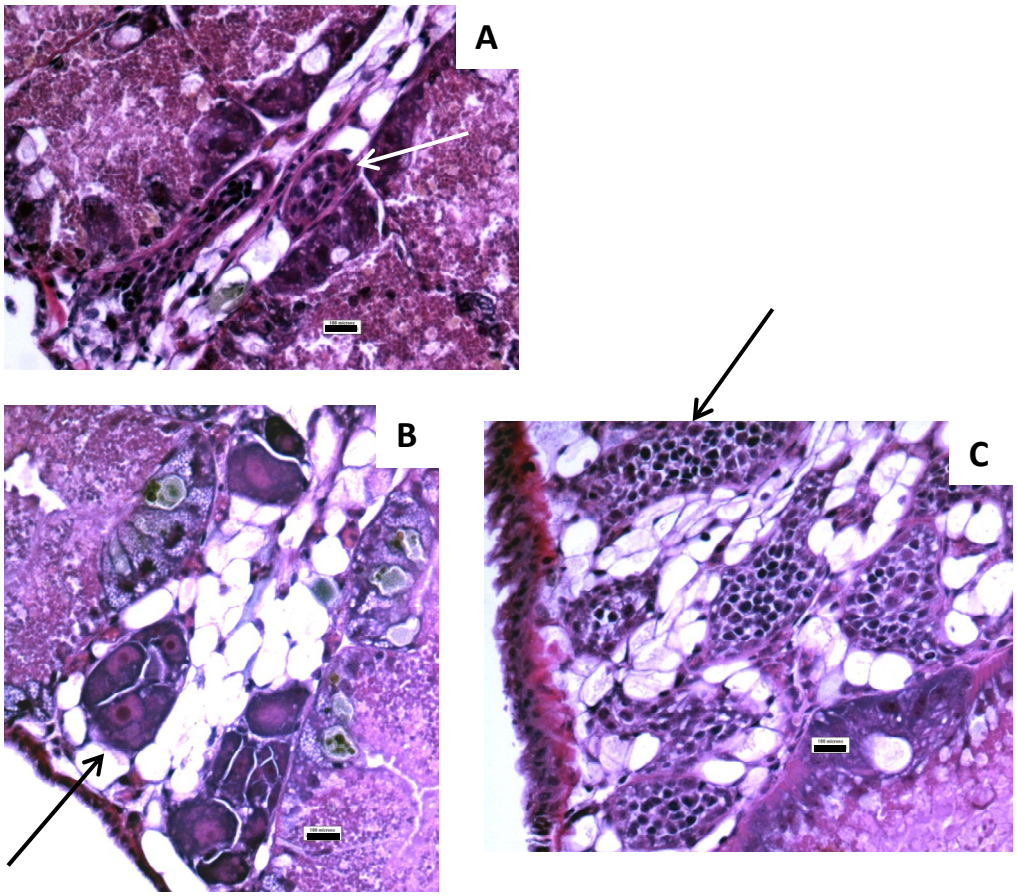


Figure 7- 5. **The mean number of acini per thick section of snail gonad (A, C) and the mean number of acini with oocytes per thick section of female gonad (B, D) for all snails in a size class collected during a particular month during either 2009 (A, B) or 2009-2010 (C, D). Both generations of *Leptoxis carinata* were collected from Briery Branch (reference site).**

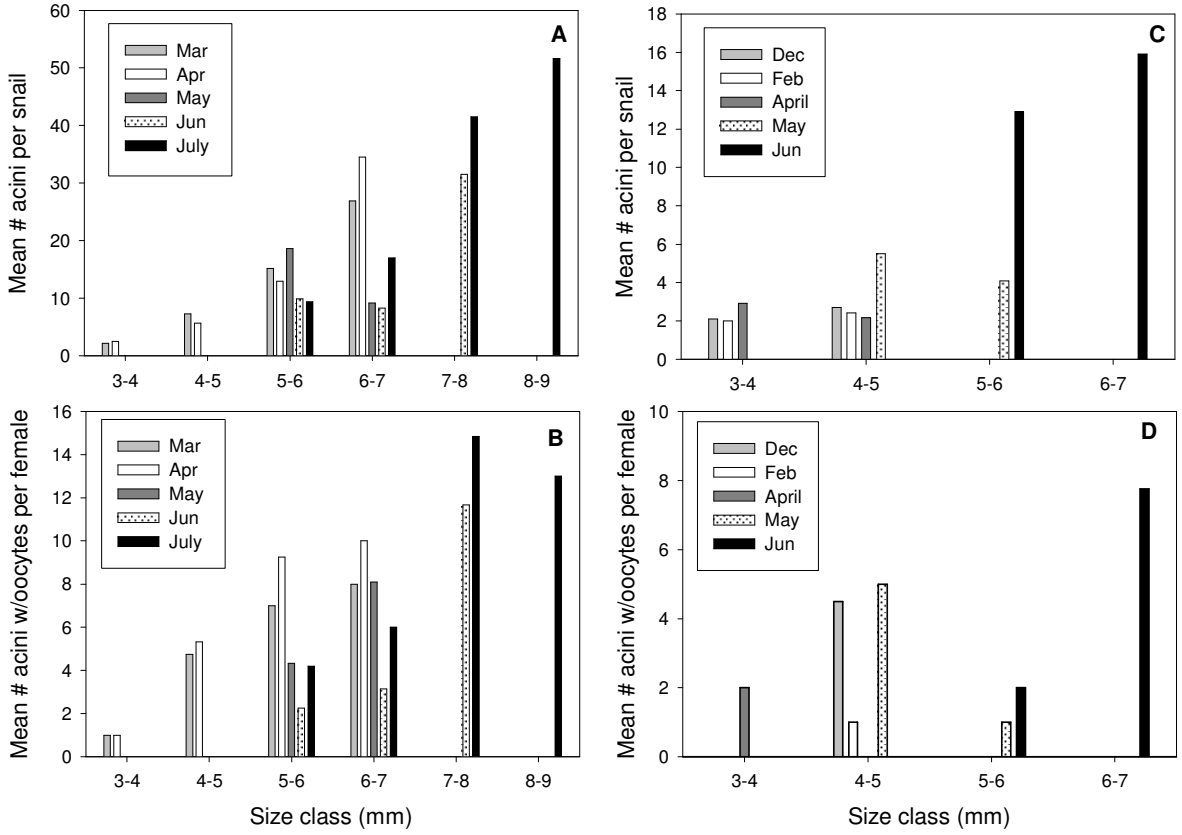


Figure 7- 6. Histological comparison of oocytes in 2008 generation female snails from Briery Branch (reference site; A, C) and Long Meadow Run (impacted site; B, D). Scale bars represent 100 µm, for observations at 20x (A, B) and 10x (C, D). Females in A and B were collected in October 2009 (15-17 months old) and were in the 7-8 mm size class. There was an apparent size difference in oocytes between Briery Branch (A) and Long Meadow Run (B). Females in C and D were collected in December 2009 (17-19 months old) and were in the 7-8 mm size class. Oocytes comprised 100% of the gonad in females from Briery Branch (C), but in females from Long Meadow Run (D), the digestive gland (dg) was visible and oocytes appeared to be in a different developmental stage.

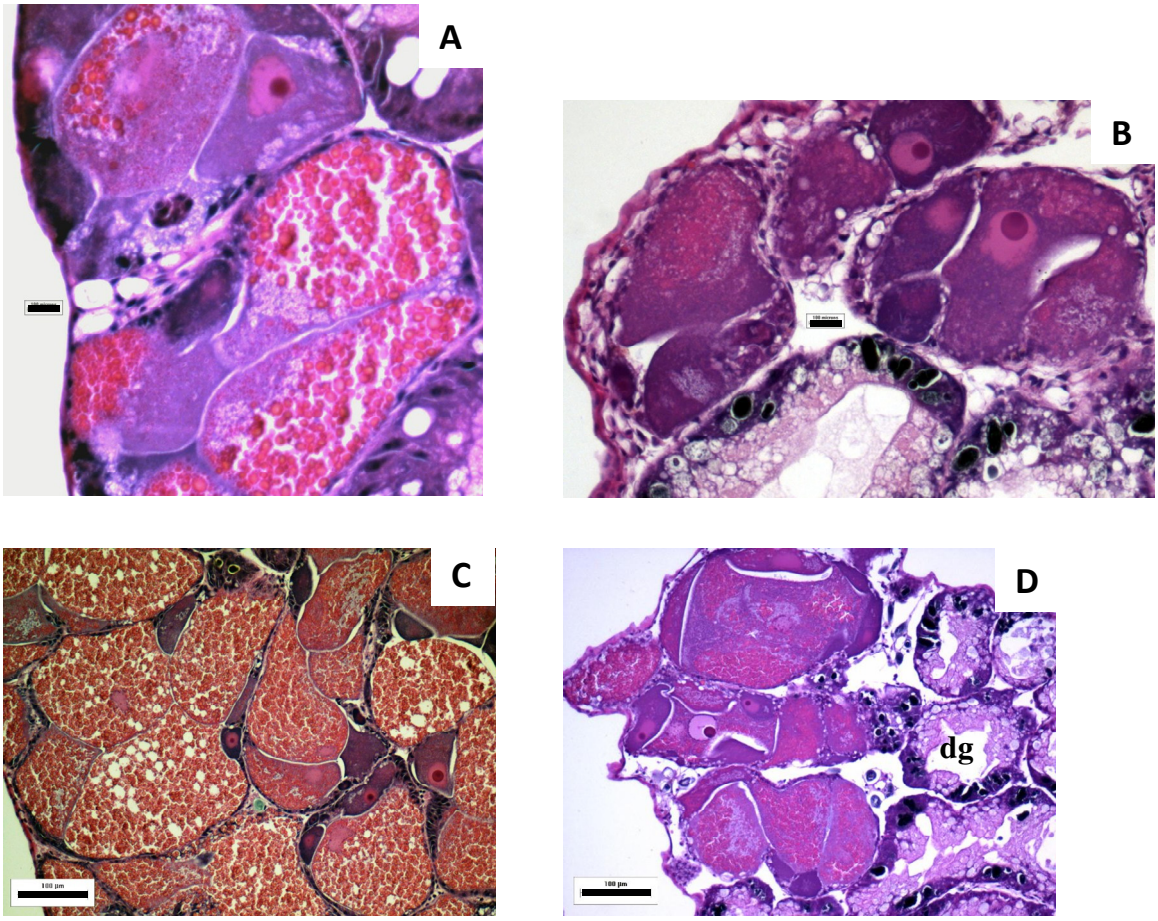
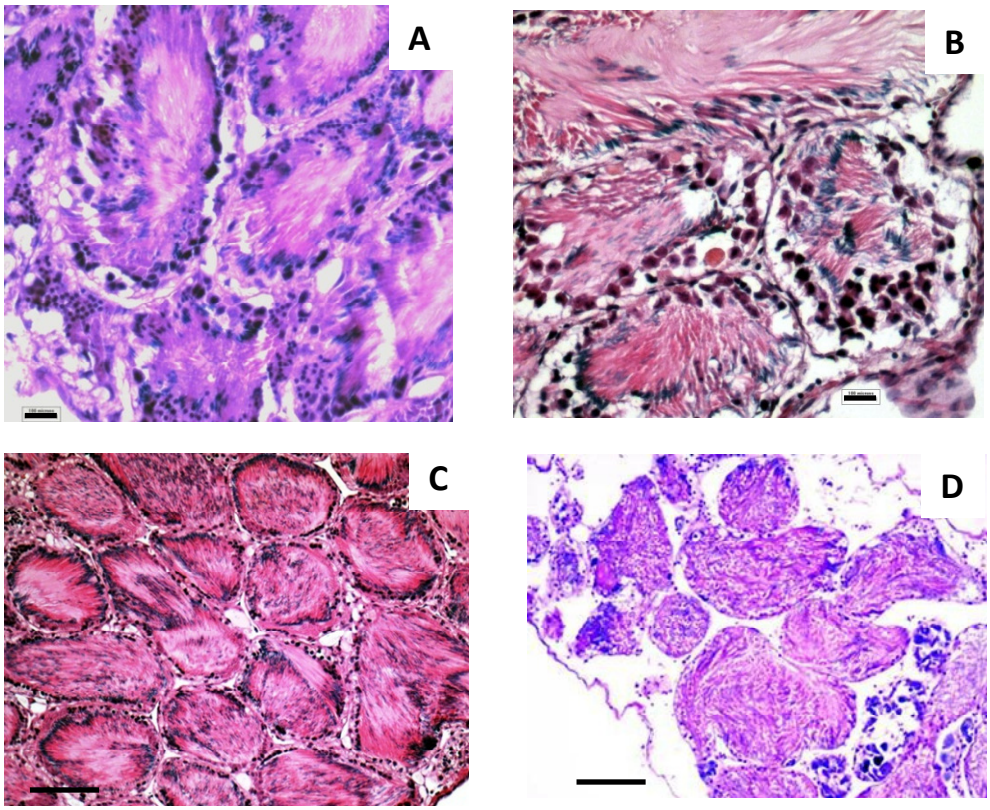


Figure 7- 7. Histological comparison of acini with sperm in 2008 generation male snails from Briery Branch (reference site; A, C) and Long Meadow Run (impacted site; B, D). Scale bars represent 100 µm, for observations at 20x (A, B) and 10x (C, D). Males in A and B were collected in October 2009 (15-17 months old) and were in the 7-8 mm size class. Males in C and D were collected in December 2009 (17-19 months old) and were in the 7-8 mm size class. Sperm from both sites were present in large acini, but the shape of the acini and appearance of the sperm was different at LOMR (B, D) compared to BRIR (A, C).



CHAPTER 8

Summary and conclusions

This study utilized a landscape-scale regression-based design to assess the effects of agricultural land use on aquatic environments in the Shenandoah River watershed. The first objective of this study was to determine whether increasing watershed densities of animal feeding operations (AFOs) resulted in increasing concentrations of contaminants in streams, due to suspected cumulative effects of application of manure from these operations on agricultural land as fertilizer. Densities of AFOs in watersheds of small streams were not directly related to concentrations of trace elements in sediment and tissue of resident mollusks, as evaluated in Chapter 3. The relatively high concentrations of arsenic in sediment and mollusk tissue suggest that sources other than AFOs, such as black shale and historic usage of lead arsenate, and the interactions between these sources and phosphate from manure applications, warrant further study. Given the potential for arsenic to interact with immune and endocrine system function at low concentrations, these studies are necessary to protect the health of aquatic organisms in the Shenandoah River watershed. Densities of AFOs in watersheds of small streams were directly related to concentrations of aqueous contaminants, including nutrients and estrogenic compounds, as evaluated in Chapter 4. The negative effects of excess nutrients in aquatic environments are well documented and eutrophication is a recognized problem in Chesapeake Bay, which is the ultimate receiving water body for the Shenandoah River. The high concentrations of dissolved inorganic nitrogen in Shenandoah River tributaries coupled with the apparently cumulative effect of watershed densities of AFOs on these concentrations, indicate that manure management strategies must be changed to address excess nitrogen. At this point, it is unclear whether estrogenic compounds should be considered in development of future manure management strategies. Future studies should focus on determining the component compounds and possible biological effects of concentrations observed in this study.

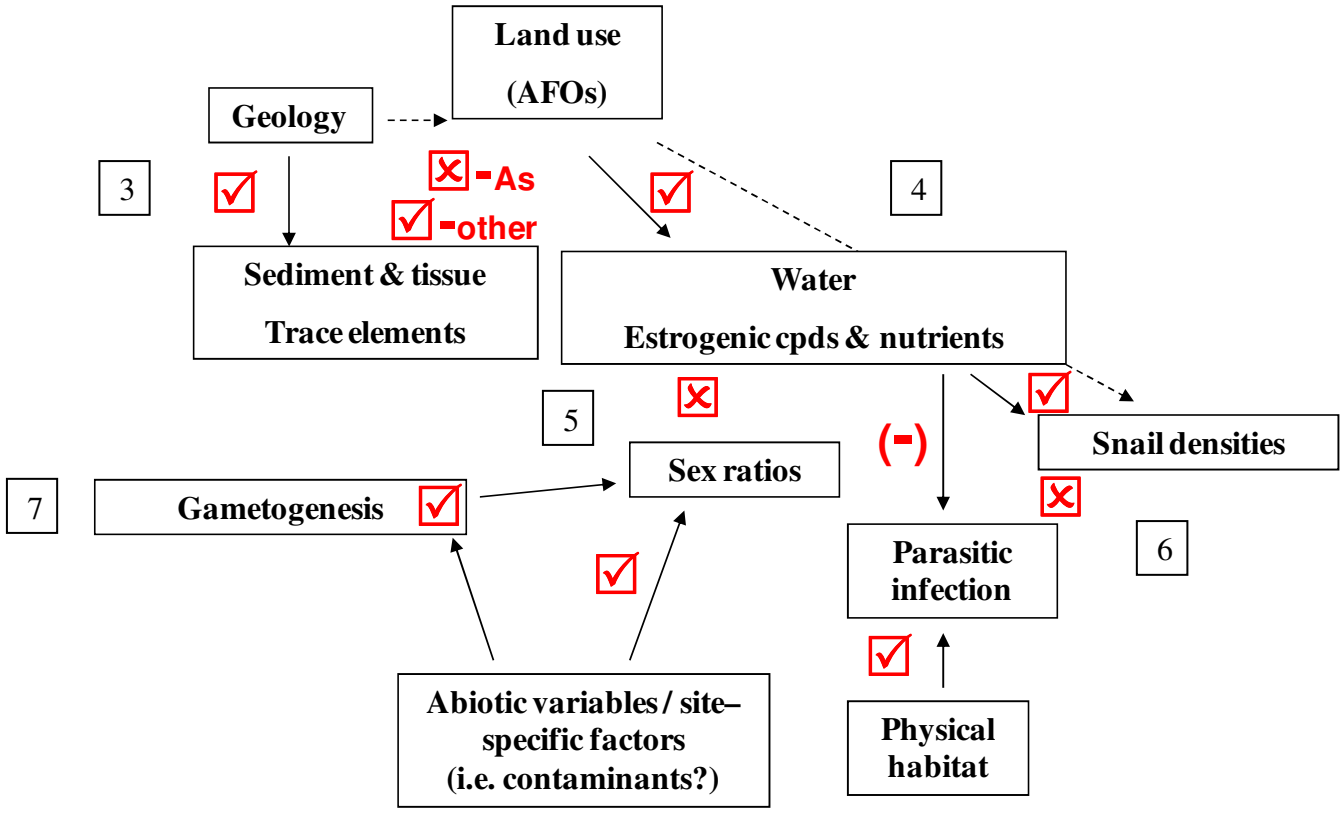
The second objective of this study was to evaluate whether population characteristics of the pleurocerid snail, *Leptoxis carinata*, were suitable indicators of biological effects related to landscape sources and/or measured concentrations of aqueous contaminants. Population sex ratios of *L. carinata* ranged from balanced to female-biased across study sites, but were unrelated to measured concentrations of estrogenic compounds or watershed densities of AFOs, as determined in Chapter 5. These results suggest that population sex ratios of *L. carinata* are not suitable indicators of effects of exposure to estrogenic compounds. However, the spatial variability of the population sex ratios and the lack of variability in site-specific population sex ratios across seasons and generations of *L. carinata*,

suggest that environmental conditions influence sexual development in this species. Population sex ratios may be an indicator of exposure to a specific class of contaminants or stressors other than estrogenic compounds, but further research on the mechanisms of sex determination and differentiation in this species is necessary before population sex ratios can be used in biomonitoring applications. Population densities of *L. carinata* were positively related to nutrient concentrations and landscape sources of nutrients, as determined in Chapter 6. Population densities of this species may be a suitable endpoint for monitoring the effects of eutrophication over a broad spatial scale. However, the incidence of parasitic infection in *L. carinata* populations does not appear to be a suitable indicator of the effects of eutrophic conditions on the incidence of disease in aquatic organisms, as digenetic trematode infection rates of populations were not positively related to nutrient concentrations or landscape sources of nutrients. Trematode parasites utilize multiple hosts to complete their life cycle and different relationships between the infection prevalence and physical and biological variables observed for different types of trematodes reflects the complexity of a multi-host system. This study was the first to examine trematode infection rates in *L. carinata* populations over a broad spatial scale. The identification of trematodes and probable secondary and definitive hosts will allow further use of *L. carinata* in investigations of trematode transmission dynamics in lotic systems.

The third objective of this study was to examine characteristics of individual *L. carinata* in an attempt to explain observed population-level responses and to evaluate these individual characteristics for potential applications in biomonitoring. This study was the first to evaluate the timing of gamete development in *L. carinata*, as described in Chapter 7. Results demonstrated that the delay (approximately eight months) between hatching and gametogenesis in *L. carinata* creates a "window of opportunity" for contaminants to disrupt sexual differentiation in this species and that controlled exposures to test this hypothesis need to occur within the first eight months after hatching. The production of gametes differed between a reference and an impacted site which supports the idea that gametogenesis in *L. carinata* is a viable individual-level endpoint for biomonitoring studies, although methods for quantification of differences between sites require further development. Immune function of *L. carinata* could also be a viable individual-level endpoint for biomonitoring studies, but standard phagocytosis assays do not appear to be suitable for this small species of snail, as described in Appendix A.

Figure 8-1 summarizes the results of this study in relation to the initial objectives presented in Chapter 1.

Figure 8- 1. Summary of results in relation to the objectives presented in the original conceptual model. Boxes with a check represent relationships that were in agreement with working hypotheses, boxes with an "x" represent no apparent relationship, and the negative sign represents a relationship opposite that of the working hypothesis. Numbers represent chapters of the dissertation.



APPENDIX A

Attempt to develop an immune function assay for *Leptoxis carinata*

Background

In mollusks, hemocytes circulate in the hemolymph (blood) and are the primary immune defense against foreign material. Hemocytes phagocytize microorganisms (e.g. bacteria) and encapsulate larger ones (e.g. trematodes). The generation of reactive oxygen intermediates (ROS) via respiratory burst is the primary killing mechanism of hemocytes (Bayne et al., 2001). Research suggests that the ability of trematodes to infect snails is a result of their ability to avoid detection by hemocytes, inhibit or alter the metabolism of hemocytes, detoxify the ROS response, or a combination of all three mechanisms (Bayne et al., 2001). Coevolution of trematodes and host snails has occurred. As a result, certain species of snails are only susceptible to specific species of trematodes. However, even under controlled laboratory conditions, infectivity of trematodes to susceptible snails is rarely 100% (Osnas and Lively, 2005). Therefore, environmental factors that modulate hemocyte function may increase the susceptibility of snails to trematode parasites. In addition, hemocyte function in itself may be a valuable measure of environmental stress. Hemocyte function is functionally homologous to the phagocytic immunocytes of the innate immune system of vertebrates (Galloway and Depledge, 2001) and conditions that suppress hemocyte function in invertebrates may also result in immune suppression in vertebrates inhabiting the same area.

Phagocytic activity is the most common measure of immune function in mollusks. Previous studies have shown that phagocytic activity of hemocytes in marine and freshwater bivalves (Blaise et al., 2002; Suave et al., 2002) and pond snails (Russo and Lagadic, 2004; Russo et al., 2007; Russo et al., 2008), can be significantly affected by a variety of contaminants. However, these relationships have not been studied in pleurocerid snails. The purpose of this study was to develop a phagocytosis assay to evaluate functional immune status of *Leptoxis carinata.* A successful assay would have been tested at a subset of sites in the Shenandoah River watershed, selected to represent a gradient of parasitism and stress (e.g. nutrients, sediment, estrogenic compounds), to determine if differences in phagocytic activity could be measured and related to variables representing either cause (stressors) or effect (parasitism). Unfortunately, assay development was not successful due to low yields of hemocytes. Therefore, the proposed methodology, actual methodology, and recommendations for future studies are presented below.

Proposed methodology

Hemolymph extraction and selection of a dilution medium

Extraction of hemolymph from freshwater pulmonate snails generally involves cardiac puncture (through the shell) or collection of hemolymph exuded through the hemal pore after stimulation of the head/foot or withdrawal into the shell (Bender et al., 2005; Humphries et al., 2008; Russo et al., 2007). These techniques were not viable for *L. carinata* due to their small size, thick shell, and the apparent lack of a hemal pore in Pleuroceridae. Iakovleva et al. (2006) obtained hemolymph from marine periwinkles (*Littorina littorea*) through puncture of the buccal sinus with a 25-gauge needle. This method was applied to *L. carinata* (28-gauge needle) and it was determined that approximately 1.5-3 µl of hemolymph could be obtained from individual snails. This was a 100-fold lower volume than can typically be obtained from one pond snail (150 µl; Russo et al., 2007). In addition, hemolymph from different individuals could not be pooled because it caused aggregation and cell death (personal observation).

Once hemolymph was successfully extracted, the first goal of this study was to select a dilution medium and determine an appropriate dilution volume. The proposed methodology included: placement of hemolymph on a sterile plastic dish, removal of 1-2 µl using a glass micropipette, and subsequent dilution in different media, adjusted to a similar osmolality as reported in other studies of freshwater mollusk hemolymph (85-100 mOsm; Ewald et al., 2009; Nascimento et al., 1982). The effects of different media on cell aggregation, cell viability, and hemocyte adherence were going to be tested by placing diluted hemolymph on a cover slip with a laser-etched 5 mm diameter centering ring and a 1 mm counting grid. After incubation for 1.5 hours and subsequent washing with the dilution medium, adhered cells were going to be counted using an inverted microscope. Cell viability was going to be assessed using a trypan blue exclusion test (Russo et al., 2008).

Phagocytosis assay

Determination of phagocytic activity generally involves cellular exposure to small particles such as bacteria (*E. coli*) or zymosan (from yeast) or followed by quantification of the number of particles ingested using microscopic observation (Iakovleva et al., 2006), or using flow cytometry and microplate readers if fluorescent particles are used (Blaise et al., 2002; Russo et al., 2007). Initially, quantitation of phagocytosis was going to be

conducted with an inverted microscope capable of fluorescence loosely following methods of Gorbushin and Iakovleva (2007), and would have included: a hemocyte adherence period of 1.5 hours, incubation with fluorescein-labeled particles, rinsing of hemocytes with dilution medium to remove non-ingested particles, and quenching of fluorescence by non-ingested cells using acidic trypan blue (Blaise et al., 2002). The phagocytic index was going to be determined as the percentage of hemocytes that ingested at least one particle. Both *E. coli* and zymosan particles would have been tested to determine if size difference facilitates determination of the phagocytic index. After a method for determining the phagocytic index was determined, serial dilutions of a phagocytosis inhibitor such as sodium azide (Suave et al., 2002) were going to be used to determine if a dose-response relationship existed. Next, development of a microplate-based assay was going to be attempted, following the methods of Blaise et al. (2002), but with modifications to accommodate smaller well sizes (384 well plate vs. 96 well plate).

Methodology

Assay development was attempted at the U.S. Geological Survey National Fish Health Laboratory (Kearneysville, WV). Adult Leptoxis carinata (7-9 mm) were collected by hand from the Opequon River (39°22'01.35" N, 77°57'29.81 W). Snails were collected daily from 12-16 July 2010 and were returned to the laboratory in site water. Asian clams (*Corbicula fluminea*) were also collected as positive controls in assessment of phagocytic activity of *L. carinata* hemocytes.

Hemolymph extraction and selection of a dilution medium

Several media were prepared as candidates for dilution of hemolymph, including: RPMI-1640, phosphate buffered saline (PBS), Hank's Balanced Salt Solution (HBSS), OptiMem®, and Liebovitz's L-15. These media were diluted to 36% with sterile water to achieve an osmolality range of 96-109 mOsm, as determined using an osmometer (Precision Systems Micro-Osmette, Natick, MA).

Prior to hemolymph extraction, *L. carinata* were blotted dry and their shells were cracked with vice grip pliers. A 28-gauge sterile syringe was inserted into the buccal sinus through the dorsal surface of the "neck" after withdrawing the leading edge of the mantle. The repeatability of this method for hemolymph extraction was tested on 150 snails. A usable volume of hemolymph (1-2 µl) was obtained from less than 15 snails. From the majority of

snails, no hemolymph was extracted. In some cases, hemolymph was visible in the syringe, but the volume was not great enough to form a bead at the end of the needle. Rinsing the syringe with dilution media was unsuccessful. The low repeatability of this method rendered it unsuitable for assay development. Therefore, a number of other methods were also attempted.

Ewald et al., (2009) obtained hemolymph from another species of pleurocerid snail, *Elimia flava*, by making an incision on the foot ventral to the operculum. This method was attempted for *L. carinata*, but was unsuccessful. This is likely due to the small size of *L. carinata*, as Ewald et al. (2009) noted difficulty in extracting hemolymph from smaller *E. flava* and limited their study to only large snails.

Eyambe et al. (1991) developed a method for non-invasive extraction of earthworm leukocytes that involved immersing the worms in a solution of ethanol, saline, EDTA, and the mucolytic agent guaiacol glyceryl ether. A similar solution was created for *L. carinata*, including: 5 ml of 100% ethanol, 10 ml EDTA, 25 ml sterile water, 60 ml of 36% HBSS, and 1,000 mg of guaiacol glyceryl ether (solution # 1). Eight snails were blotted dry and removed from their shells as previously described and placed in 0.3 ml of solution # 1 in 8-well chamber slides (one snail per well) for 15 min. Upon removal of the snails and examination of the slides, it was determined that *L. carinata* released a large number of "round cells". It was unclear if these cells were algae or hemocytes. In order for hemocytes to adhere to surfaces, they need to be amebocytes, or "spreading" cells. It was thought that the very high osmolality of solution #1 (988 mOsm) could have negatively affected any extracted hemocytes.

Next, a dilute solution of 5 mg/ml $MgCl_2$, 0.5 mg/ml guaiacol glyceryl ether, and 0.5% ethanol was prepared (solution # 2). Snails were placed in this solution in chamber slides as described above for 15 min. After removal of the snails and examination of the slides, it was determined that *L. carinata* released substantial numbers of amebocytes. A number of other cell types were also present in solution. All of the wells contained diatoms and general detritus and one well contained trematode miracidia (*Notocotylus spp*.).

Phagocytic activity

Due to the apparent success of solution # 2 in hemocyte extraction, and potential to replace dilution media, the phagocytic activity of the extracted cells was tested following the initial steps of the microplate method developed by Blaise et al. (2002). Ten snails were removed from their shells as previously described and were

placed directly into wells of a 96-well sterile clear-bottomed black polystyrene microplate (Costar, Corning, Inc., Corning, NY) and immersed in 0.2 ml of solution # 2. Snails were left in the solution for 15 min., removed, and hemocytes in the solution were allowed to adhere to the plate for 1.5 hours. Asian clam hemolymph (100 µl), extracted by insertion of a 28-gauge syringe into the adductor muscle, was placed in an adjacent well for 1.5 hours as a positive control.

After 1.5 hours, solution # 2 (or clam hemolymph) was removed and each well was gently washed three times with 36% HBSS. Fluorescein-labeled *E. coli* K-12 BioParticles (Molecular Probes, Eugene, OR) were prepared according to the manufacturer's instructions and 20 µl of prepared solution was added to each well in 100 µl of HBSS. The solution was incubated at 20^{o}C for 1 hour to allow phagocytosis to occur. After one hour, the solution was removed, wells were rinsed once with 36% HBSS, and 5% acidic trypan blue was added to each well to quench fluorescence from uningested particles. After prompt removal of trypan blue, wells were examined using an inverted microscope with both white and fluorescent light. The bottom of the well that received clam hemolymph was covered with hemocytes and almost all of them had phagocytized multiple particles. In contrast, only a few of the wells that received snails had any fluorescent activity, and this was due to one or two hemocytes. It was concluded that hemocytes extracted from the snails had not adhered to the plates.

The process of adding snails directly to microplate wells was repeated with two modifications: wells with snails and HBSS only were added and for all wells, solutions were removed, but wells were not washed in between steps. These modifications produced the same results as the original attempts – hemocytes failed to adhere to the wells.

Two sets of final attempts at developing a method for extracting viable hemocytes were conducted. Four snails were removed from their shells as previously described and were placed in sterile microcentrifuge tubes with either 0.2 ml of solution # 2 or 0.2 ml of HBSS for 15 min (eight snails total). Two sets of eight snails were prepared. Four tubes containing 50 µl of clam hemolymph and 50 µl HBSS were prepared as positive controls.

For one full set of tubes (eight snails, 2 clams), solutions containing extracted hemolymph and media were transferred to wells (50 µl per well) within a 384-well, white clear-bottomed polystyrene microplate (Costar, Corning, Inc., Corning, NY). The plates were centrifuged at 50 RCF for 10 min. and incubated at 20^{o}C for 1.5 hours to allow cells to adhere to the wells. The media and hemolymph solutions were removed by quickly inverting the plate, and 50 µl of diluted *E. coli* solution (50 µl of prepared solution in 200 µl of HBSS) was added to each well.

The *E coli*. solution remained in the wells for 45 min. (at 20°C) to allow for phagocytosis. The solution was removed by inversion, 20µl of acidic trypan blue was added to the wells to quench extraneous fluorescence, and was promptly removed by inversion of the plate. When the plates were examined, there was noticeable material adhered to the wells that had received extracted snail hemolymph, but there was little fluorescent activity and it appeared as if most of the adhered material was detritus. In contrast, wells that received clam hemolymph were coated in hemocytes that had phagocytized numerous particles.

For the second set of tubes (eight snails, 2 clams), solutions were left in the tubes and 200 µl of diluted *E. coli* solution was added and incubated for 45 min. The tubes were then centrifuged at 50 RCF for 10 min, the solutions were removed, 200 µl of HBSS was added, and the cycle was repeated two more times. After the final addition of HBSS, the solution was transferred to wells (50 µl per well) within a 384-well, white clear-bottomed polystyrene microplate (Costar, Corning, Inc., Corning, NY). The plates were centrifuged at 50 RCF for 10 min., the HBSS was removed by inversion, and acidic trypan blue (20 µl) was added to the wells and promptly removed. Upon examination of the wells, the same pattern was observed as for the hemolymph solutions added directly to the plates, the adhered material was primarily detritus.

Recommendations for future studies

In summary, all attempted methods of extraction of hemolymph from *L. carinata* failed to reliably produce viable hemocytes. The small size of *L. carinata*, coupled with the inability to "clean" live organisms, may preclude direct measurements of phagocytic activity in this species. However, it may be possible to assess the effect of contaminant exposure on parasite susceptibility through other methods. Snails exposed to contaminants of interest could be evaluated histologically for potential differences in hemocyte activity in major hemocoels like the buccal sinus, compared to unexposed snails. Expression of relevant physiological and immunological genes could also potentially be compared between exposed and unexposed snails. Alternatively, snails exposed to contaminants of interest could be subsequently exposed to trematode miracidia and infection rates compared to unexposed snails as a measure of susceptibility.

Literature cited

Bayne, C. J., Hahn, U. K., & Bender, R. C. (2001). Mechanisms of molluscan host resistance and of parasite strategies for survival. *Parasitology, 123*, S159-S167.

Bender, R. C., Broderick, E. J., Goodall, C. P., & Bayne, C. J. (2005). Respiratory burst of *Biomphalaria glabrata* hemocytes: *Schistosoma mansoni*-resistant snails produce more extracellular H_2O_2 than susceptible snails. *Journal of Parasitology, 91*, 275-279.

Blaise, C., Trottier, S., Gagne, F., Lallement, C., & Hansen, P. D. (2002). Immunocompetence of bivalve hemocytes as evaluated by a miniature phagocytosis assay. *Environmental Toxicology, 17*, 160-169.

Ewald, M. L., Feminella, J. W., Lenertz, K. K., & Henry, R. P. (2009). Acute physiological responses of the freshwater snail *Elimia flava* (Mollusca: Pleuroceridae) to environmental pH and calcium. *Comparative Biochemistry and Physiology, Part C, 150*, 237-245.

Eyambe, G. S., Goven, A. J., Fitzpatrick, L. C., Venables, B. J., & Cooper, E. L. (1991). A non-invasive technique for sequential collection of earthworm (*Lumbriculus terrestris)* leukocytes during subchronic immonotoxicity studies. *Laboratory Animals, 25*, 61-67.

Galloway, T. S., & Depledge, M. H. (2001). Immunotoxicity in invertebrates: Measurement and ecotoxicological relevance. *Ecotoxicology, 10*, 5-23.

Gorbushin, A. M., & Iakovleva, N. V. (2007). Functional characterization of *Littorina littorea* (Gastropoda: Prosobranchia) blood cells. *Journal of the Marine Biological Association of the United Kingdom, 87*, 741-746.

Humphries, J. E., & Yoshino, T. P. (2008). Regulation of hydrogen peroxide release in circulating hemocytes of the planorbid snail *Biomphalaria glabrata*. *Developmental & Comparative Immunology, 32*, 554-562.

Iakovleva, N. V., Shaposhnikova, T. G., & Gorbushin, A. M. (2006). Rediae of echinostomatid and heterophyid trematodes suppress phagocytosis of haemocytes in *Littorina littorea* (Gastropoda: Prosobranchia). *Experimental Parasitology, 113*, 24-29.

Nasciemento, M. C. S., Daniel, J. P., & Heneine, I. F. (1982). The hemoglobin of the snail *Biomphalaria glabrata*. The absence of sulfhydryl groups (SH) the presence of disulfide bonds (SS), and their relation to ligand properties. *Comparative Biochemistry and Physiology, Part B: Biochemistry and Molecular Biology, 73B*, 251-256.

Osnas, E. E., & Lively, C. M. (2005). Immune response to sympatric and allopatric parasites in a snail-trematode interaction. *Frontiers in Zoology, 2*, 8.

Russo, J., & Lagadic, L. (2004). Effects of environmental concentrations of atrazine on hemocyte density and phagocytic activity in the pond snail *Lymnaea stagnalis* (Gastropoda, Pulmonata). *Environmental Pollution, 127*, 303-311.

Russo, J., Lefeuvre-Orfila, L., & Lagadic, L. (2000). Hemocyte-specific responses to the peroxidizing herbicide formesafen in the pond snail L*ymnaea stagnalis* (Gastropoda, Pulmonata). *Environmental Pollution, 146*, 420-427.

Russo, J., Madec, L., & Brehelin, M. (2008). Effects of a toxicant on phagocytosis pathways in the freshwater snail, *Lymnaea stagnalis*. *Cell and Tissue Research, 333*, 147-158.

Suave, S., Brousseau, P., Pellerin, J., Morin, Y., Senecal, L., Goudreau, P., et al., (2002). Phagocytic activity of marine and freshwater bivalves: In vitro exposure of hemocytes to metals (Ag, Cd, Hg, and Zn). *Aquatic Toxicology, 58*, 189-200.