8-2007

A Statewide Biomarker Approach to Estimate Contaminant Effects in the Wadeable Streams of South Carolina

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A STATEWIDE BIOMARKER APPROACH TO ESTIMATE CONTAMINANT EFFECTS IN THE WADEABLE STREAMS OF SOUTH CAROLINA

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Environmental Toxicology

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by
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August 2007

Accepted by:
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ABSTRACT

Freshwater species in South Carolina have been declining for many years. However, the threats that these organisms face from point and non-point source pollution are largely unknown. The South Carolina Department of Natural Resources (SCDNR) began a five year survey of the wadeable streams of South Carolina in May 2006. One of the purposes of this study was to use molecular biomarkers of contaminant exposure to fully assess the health of fish in South Carolina's freshwater streams. During the first year of the study, sunfish (*Lepomis* sp.) were sampled from May through September 2006 at randomly selected sites in three ecobasins in South Carolina (the Saluda Sandhills, the Savannah Sandhills, and the Pee Dee Atlantic Southern Loam Plains). Somatic indices, including hepatosomatic index (HSI), spleen somatic index (SSI), gonadosomatic index (GSI), and Fulton's condition factor (K) were measured to determine the overall bodily condition of the fish. Cytochrome p4501A induction (as measured by the EROD assay) and bile fluorescence were measured to estimate exposure to polycyclic aromatic hydrocarbons (PAHs). Glutathione-s-transferase (GST) activity was measured to estimate oxidative stress. Additionally, measurements of land use and fish assemblage structure were used to determine relationships between land use and biomarker measurements. Results indicate that the HSI, SSI, and GSI were influenced by several factors, including sex, season, and the presence of parasitic infection. Though both EROD and GST were not induced beyond basal levels for the fish sampled, bile fluorescence was significantly elevated at fish from specific sites within the Saluda Sandhills and Pee Dee Atlantic Southern Loam Plains, indicating possible transient exposure to and metabolism of PAHs

at these sites. Fish from these ecobasins also had higher EROD activities, bile fluorescence, and HSIs than the Savannah Sandhills, again suggesting possible exposure to higher levels of PAHs than the Savannah Sandhills. Additionally, bile fluorescence and HSI were significantly correlated with the percentage of impervious surface cover within the watershed area at sites, demonstrating a possible link between PAH exposure and land use. This study provided a first estimate of the health of fish in specific ecobasins of South Carolina as part of a five year survey of the wadeable streams of South Carolina.

DEDICATION

I dedicate this thesis to my parents, Doug and Elizabeth Keaton. Without their encouragement, love, and support, I would not have been able to complete this work. I thank them for listening to my complaints and reassuring me when I was worried. I could not have finished Clemson without them.

ACKNOWLEDGMENTS

I would first like to thank my advisor, Dr. Peter van den Hurk. I am very thankful for his help, guidance, and patience throughout the past two years. I would also like to thank my committee members, Dr. Stephen Klaine and Dr. Charles Rice for all of their guidance, assistance and help with my project. I would also like to thank everyone at the Clemson Institute of Environmental Toxicology and Dr. Sandra Gray and Jane Owenby at the Clemson endocrine lab for all of their support as well. I thank Dr. Dennis Haney at Furman University for encouraging me to pursue environmental research as an undergraduate.

I would like to thank the entire Stream Team (Kevin Kubach, Troy Cribb, Cathy Marion, Bill Poly, Alan Jones, Drew Gelder, and Brandon Seda) that has worked with me on this project. I apologize for all of my complaints during the past year and could not have finished this project without their help. I would like to specifically acknowledge Kevin Kubach, Cathy Marion, and Bill Poly for providing me with information and resources that greatly helped me in writing this thesis.

I would like to thank Kristen Gaworecki for all of her assistance and help during my time at Clemson. I could not have finished this thesis without her advice and suggestions. I would also like to thank Erika Schreiber for her help and advice. I thank Pam Truman, Annie Bowling, and Becca Haynie for providing me with support and friendship during the past two years.

Finally, I would like to thank the South Carolina Water Resources Center for funding this work. Without this funding, this research would not have been possible.

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LITERATURE REVIEW AND INTRODUCTION

State of South Carolina and Project Overview

The state of South Carolina comprises 32,965 km^2 and is the 40^{th} state in the nation in terms of land area. Despite its small size, South Carolina contains over 11,000 miles of streams divided into seven river basins (Savannah, ACE [Ashepoo-Combahee-Edisto], Saluda, Broad, Catawba/Wateree, Congaree/Lower Santee, and Pee Dee) (SCDNR 2006). These river basins can be further subdivided according to the ecoregions that they intersect. Ecoregions are areas of land that are similar in the types of environmental resources that they contain (Griffith 2002). The United States Environmental Protection Agency (EPA) further subdivides ecoregions into smaller, more manageable subdivisions called Level IV ecoregions, of which South Carolina has seven (Blue Ridge, Inner Piedmont, Outer Piedmont, Slate Belt, Sand Hills, Atlantic Southern Loam Plains, and Carolina Flatwoods). The resulting combination of Level IV ecoregions and river basins results in 30 units within South Carolina called ecobasins (SCDNR 2006). The vastly different physical characteristics of the state's ecobasins enable South Carolina to support an enormous variety of aquatic life.

Aquatic species in South Carolina are continually threatened by stressors from anthropogenic sources. South Carolina is currently home to over 4.5 million people living in 1.5 million households, and the population of the state is ever expanding, increasing by 6.1% from 2000 to 2005 (U.S. Census 2000). Land is continually being developed to meet the needs of an expanding population. This results in an increase in houses,

businesses, and roads in developed areas. Unfortunately, this necessary development results in an increase the percentage of impervious surfaces and amount of runoff from urban areas (van Metre et al. 2003). Accordingly, non-point source pollution from runoff is now the leading cause of water pollution in South Carolina (SCDHEC 2006). Most pollution results from bacteria, fertilizers from agricultural uses, nutrient loading, chemicals from industrial and personal use, and oils from transportation (SCDHEC 2006). The South Carolina Department of Natural Resources (2006) contends that these sources are widespread, numerous, and largely unknown, making these threats difficult to control. A survey performed by the South Carolina Department of Health and Environmental Control (2006) has found that while 65.1% of streams in South Carolina are fully supportive of aquatic life, approximately 10 to 15% of surface waters assessed are impaired by toxicants. Additionally, 17 to 22% of fish kills in 2004 and 2005 occurred because of unnatural causes such as chemical spills, runoff, and herbicide spraying (SCDHEC 2006).

No studies have been performed to determine the overall condition of streams in South Carolina. In 2006 the South Carolina Department of Natural Resources began a state-wide, ecobasin-based survey of wadeable streams in South Carolina. The project is the first to use both abiotic measurements of land use, water and sediment chemistry, and biotic measurements of fish assemblage structure and fish health to assess the overall community structure of fish in South Carolina. This study will determine the exposure of fish to various environmental toxicants using biomarker techniques and will provide a valuable assessment of the physical health of fish in South Carolina streams.

Biomarkers

Aquatic systems have long been the ultimate sink for chemicals and compounds such as metals, pesticides, polycyclic aromatic hydrocarbons (PAHs), pharmaceuticals, and personal care products (McCarthy and Shuggart 1990). As a result, researchers are faced with the question of how to assess the bioavailability of these contaminants to aquatic organisms and also determine the effects that these chemicals have once absorbed into the animal. The field of biomarkers has rapidly developed as a relatively quick, cost-effective alternative to abiotic environmental chemical analyses and is a valuable tool used to estimate the physical health of an organism.

Descriptions of the term "biomarker" have been formulated in many publications. Generally, a biomarker is a change in a cellular or biochemical structure, function, or behavior that is measurable in an organism or part of an organism such as organs, feces, urine or bile (Hansen 2003, van der Oost et al. 2003). These measurements are used to evaluate a change from basal levels that can be attributed to exposure to a certain set of conditions. Biomarkers can be used to assess exposure to chemical, physical, or biological stressors (van der Oost et al. 2003).

The National Research Council (1989) has broadly classified biomarkers into three subcategories: biomarkers of exposure, effect, or susceptibility. A biomarker of exposure is a chemical, xenobiotic, or metabolite that is measured within an organism (NRC 1989). It can also be a product of an interaction between a chemical and a biological target. These biomarkers reveal that a toxicant is present and bioavailable in the system of interest, but do not necessarily indicate that biological harm is occurring

inside the organism. A biomarker of effect is a physiological or biological parameter that is used as a sign that there is a possible health impairment occurring because of exposure to a toxicant (NRC 1989). Biomarkers of effect provide evidence that a toxicant of interest has had some kind of impact on the health of the organism. Lastly, a biomarker of susceptibility is a biomarker that measures the ability of an organism to respond to a change in exposure to an agent (NRC 1989). Many studies integrate a combination of biomarkers to fully assess the impact of a chemical or chemicals in an environmental system.

Biomarkers have been widely used in both laboratory and field settings to determine the biological impact of contaminants. Most biomarkers utilize the liver or kidney in assays, as these organs typically have high blood flow and detoxification capacities, resulting in increased interaction with the toxicant (Pritchard 1993). However, biomarker measurements are not limited to these organs and have been made in most bodily tissues. Among the biomarkers that can be measured are biotransformation enzymes and their products, activities of oxidative stress enzymes, hematological and immunological parameters, reproductive and endocrine parameters, genotoxic measurements such as DNA adducts and DNA damage, and gross morphological, physiological, or histological estimations (van der Oost 2003). Each exposure situation will require a unique suite of biomarkers for appropriate assessment.

Biomarkers have proven to be a beneficial advance in the monitoring of environmental conditions. First, they can be highly sensitive measurements that provide information on the biological effects of pollution. Frequently, biomarkers are as valuable

in field settings as measurements of sediment or water chemistry because they indicate the actual exposure of an organism to the contaminant, information that cannot be gained easily from measuring contaminant levels alone (McCarthy and Shuggart 1990). Additionally, biomarkers can indicate exposure to contaminants that are quickly metabolized yet still negatively impact the organism's health. Such measurements can also provide a measure of toxicant interactions with tissues (Adams et al. 1990). Biomarker assays are rapid and efficient, as numerous samples can be run quickly and inexpensively. Increasingly, biomarkers are being investigated as early warning tools by risk assessors for surveillance, hazard assessment, regulatory needs, and for the evaluation of remediation techniques performed in contaminated systems (Markert 2003).

While biomarkers are important in the determination of the health of an organism, several important considerations must be noted before biomarkers can be applied in specific situations. Certain biomarkers (such as EROD activity measuring CYP1A induction) can be induced by a large number of structurally similar contaminants, which makes determining the exact cause of enzyme induction difficult (van der Oost 2003). Organisms are often exposed to mixtures of stressors at a given time, and determining the effect of a single stressor using biomarkers is frequently difficult. Many environmental parameters (salinity, turbidity, temperature, and season) and biotic factors (sex, size, genotype, diet, and reproductive status) can impact the results of biomarker assays, as organisms might be more or less susceptible to a particular stimulant in different environmental conditions (van der Oost et al. 2003). Basal levels and natural variability

in biomarker measurements must be determined before biomarkers are used in any specific situation.

In the current study hepatic and somatic biomarkers were used to determine the exposure of sunfish in the wadeable streams of South Carolina to xenobiotics. Specific biomarkers were used to estimate exposure to PAHs and endocrine disrupting compounds, and somatic measurements were taken to provide general idea of the overall health of the fish. Land use parameters were also determined to estimate the relationships between land use and biomarkers of contaminant exposure. Biomarkers have been widely used to provide information about the biological health of aquatic organisms, and the measurements performed in this study can provide an early warning of potentially deleterious effect on individual fish from xenobiotic exposure or provide information about the basal level of biomarker expression of sunfish in South Carolina.

EROD Assay

Hepatic biomarkers are typically the most commonly estimated biomarkers of exposure, as the liver is the site of many detoxification reactions in the body. Among the most frequently measured hepatic biomarkers of PAH exposure is cytochrome p450 induction, which is measured through the EROD assay. The cytochrome p450 (CYP) enzyme system is a large group of monooxygenases that plays an important role in the biotransformation of xenobiotics and endogenous substrates (Rose and Hodgson 2004). The group is classified as a superfamily of microsomal heme proteins that catalyzes primarily oxidative reactions that are part of phase I metabolism of a lipophilic

compound (Stegeman and Hahn 1994). Since the 1960's, this family has been studied in mammals, birds, reptiles, amphibians, and fish (Ohelmann and Schotte-Ohelmann 2003, Whyte et al. 2000, Stegeman and Hahn 1994). Commonly, the CYP1A subfamily is the focus of much investigation because of its role in the metabolism of contaminants and pollutants (Hansen 2003). CYP1A activity has been observed in over 30 species of fish from 10 families and is primarily induced by polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) (Whyte et al. 2000).

CYP1A is induced through the aryl hydrocarbon receptor (Ah receptor) and involves an increase in production of CYP1A enzyme (Stegeman and Hahn 1994). The Ah receptor is activated by aromatic compounds such as PAHs, polybrominated biphenyls (PBBs), 3-methylcholanthrene, B-naphthoflavone, and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) which enter the cell through the plasma membrane (Buhler and Williams 1989). After the activating compound binds to the receptor, the receptor-compound complex moves into the cell nucleus where it interacts with another protein known as the aryl hydrocarbon receptor nuclear translocator (ARNT) (Rose and Hodgson 2004). The complex then binds to the xenobiotic responsive elements (XREs) on DNA strands, which allows for increased transcription of CYP1A mRNAs and increased enzyme synthesis (Rose and Hodgson 2004). This induction allows organisms to mount a more rapid detoxification defense against potentially harmful xenobiotics upon exposure.

CYP1A induction is considered to be biologically important as it is a sign of exposure to larger PAHs, PCBs, and other chlorinated aromatics. This induction can be

an early indication of potential adverse effects or a form of resistance or adaptation to environmental pollution (Hansen 2003). Additionally, CYP1A induction is biologically important because PAHs can be metabolized via oxidation reactions to more reactive intermediates such as epoxides, arene oxides, and dihydrodiols that can react with macromolecules such as DNA or proteins (James et al. 1991, Gmur and Varanasi 1981, Gelboin 1980). The production of reactive metabolites and the reaction of these metabolites with macromolecules frequently precludes the development of DNA adducts and tumors. James and Kleinow (1994) note that fish from polluted waters and sediments show neoplasms in a number of organs, including the liver, mouth, and bile duct, and many studies have noted an increase in DNA adducts with increasing PAH contamination, with or without the accompanying neoplasms (Aas et al. 2000, van der Oost et al. 1998).

Before CYP1A induction can be properly applied as a biomarker of PAH and PCB exposure, several important factors must be considered. First, gender differences and reproductive status have been shown to impact the extent of induction seen in fishes from contaminated sites. Studies have found that reproductively active female fishes show decreased CYP1A activities, particularly in the spring and summer months, while activities are higher in reproductively active males, reproductively inactive females, and reproductively inactive males (Mayon et al. 2006, Flammarion and Garric 1997, Stegeman and Hahn 1994). This difference is largely attributed to the presence of elevated 17-β-estradiol levels in reproductively active female fishes, as estradiol can regulate CYP1A activity by altering the content of CYP1A in fish at the pre-translational

level (Navas and Segner 2001). Likely, an interaction between the estrogen receptor bound with estradiol and the Ah receptor, or potentially an interference created by the estrogen receptor and estradiol is the reason for the EROD suppression seen in female fishes (Navas and Segner 2001).

Secondly, a number of chemicals act as CYP1A inhibitors. Whyte et al. (2000), in their review of EROD activities in fish, cite numerous studies indicating that mixtures of polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans, polychlorinated biphenyls, organotins, endogenous compounds, and elevated levels of PAHs were potent inhibitors of CYP1A activity. Stegeman and Hahn (1994), in their review of phase I enzymes, found that most of these inhibitors work by either competitive inhibition or by "suicide" inactivation in which the inhibitor is metabolized by CYP1A into a metabolite that modifies the actual enzyme, thus rendering it ineffective. As organisms in polluted settings are commonly exposed to mixtures of chemicals, CYP1A induction may be masked by the presence of inhibitors, leading to interference in the results.

Thirdly, water quality characteristics can markedly influence CYP1A induction. Most studies indicate a negative relationship between CYP1A activities and temperature. Temperature is hypothesized to influence CYP1A by its effects on the saturation of membrane lipids, and also in the rate and magnitude of enzyme induction (Stegeman and Hahn 1994). Some studies have found temperature to be a more important characteristic than contaminant level in determining the extent of CYP1A induction (see Whyte et al. 2000, Sleiderlink et al. 1995). Additionally, pH can alter hepatic CYP1A induction with increasing acidic treatments potentially affecting baseline activity levels (Munkittrick et

al. 1993). Sediment composition and the amount of organic matter in the water can also impact the bioavailability PAHs and CYP1A inducers. Differences in sediment composition can affect bioavailability, as hydrophobic compounds and CYP1A inducers are much more likely to adsorb to organic matter than clay, thereby altering their bioavailability and absorption by fish (Neff 1979).

In addition to environmental factors, individual characteristics of fish and fish species can impact CYP1A levels. For example, one individual fish might be more or less susceptible to CYP1A induction or detrimental effects from PAHs or PCBs, with Stegeman and Hahn (1994) attributing this to allelic variations in individuals, resulting in different patterns of metabolites formed. Additionally, patterns of feeding behavior, trophic levels, levels of feeding, and habitat can alter CYP1A activities, even among fish at the same contaminated site (Escartin and Porte 1999). Controlling for factors like species, sex, age, reproductive status, and habitat can be helpful in using CYP1A induction to determine piscine exposure to PAHs.

The EROD assay is among the most sensitive methods for detecting induction of CYP1A and is a classical method for establishing levels of this phase I biotransformation pathway in numerous species and organisms (Whyte et al. 2000). While other studies have noted CYP1A activities in the gill, kidney, gut, and heart, the liver generally has the highest content of CYP1A in vertebrates and is considered to be among the most active organs in phase I biotransformation (see Mgdella et al. 2006, Hodgson and Rose 2004, Sarasquete and Segner 2000, Whyte et al. 2000, Stegeman and Hahn 1994). Accordingly, the EROD assay is typically performed on subcellular fractions of the liver. The assay

measures the conversion of the non-fluorescent substrate 7-ethoxyresorufin to the fluorescent resorufin (Pohl and Fouts 1980). The production of resorufin is measured using spectrophotomic methods (Klotz 1984). Both laboratory and field studies have found relationships between EROD activities and environmental levels of CYP1A inducers (Martinez-Gomez et al. 2006, Schmitt et al. 2005, Ohelmann and Schotte-Ohelmann 2003, Sarasquete and Segner 2000, van der Oost et al. 1998, Goksoyr and Forlin 1992, Bucheli and Fent (1995) found correlations between CYP1A induction and contaminant exposure in 93% of all field studied reviewed. This induction is generally rapid and can exist for days or weeks (Soimasuo et al. 1995).

CYP1A induction has historically been a valuable tool for assessing exposure to specific types of environmental contaminants and has become a classical biomarker demonstrating exposure to anthropogenic pollution. CYP1A induction is used in this study to determine the exposure of fish in South Carolina streams to contaminants such as PAHs. If care is taken to ensure that the factors that affect CYP1A induction are kept constant, the EROD assay can be used to tell much about the recent exposure of fish to PAHs.

Bile Fluorescence Assay

Bile fluorescence is increasingly utilized to assess the exposure of fish to polycyclic aromatic hydrocarbons (PAHs). In fish, lipophilic PAHs undergo a first phase I reaction in which a reactive functional group is added to the compound (Rose and Hodgson 2004). Following this oxidation reaction, phase II enzymes add hydrophilic

groups such as sugars and sulfates to the molecule, better enabling its excretion (George 1994). Generally, compounds that are of low molecular weight are poorly removed by biliary excretion into the gall bladder, but because many phase II conjugation metabolites, such as glucuronides and glutathione conjugates, are of higher molecular weight, they are typically excreted into the bile and removed from the body by fecal elimination (Klaassen and Rozman 1992). PAH metabolites excreted into bile are highly fluorescent, and bile fluorescence can be measured to obtain an estimate of exposure, uptake, and metabolism of PAHs (Aas et al. 2000, Lin et al.1996, Krahn et al.1987). Bile fluorescence has been measured in both laboratory and field studies and is frequently used in combination with other biomarker assays to gain a clearer picture of PAH exposure in fish (Reynolds et al. 2003, Aas et al. 2000, Gagnon and Holdway, 2000, Escartin and Porte 1999).

Assessing bile fluorescence in fish is a simple procedure that can by quickly and easily performed. Several methods exist for the detection of fluorescence, including high performance liquid chromatography with fluorescence detection (HPLC-F), synchronous fluorescence spectrometry, and fixed wavelength fluorescence (Aas et al. 2000). Fixed-wavelength fluorescence has been shown to be a comparable method to HPLC-F and is a simple alternative to the HPLC-F (Lin et al. 1996). Using this method, fluorescence of diluted bile metabolites can be measured at different wavelength pairs corresponding to the different compounds being investigated. For example, 2-ringed PAH metabolites show maximal fluorescence at 290nm excitation/335 nm emission, while 4-ringed metabolites fluoresce at 341nm/383nm, and 5-ringed metabolites fluoresce at

380nm/430nm (Aas et al. 2000, Lin et al.1996, Krahn et al.1987). Measurement of metabolites in bile is a measure of recent exposure, as metabolites have been shown to appear in the bile within hours of PAH absorption (Gagnon and Holdway 2000, DiGiulio et al. 1995).

Despite the relative ease with which bile fluorescence can be measured, there are certain variables that need to be considered when assessing the presence of fluorescent aromatic compounds (FACs) in bile. First, the feeding status of an animal has been shown to markedly affect fluorescence values seen. Collier and Varanasi (1991) found that fluorescence per milliliter of bile decreased significantly in English sole (*Paraphrys vetulus*) that had begun feeding but increased in animals that had not been fed. They hypothesized that this could be due to a concentrating effect in the gall bladder of the non-feeding fish from the active transport of water across the gall bladder epithelium. Several normalizations have been proposed to correct for this discrepancy due to feeding status, namely expressing fluorescence units per volume of bile, per milligram of bile protein, or per milligram of biliverdin in bile. Recent evidence has shown that normalization to bile protein is a more appropriate correction. Van den Hurk (2006) found that normalization to bile volume can be highly variable and difficult to measure because of the very small volumes of bile involved in bile fluorescence assays, while normalization to biliverdin absorbance is inappropriate because chemical stress from environmental pollution can lead to heme oxygenase induction and increased biliverdin excretion. Because biliverdin levels are more variable than bile protein levels under

chemical stress, bile protein is a more appropriate normalization factor for bile fluorescence measurements.

Additional sources of variation arise from interspecies and individual variation. Leadly et al. (1999) found FACs in bile to be highly variable for replicate fishes of brown bullheads (*Ameiurus nebulosus*), even in laboratory settings in which variables such as size and feeding status were controlled. Similarly, Escartin and Forte (1999), in their study of xenobiotics in deep sea fish, found definite species differences and variations in metabolic patterns in five separate species of fish. These observed variations indicate different exposures due to habitat, trophic strategy, or basal levels of xenobiotic metabolizing enzymes, and it is important to consider these factors when using bile fluorescence as a measure of exposure to environmental contaminants.

The measurement of bile fluorescence is a valuable tool to assess exposure and metabolism of PAHs in fishes. It is a simple, cost-effective, informative method of evaluating PAH exposure and metabolism in aquatic systems. While several factors can affect the levels of fluorescence seen in fishes, this does not discount the use of bile fluorescence as an appropriate biomarker of exposure in PAH contaminated aquatic systems. The assay was used in the present study in combination with the EROD assay to fully determine the exposure of sunfish to PAHs in South Carolina streams.

GST Assay

Glutathione-s-transferases (GSTs) are ubiquitous cytosolic enzymes that have been described in numerous organisms, including bacteria, plants, yeasts, mollusks, arthropods, and vertebrates such as fish, birds, and mammals (Pemble and Taylor 1992). These enzymes are phase II enzymes that have varied functions within an organism. Many GSTs are believed to act as "housekeeping" enzymes in animals (Leaver et al. 1993). For example, GSTs are important in intracellular transport of compounds, biosynthesis of metabolites such as leukotrienes and prostaglandins, and the isomerization of steroids (George 1994). GSTs also act in the detoxification of electrophilic xenobiotics and compounds, working to prevent oxidative damage to macromolecules and protect nucleophilic groups such as proteins and nucleic acids (Rose and Hodgson 2004). Elevated levels of GSTs in common detoxification organs such as the liver, kidney, and gill provide further evidence of the defensive nature of GSTs (Leaver et al. 1993).

GSTs work by catalyzing the reaction of glutathione, a tripeptide composed of glycine, glutamic acid, and cysteine, with an electrophilic molecule or reactive oxygen species (Rose and Hodgson 2004). Typical substrates for GSTs are lipophilic with electrophilc carbon atoms (Sipes and Gandolfi 1992). The conjugation of xenobiotics with glutathione by GSTs increases the water solubility of a compound and is typically a second line of defense (following the initial reactions of superoxide dismutase and catalase) against reactive oxygen species (Hayes and McLellan 1999).

Several GST isoforms exist in fish, each with different subunit composition and substrate selectivity (Sipes and Gandolfi 1992). These isoforms are phylogenetically divergent from the frequently studied mammalian isoforms, but do bear some similarity to the π and θ families seen in mammals (Hayes and McLellan 1999). While GST activity can increase in response to pollutants that result in oxidative stress, it is generally a much smaller increase than is seen with CYP1A induction (Figueiredo-Fernandes et al. 2006, Collier and Varanasi 1991). For example, a 2-5 fold induction of GST has been noted with fish injected with PAHs, in contrast to the orders of magnitude induction seen in EROD assays used to measure CYP1A induction (George 1994).

Relatively few studies of the actual mechanism of GST induction have been performed in fish, as much research has largely occurred in mammalian systems. GST induction is complex and is currently poorly understood in fish. Studies have noticed that CYP1A inducers such as 3-methylcholanthrene and β-naphthoflavone have caused no effect or led to decreased expression of GST enzymes, indicating a different pattern of induction from CYP1A (Lemaire et al. 1996, Scott et al. 1992, Goksøyr et al. 1987). Likely, unlike CYP1A indication which acts through XREs, GST induction occurs through nuclear response elements such as antioxidant response element (AREs) and elecrophile response elements (EpREs). Support for this comes from studies observing GST induction without PAH exposure or CYP1A induction. Hayes and McLellan (1999) note that certain soluble GST forms are induced as an adaptation to oxidative stress, likely through these response elements, and provides the example of increased GST production in response to *trans*-stilbene oxide. Leaver et al. (1993) observed that the

administration of epoxides increases GST mRNA levels in the plaice (*Pleuronectes platessa*) by several-fold, a response also seen in the flounder (*Platichthys flesus)*, whereas PAH inducers acting through the XRE may actually suppress this response.

GST induction is variable and can be affected by interspecies differences and individual variation. The presence of different GST isoforms in fish, each of which is differentially responsive to different substrates, also contributes to the variation in activities observed in different species of fish (Foerlin et al. 1996). Despite the presence of several GST isoforms in fish, each shows reactivity toward 1-chloro-2,4-dinitrobenzene (CDNB) (George 1994). It is this conjugation that forms the basis of the GST assay. The assay measures the conjugation of CDNB with glutathione, and the absorption of the conjugated product is measured at 344nm. The GST assay was used in this study to determine if fish in South Carolina streams were subject to oxidative stress and provide a biomarker measurement complimentary to other measurements of contaminant exposure.

Estrogen Binding Assay

The endocrine system functions to regulate many other organ systems and maintains a physiological balance within an organism. Because the endocrine system is closely tied to other organ systems, toxicants that act on the endocrine system can have far-reaching results, resulting in developmental, growth, and reproductive abnormalities (Leblanc 2004). Chemicals that impair normal endocrine function are classified as endocrine disrupting contaminants (EDCs). The U.S. EPA defines EDCs as “exogenous

agents that interfere with the production, release, transport, metabolism, binding, action, or elimination of the natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes (US EPA 2006)." Because of the consequences that EDCs have on reproductive health, the EPA has identified endocrine disruption as a high priority topic in the Office of Research and Development (US EPA 2006).

Among the most important topics studied involving endocrine disruption is the effects of environmental estrogens in environmental systems. Environmental estrogens are exogenous compounds that interact with the estrogen receptor to cause measurable effects (Tapiero et al. 2002). The determination of the estrogenicity of chemicals has proven to be difficult. The endogenous ligand for the estrogen receptor, 17-β estradiol, is composed of several rings and is lipophilic. However, environmental estrogens are of varied structures, so it is difficult to determine if a chemical is estrogenic based on chemical structure alone (Soto and Sonnenschein 2005, Tapiero et al. 2002,). Typically, environmental estrogens tend to be hydrophobic in nature, and most are characterized by a lipophilic region absent in non-estrogens (Cunningham et al. 1997). Many environmental estrogens share a phenol ring structure within this hydrophobic region (Witorsch 2000). The size and orientation of functional groups on the molecule is important in predicting estrogen receptor binding, and a rigid ring structure favors strong receptor interaction (Fang et al. 2001). These properties, in combination with other steric and electrostatic properties influence if a molecule can bind to the estrogen receptor (Leblanc 2004).

Environmental estrogens have numerous uses and are widespread in the environment. The primary source of estrogenic chemicals in the environment comes from pharmaceuticals, which are generally the strongest estrogen mimics (Bhat 2000). The use of estrogens such as those found in hormone replacement therapies and birth control pills have gained popularity in human and agricultural uses and are increasingly becoming abundant in environmental systems. Common examples include ethinylestradiol and the now discontinued diethylstilbestrol (Tapiero et al. 2002). Other xenoestrogens include chemicals used in home and industrial applications such as plasticizers such as bisphenols, pesticides (endosulfan, kepone, toxaphene, chlordane), herbicides (alachor, atrazine), industrial chemicals, heavy metals (lead, mercury, and cadmium), food additives, cosmetics, paints, detergents, and cosmetics (Soto and Sonnenschein 2005, Bhat 2000). Additionally, there are naturally occurring compounds called phytoestrogens and mycoestrogens found in plants and fungi that have some estrogen-like activity (Bhat 2000).

For an estrogenic compound to cause an effect, the molecule must first interact with an estrogen receptor (ER). In fish, there are three isoforms of the estrogen receptor, ERα, ERβ, and ERγ, each of which functions as a nuclear receptor in the extracellular matrix of the cell (Leblanc 2004, Hawkins et al. 2000). The isoforms exist in different tissues in the fish. For example the prevalence of ERγ in the testes of male fish might be indicative of its role in endocrine disruption in males (Hawkins et al. 2000). Once the estrogen receptor is bound with the ligand, the receptor dimerizes with a second bound receptor and moves into the nucleus. Following this movement into the nucleus, the

complex stimulates the transcription of estrogen responsive genes (Leblanc 2004). It is this mechanism by which most of the effects of environmental estrogens occur.

Environmental estrogens can have a variety of effects in fish. Gonad size is decreased in both male and female fish by exposure to environmental estrogens because of disruptions from estrogens in signaling processes within the fish. In severe cases, environmental estrogens can result in hermaphroditic fish. Jobling et al. (1995) found that up to 100% of fish living downstream of wastewater treatment plants in the United Kingdom were hermaphroditic, possessing both male and female characteristics. This condition has been seen in other species, including the gudgeon (*Gobio gobio*), flounder (*Platichthys flesus)*, sheepshead minnow (*Cyprinodon variegates lacepede*), resulting in spermatogonia in ovaries and the presence of both gonad structures in the fish (Kirby et al. 2004, van Aerle et al. 2001, Zilloux et al. 2001). Structural changes in gonad morphology often reduce fecundity in the fish, as many of fish are less successful reproductively because of the altered gonad structures (Jobling et al. 1995).

Estrogenic compounds present in animal tissues can be measured in several different ways, including reporter gene assays and cell proliferation assays using estrogen-dependent cells. A developing method for determining exposure to estrogenic compounds uses a competitive estrogen receptor binding assay. Once absorbed into the body, estrogens are typically conjugated by phase II reactions and excreted via biliary excretion (Houtman et al. 2005). This results in an accumulation of estrogenic compounds in the bile. These compounds can be extracted from the bile and deconjugated, restoring original estrogenic potency (Legler et al. 2005). The extracts are

run through a radioactive estrogen binding assay to determine the relative potency of compounds present in the bile. In this assay, a radioactive molecule (such as ^{3}H-17-β-estradiol) is bound *in vitro* to the estrogen receptor (α or β). The test compounds (such as the bile extracts) are administered, and if the bile contains estrogenic compounds, these will competitively displace the radioactive molecule from the receptor. The resulting radioactivity is measured to determine the interaction of the test compound with the receptor (Korach et al. 1978). The competitive binding assay was used in this study to determine the exposure of sunfish to EDCs and to provide one of the first measurements of the exposure of feral fish in South Carolina to environmental estrogens.

Somatic Indices

Biomarkers of exposure and effect can be measured at different levels of biological organization. Gross physical measurements can be indicative of a variety of conditions, including energy availability, reproductive status, and age (Adams and McLean 1985). Additionally, organo-somatic indices can be reflective of toxicant exposure. These indices, such as Fulton's condition factor (K), hepatosomatic index (HSI), spleen-somatic index (SSI), and gonadosomatic index (GSI), along with other biochemical and physiological parameters, provide evidence of overall health of fish and are a measure of biological responses at the individual level.

Fulton's condition factor (K) has been described in many species and is a way to quantitatively compare the health of fish from the same site or two different sites. Condition is commonly described as the energy that is available to perform common

activities such as reproduction, feeding, predator avoidance, and survival (Neff and Cargnelli 2004). The easiest method for measuring condition is through Fulton's condition factor, which relates the mass of the fish to the length (Fulton 1904). It is generally observed that the higher the condition factor, the larger the fat content of the fish and the more energy available (Neff and Cargnelli 2004). Typically, any stress will decrease the condition factor. Studies using the gudgeon (*Gobio gobio*) have found that condition factor decreased with elevated heavy metal burden in fish exposed along a contaminant gradient (Berovets and Blust 2003). Similar results were seen in redear sunfish (*Lepomis microlophus*), as fish that were exposed to the highest levels of selenium had the lowest condition factors (Sorrensen and Bouer 1984). It is believed that toxicant stress will cause a re-allocation of resources, anorexia, stunting, or poor feed conversion, each of which decrease the condition factor (Sorrensen and Bouer 1984). Biotic stressors such as parasitic infection and habitat disturbance have also been shown to decrease the condition factor of sunfish (Mouritsen and Poulin 2002, Colle and Shireman 1980). It is important to note that condition factors are influenced by any parameter that will influence growth and energy allocation in the fish, such as sex, season, water temperature, diet, musculature, and reproductive activity (Barnham and Alexander 1998). However, the condition factor is still perhaps the easiest non-invasive somatic index to measure and is a good preliminary assessment of the energy availability and overall health of the fish.

The liver is the site of a majority of detoxification reactions, and it is frequently the site of action for the toxicant or the toxic metabolites produced during metabolism.

Liver injury can occur by an increase of fat in the liver (steatosis), degeneration of the liver (necrosis), lipid peroxidation, suppression of bile flow (cholestasis), accumulation of collagen in the liver (cirrhosis), and carcinogenesis (Hodgson and Levi 2004, Plaa 1992). The type of damage that occurs is largely dependent upon the type of chemical and duration of exposure (Hodgson and Levi 2004). Many of these conditions affect the size, shape, and appearance of the liver, in addition to liver functions. As a result, the hepatosomatic index (HSI) is used as a gross measurement of liver impairment and exposure to toxicants.

The size of the liver can change due to toxicant stress. Lipid peroxidation from free radical damage can impact the removal of fat from the liver, as free radical species can affect the endoplasmic reticulum of hepatocytes, damaging the ability of the liver to form and excrete low-density lipoproteins (Recknagel and Ghoshal 1966). Furthermore, Slooff et al. (1983) found that liver size increases in response to pollutants due to hypertrophy (an increase in the size of hepatocytes), while Poels et al. (1989) attributed the morphological changes to hyperplasia (an increase in cell number).

Liver size is dependent upon many factors independent of exposure to toxicants, including temperature, season, feeding status, age, reproductive status, and energy status, so several reasons for elevated HSIs may exist in a particular location (Adams and McLean 1985). Also, exposure to toxicants might not be reflected in changes in the HSI. Reviews of biomarkers have noted that lab studies show indicate an increase in HSI with toxicant exposure in 38% of reports, while field studies indicate an increase in 43% of evaluations (van der Oost et al. 2003).

The spleen somatic index (SSI) is used to assess the immunological status of an organism. The spleen is a major filter for blood and is where some immunological responses to blood-borne antigens occurs (Selgrade 2004, Dean and Murray 1992). The spleen also stores red blood cells and aids in the breakdown of old red blood cells. Interpretation of the SSI can reveal much about not only exposure to contaminants but also much about the immunological status of the fish. The mass and size of the spleen can either decrease or increase depending upon the conditions to which the fish is exposed.

Generally, exposure to toxicants will cause stress in the organism and weaken the immune system. As a result, spleen weights will decrease because of lymphoid depletion, indicating immune dysfunction due to contaminant stress (Dean and Murray 1992). Teh et al. (1997) found little difference in the size of spleens of sunfish and bass taken from contaminated and reference sites, though noted that lymphoid cell depletion, vascular congestion, and cellular necrosis in fish from polluted waters. Additionally, van den Heuvel et al. (2005) found a decrease in spleen size correlated with a decreased number of white blood cells in the blood of rainbow trout (*Oncorynchus mykiss*) exposed to bleached-pulp mill effluent, attributing this to the release of erythrocytes from the spleen in response to a decrease in circulating blood cells from toxicant stress. Impaired immune function can leave the organism open to viral or parasitic infection, which will further stress the fish and make them more susceptible to the effects of pollutants (Schwaiger 2001).

Conversely, splenomegaly (enlargement of the spleen) will occur in response to infections that are viral, bacterial, or parasitic in nature. Parasitic infection can cause fish

to develop immunity to resist infection (Dogiel et al. 1970). The resulting immune response produces an increase in spleen size by increasing the amount of lymphoid tissues, inflammation, or macrophages and phagocytes in the spleen (Dogiel et al. 1970). Splenomegaly is common in infected fish, with the spleen of infected fish 2-5 times larger than uninfected (Arnott et al. 2000). Parasitic infection can reduce fish population numbers if the infections lead to increased mortality. Accordingly, splenomegaly is among the earliest warning signs of infection in fish.

The gonadosomatic index (GSI) is a biomarker for reproductive effects of toxicants. Increasingly, aquatic animals are exposed to elevated concentrations of endocrine disrupting compounds, including hormones, plasticizers, pesticides, and naturally occurring estrogenic compounds such as phytoestrogens. These compounds can impair the structure and function of reproductive organs, which can lead to effects on the population, community, or ecosystem levels if endocrine disruption is severe. A common physical measurement used to assess reproductive health is to measure the size of the testes in male fish or the ovaries in female fish.

Both male and female fish are vulnerable to the effects of endocrine disrupting compounds that affect the morphology of reproductive organs. In both sexes gonad size will decrease upon exposure. In males studies have found a decrease in testis mass when fishes are exposed to estrogens (Chang et al. 1995, Billard et al. 1981). Christansen, in a histological investigation of male eelpout (*Zoarces viviparous*) treated with the estrogenic plasticizer 4-nonylphenol and the hormone 17-β-estradiol, found that exposed male fish had small, gray, firm testes as opposed to the large, white testes of the control fishes

(1998). Jobling (1995) furthermore found a correlation between estrogenic potency and a decrease in the GSI in male rainbow trout (*Oncorhnchus mykiss*) exposed to 4-nonylphenol and 17-β-estradiol for three weeks. In severe cases hermaphroditic fish have been discovered in sites contaminated with high levels of endocrine disruption, as was found downstream of wastewater treatment plants in the United Kingdom (Purdom et al. 1994).The decrease in gonad size can be attributed to a variety of causes, including an inhibition of androgen synthesis, a direct effect on Sertoli cells, or a direct effect on somatic and germ cells (Flammarion et al. 2000).

Ovary size is similarly reduced in female fishes exposed to estrogenic compounds. Van den Belt et al. (2001) found that estrogenic compounds interfere with oocyte maturation in female non-spawning zebrafish (*Danio rerio)* and accordingly decreasing the GSI in these fish when compared to control fish. In a separate study, ethinylestradiol was found to reduce the ovary-somatic index in zebrafish by reducing the number of mature, vitellogenic oocytes, which normally comprise a large part of the ovary volume (van den Belt et al. 2001). This is ascribed to a change in the complex processes that govern reproduction in fishes, with the elevated levels of ethinylestradiol suppressing the pituitary output of gonadotropin, which influences the formation of eggs and reproduction.

Because somatic indices are among the easiest biomarkers to measure in aquatic organisms, they were used in this study to provide an estimate of the reproductive health of sunfish in South Carolina streams. These indices are frequently used in combination with other biomarker measurements and were performed here to support other more

specific biomarkers of exposure and effect. These measurements can also provide a crude estimate of contaminant exposure to estrogenic and hormonal compounds.

Lepomis sp.

The Centrarchidae family is among the largest native families of freshwater fishes in North America, second only to bullhead catfish (Ictaluridae) in abundance and dominance. The Centrarchidae family comprises thirty species in eight genera. The United States Environmental Protection Agency has integrated the Centrarchidae family into their Index of Biological Integrity scoring (IBI) and has used it as a part of assessing aquatic community health, indicating its importance in ecosystem structure (US EPA 2006). In addition to their use in ecological studies, Centrarchids are common sport and game fishes, with the black basses (*Micropterus* sp.) and crappies (*Pomoxis* sp.) included in this family (Brunson and Morris 2003). Sunfishes (*Lepomis* sp.) comprise nearly half of all species within the Centrarchidae family.

There are eleven living species of sunfish that are widely distributed throughout the United States, most of which are present in South Carolina. Among these are the bluegill (*Lepomis macrochirus*), redbreast (*Lepomis auritus*), dollar (*Lepomis marginatus*) warmouth (*Lepomis gulosus*), redear (*Lepomis microlophus*) and pumpkinseed (*Lepomis gibbosus*). Sunfish generally inhabit slower moving bodies of water with large amounts of cover (Vadas and Orth 2000), and many are present in quiet, sluggish bodies of water and in larger, deeper streams (Table 1). Frequently, species are associated with dense vegetation, large woody debris, shoreline roots, brush piles, and

rubble (Marcy et al. 2005). Most species consume small invertebrates, gastropods, sediment dwelling organisms, and zooplankton, with some species such as the warmouth becoming increasingly piscivorous with age (Marcy et al. 2005). The actual organisms consumed are dependent upon the size, age and species of the sunfish (Mittelbach 1984).

Sunfish typically spawn during the summer months, with reproduction occurring from May through August depending upon species and the geographic location of the fish (Marcy et al. 2005). They are nest-builders that generally spawn in shallow waters. The male fish prepares the nest at the appropriate water depth, then circles the nest and grunts to attract the female. The females deposit eggs, and male fish immediately fertilize them. The males will then defend the eggs until hatching (Brunson and Morris 2000). Female fishes can deposit eggs in more than one nest and can spawn multiple times throughout a season.

Sunfish were chosen as the target genus for this study for several reasons. First, they are highly abundant in South Carolina. Previous sampling efforts by the South Carolina Department of Natural Resources have found that the redbreast sunfish is present in approximately 80% of all sites sampled, with other sunfish species slightly less abundant but still a numerically dominant family. Additionally, sunfish are generally sedentary with small home ranges. Gatz and Adams (1984) found that redbreast, bluegill, and warmouth rarely moved over large distances during their three year monitoring study in Tennessee. The immobile nature of sunfish indicates that they will be better reflective of the water and sediment quality at a particular site, and thus provides stronger evidence for the historical nature of exposure at a sampling location. Most sunfish are moderately

tolerant of pollution and have historically been used in numerous toxicity experiments (US EPA 2006). Bluegill sunfish have been widely used to test chemicals such as detergents, insecticides, pharmaceuticals, metals, and raw effluent (Vittozzi and DeAngelis 1991, Phipps and Holcombe 1985). This demonstrates their use as sentinel species in determining the harmful effects of xenobiotics and chemicals and further makes them an important family for selection of sentinel species for study.

OBJECTIVES

The purpose of this study was to use biomarkers of xenobiotic exposure and metabolism to determine the bioavailability of xenobiotics to fish in the wadeable streams of South Carolina using sunfish (*Lepomis* sp.) as sentinel species. This was performed using the following objectives:

1. To use biomarkers of exposure (bile fluorescence and bile estrogenicity) and effects (EROD induction, GST induction, and somatic indices) to determine the exposure and effects of PAHs and estrogenic compounds to *Lepomis* sp.
2. To compare xenobiotic contamination of three ecobasins in South Carolina using biomarker measurements and relate these results to land use around each sampling site and within each ecobasin.
3. To determine relationships between biomarker measurements using correlation analyses.
4. To relate results of biomarkers of effect to population and community structure.

MATERIALS AND METHODS

Chemicals

All chemicals were obtained from Fisher Scientific (Atlanta, Georgia) and Sigma Aldrich (St. Louis, MO) unless otherwise noted. Biliverdin dihydrochloride was purchased from MP Biomedicals (Aurora, Ohio).

Study Area, Site Selection and Sampling

Sampling sites were selected from within three ecobasins: the Saluda Sandhills, the Savannah Sandhills, and the Pee Dee Atlantic Southern Loam Plains (PDALSP) (Figure 1). Each ecobasin is composed of its own river basin (Saluda, Savannah, and Pee Dee) and Level IV ecoregion (the Sandhills or Atlantic Southern Loam Plains). The Sandhills generally consist of hilly regions with low-nutrient soils primarily formed in sand, which makes this ecoregion a poor area for farming (Griffith 2001). Most portions are wooded, with some areas developed for other uses such as golf courses or peach orchards (Griffith 2001). The Atlantic Southern Loam Plains tends to be flatter than the Sandhills and is primarily used for agricultural uses because of its finely-textured and well-drained soils (Griffith 2001).

For this study sites were randomly selected using GIS (geographic information systems). The chosen sites consisted of wadeable streams ranging from 4.1 to 129.7 km^2 in stream area located in areas that were not prone to flooding, drying, or braiding.

Sampling occurred during the summer months (from May to September) for ease of sampling and lower water flow (Table 2).

At each site a transect was selected to cover approximately twenty times the average width of the stream for a minimum of 100 meters of sampling length. Fish were collected using electrofishers and dipnets. After completion of the third and final pass through the sampling site, all captured fish were divided according to species and counted. All fish of the genus *Lepomis* were set aside from the rest of the sampled fish. Initial protocol had stated that only redbreast sunfish (*Lepomis auritus*) would be selected for biomarker analysis, but as these were unavailable at many sites, all fish from the genus *Lepomis* were selected for study. An attempt was made to capture at least 10 fish at each site. However, only a total of 5 to 10 fish of ages were caught per site (Table A-1).

After collection, fish were retained alive in water from the sampling location prior to dissection. Fish were identified according to species, the lengths of the selected fish were measured, and a wet weight of the whole fish was taken before dissection. Each individual was then sacrificed by severing the spinal cord. Following this paralysis, fish were dissected and gender was noted. The gall bladder was carefully removed intact and stored on ice in an amber microcentrifuge tube (Fisherbrand® flat top microcentrifuge tube). The organs of interest (liver, spleen, and gonads) were then sequentially removed and placed into foil packages. The packages were immediately flash frozen in liquid nitrogen until transfer to the lab. Upon arrival at the Clemson Institute of Environmental Toxicology in Pendleton, South Carolina, harvested tissues were frozen at -80°C and gall bladders at -20°C until analyzed.

Population and Land Use Assessment

All land use data and watershed areas were prepared by Cathy Marion (Clemson University Department of Forestry and Natural Resources) using ArcGIS® software (Version 9.1, Environmental Systems Research Institute) and 2001 land use data for South Carolina (USGS 2007). Land use was determined for the entire watershed area draining into each site and a 100 meter zone (buffer zone) along the stream in the watershed area. Land use is expressed as percentage of area used for a specific purpose. Land use classifications are described in Table 3 (US EPA 2007). Additionally, a measure of impervious surface cover was estimated from developed land using the land use classifications described in Table 3 (SWRC 2004). Total percent impervious surface cover was determined by averaging percent impervious cover for the four types of developed land (developed open spaces, low intensity, medium intensity, and high intensity development), multiplying this percentage by the total land area covered by each type of developed land, and using this percentage to estimate impervious surface cover at each site. This percentage was then use in correlation analyses. Human population data were determined for the watershed area at each site and for each ecobasin using United States Census data from 2000 (US Census 2000).

Somatic Indices

Fulton's condition factor was calculated from measurements taken in the field and calculated as $10^5(W/L^3)$, where weight is in grams and length is in centimeters (Fulton 1904). Hepatosomatic indices (HSIs) were calculated for each individual using the

formula (liver mass/whole body mass)*100. Gonadosomatic (GSIs) indices were calculated as (gonad mass/whole body mass)*100. Spleen-somatic indices (SSIs) were calculated as (spleen mass/whole body mass)*100.

Preparation of S9 Fractions

Livers were thawed and placed into low speed centrifuge tubes on ice. Each liver was 0.2 g to 1 g each, so approximately 5 ml of homogenization buffer was added to each liver. Homogenization buffer contained 0.25M sucrose for protection of the liver tissue during freezing, 0.05M tris-base [tris (hydroxymethyl) aminomethane] as a buffer (pH 7.4), chelating agent ethylenediaminetetraacetic acid (EDTA), reducing agent dithiothreitol (DTT), and protease inhibitor phenylmethylsulphonyl fluoride (PMSF). All tubes were homogenized using a Biospec Products, Inc. Tissue Tearer^{TM} (Bartlesville, OK) until tissue appeared upon visual inspection to be fully homogenous. The tissue homogenizer was thoroughly cleaned with water and methanol between samples.

Following homogenization of the livers, samples were spun at 9,000 rpm (10,000 x g) at 4°C for 20 minutes on a Beckman J2-21 M/E centrifuge with a JA-20.1 rotor. Tubes were carefully placed on ice and visible clumps of fat were aspirated from the supernatant. The supernatant was then divided into several 1.5 ml microcentrifuge tubes and frozen at -80°C until further analysis. This supernatant is the S9 (postmitochondrial) fraction.

An additional 25 µl aliquot was separated and used to determine the protein content of the S9 fractions. Samples were diluted 1:20 in deionized water and quantified

using the Pierce BCA Protein Assay Reagent Kit®. This assay uses bicinchoninic acid for the colorimetric detection of protein and quantifies protein in unknown samples by using bovine serum albumin as a standard.

EROD Assay

The EROD assay was used to measure CYP1A induction in fish samples. In this assay the conversion of 7-ethoxyresorufin to resorufin by CYP1A was measured using fluorescence spectrometry. The method follows procedures outlined by Klotz et al. (1984) and Pohl and Fouts (1980) and was further adjusted for 96-well plates and S9 fractions of livers rather than microsomes.

Black polystyrene flat bottomed plates (Whatman Uniplate®, Clifton, NJ) were obtained and the reaction mixtures were pipetted into each well. Individual wells contained 25 µl of 1M tris buffer [tris (hydroxymethyl)aminomethane] at pH 7.8, 25 µl of 2% bovine serum albumin, 25 µl of 50mM $MgCl_2$, 20 µl of water, 5 µl of 0.1mM ethoxyresorufin, and 100 µl of S9 fraction (diluted to 0.5 mg/ml in deionized water for a total of 50 µg of protein). Each sample was run in triplicate wells, and the reaction was started by the addition of 50 µl of freshly prepared 2.5mM NADPH (β-nicotinamide adenine dinucleotide phosphate) to each well. The plate was immediately transferred to a fluorescence plate reader (Molecular Devices Spectramax Gemini®, Sunnyvale, CA) where fluorescence readings were taken at 530 nm excitation, 585 nm emission every 10 minutes for 30 minutes to ensure linearity. These readings were compared to a standard curve containing the reaction mixture described above without the S9 fraction and

NADPH but containing 0 to 800 nM resorufin. The EROD activity was determined as picomoles of resorufin generated/mg protein/minute.

Bile Fluorescence Assay

Thawed gall bladders were pierced with small scissors and bile was collected into an amber Fisherbrand® flat top microcentrifuge tube. To each tube 250 µl of deionized water was added. Tubes were then vortexed using a Fisher Vortex Genie 2® and centrifuged at 4000 rpm for 10 minutes on an Eppendorff Centrifuge 5810R with a F45-30-11 rotor. Following centrifugation, two 25 µl aliquots were assayed for protein as described previously. To the remaining bile, 200 µl of methanol was added to each sample, which was vortexed, placed on ice for 15 minutes, and spun again at 4000 rpm for 10 minutes. Two 200 µl subsamples were transferred to a clear 96-well plate and biliverdin absorption was measured at 365 nm and 660 nm. A standard curve for biliverdin ranging from 0 mg/l to 25 mg/l was produced using biliverdin standard and a 50:50 methanol: water dilution. Following the biliverdin assay, plates were covered with plastic plate covers and stored at -20°C until use in the estrogen binding assay.

To measure the fluorescence of bile samples, a series of dilutions in from 1:100 to 1:1500 was produced for each bile sample in a black polystyrene flat bottomed plate (Whatman® Uniplate®, Clifton, NJ) using a 50:50 methanol/deionized water mixture. This was done to determine the dilution factor needed to yield the maximum fluorescence value while minimizing the innerfilter effect (Lin et al. 1996). Blanks consisted of the methanol/water mixture. Fluorescence was measured using a fluorescence plate reader

(Molecular Devices® Spectramax Gemini®, Sunnyvale, CA) for the following wavelength pairs: 290/335nm for 2-ringed (naphthalene-type and smaller) PAH metabolites, 341/383nm for 4-ringed (pyrene-type) metabolites, and 380/430nm for 5-ringed [benzo(a)pyrene-type] metabolites. Each dilution was analyzed in duplicate.

GST Assay

The GST assay was used to determine the activities of glutathione-s-transferases by measuring the conjugation of glutathione (GSH) to 1-chloro-2,4,-dinitrobenzene (CDNB). This procedure follows steps outlined by Habig et al (1974) and uses an automated plate reader to determine the maximum velocity (V_{max}) of the conjugation reaction. The absorption of the glutathione conjugate is measured continuously at 344nm for 2 minutes to determine enzymatic activities.

Clear, polystyrene, flat-bottomed 96-well plates (Fisherbrand®) were obtained and the reaction mixtures were pipetted into each well. Individual wells contained a final volume of 250 µl consisting of 100 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid)), 95 µl deionized water, 50 µl of GSH (1 mM), and 50 µl of S9 fraction (diluted to 0.5 mg/ml in deionized water for a total of 25 µg of protein). Each sample was analyzed in triplicate. To start the reaction, 30 µl of freshly prepared CDNB (50 mM solution in 100% ethanol, diluted 1:6 in deionized water) was added to each well using a multi-channel pipet. The plate was immediately transferred to a plate reader (Molecular Devices® Sunnyvale, CA) where absorption was measured every 9 seconds for 2 minutes at 344 nm. Results were reported in nmol/mg/min.

Preparation of Bile Extracts for the Estrogen Binding Assay

For the estrogen binding assays, the first extraction techniques followed procedures outlined by Truman (2006). Ten to 50 µl of diluted bile was removed from the plates and transferred to a Baxter12x75mm borosilicate glass culture tube. The tubes were then evaporated to dryness under a gentle stream of air. Bile extracts were reconstituted in 700 µl of sodium acetate buffer (pH 5.0, 100 mM) and 600 µl of distilled water. Four units of glucuronidase and 1 unit of sulfatase per µl of bile extract were added to the tubes, which were then sealed and incubated for 24 hours in a water bath at 37°C. Following incubation, 100 µl of 1N HCl were added to each tube to stop the reaction. Following the addition of HCl, 2 ml of methanol were added and tubes were incubated on ice before being centrifuged at 2000 x *g* for 20 minutes. These samples were then stored in glass tubes and refrigerated before use in the assay.

When it became evident that this extraction technique produced samples that were below the detection limit of the assay, even with samples from gravid female fish, a second extraction technique was used to decrease the dilution factor. Ten to 50 µl of bile from the biliverdin assay were again dried under air and reconstituted in 30 µl of sodium acetate buffer. Four units of β-glucurondase and 1 unit of sulfatase per µl of bile extract were then added and incubated for 2 hours instead of 24 according to Fisher guidelines for the use of these chemicals. The reaction was not stopped with 1N HCl, but instead it was believed that the excess of methanol added (approximately 4 volumes) would be sufficient to denature the enzymatic activity of the β-glucuronidase and sulfatase and stop

the reaction. After the addition of methanol, samples were placed on ice for 30 minutes, spun at 2000 x *g* and stored at -20°C until further analysis.

Estrogen Binding Assay

Duplicate samples of 50 µl of bile extracts were placed into Baxter 12 x 75 mm borosilicate tubes in a Dri-Bath™ at approximately 40°C and evaporated to dryness using filtered air. Meanwhile, additional tubes for total count (TC), non-specific binding (NSB), diethylstilbestrol (DES for a positive control) and total bound (TB) and standard curves tubes were produced from 0.031 ng to 4 ng of 17-β-estradiol/100 µl methanol. Standard curve tubes received 100 µl of the appropriate standard. NSB tubes received 200 ng (200 µl) of 17-β-estradiol, and 10 µL of DES was added to DES control tubes. All standard tubes, DES tubes, and NSB tubes were evaporated to dryness under filtered air. Once the solvent was evaporated, 50 µl of binding buffer [40 ml of 10% glycerol, 0.123 g of 2 mM dithiothreitol, 0.400 g bovine serum albumin (1 mg/ml), 4 ml of 1M tris stock (pH 7.5), 356 ml distilled water] was added to reconstitute the samples and standards. The ERβ was diluted in binding buffer to obtain approximately 0.30 pmol receptor/50 µl binding buffer. 100 µl of ^{3}H-17-β-estradiol was added to all tubes, and 50 µl of receptor was added to all tubes except TC, which received 50 µl of binding buffer. All tubes were then centrifuged at 1000 x *g* for 5 minutes. Tubes were covered with Parafilm™, incubated at room temperature for 2 hours, and then placed in a 4°C refrigerator overnight. An activated charcoal suspension (3 g/ml binding buffer) was prepared and

stirred at room temperature for at least 30 minutes before being stored at 4°C overnight as well.

The following day, the activated charcoal suspension was stirred on ice for 30 minutes. Samples was removed from the refrigerator and placed into an ice water bath. Activated charcoal suspension (1 ml) was added to all tube except TC (which received 1 ml of binding buffer), and tubes were briefly shaken. Tubes were then incubated in an ice-water bath for 10 minutes and centrifuged at 1800 x *g* for 10 minutes. The supernatant was carefully poured into 7 ml scintillation vials and 4 ml of scintillation fluid (Ultimata Gold™; Meriden, CT) was added to tubes. Tubes were capped, briefly shaken, and counted for 10 minutes per vial using a Beckman LS 1800 liquid scintillation counter (Irvine, CA). Data capture was performed using StatLIA™ Analysis software (Brendan Scientific, Carlsbad, CA).

Statistical Analyses

All data were analyzed using SigmaStat® (Systat Software™, Version 3.1, 2004) statistical software. Data are presented as mean ± the standard error of the mean. Data were tested for normality and equality of variances. Although no data met these criteria after repeated transformations, data were analyzed using a one way analysis of variance (ANOVA) because of the power of this test with small sample sizes. Additionally, sites were analyzed using the non-parametric Kruskal-Wallis one way analysis of variance on ranks to confirm the ANOVA results. Sampling sites were compared with all data combined, separated on the basis of gender, species, or both gender and species.

Significant differences were further assessed using Tukey's test when ANOVAs were used or with Dunn's multiple comparison test when the Kruskal-Wallis ANOVA on ranks was used. Additional comparisons were made by performing regression analyses on mean data at each site. For all tests, significance was determined to be $p \leq 0.05$.

RESULTS

EROD Assay

Neither sex nor species significantly influenced the results at any site. Accordingly, data were pooled and comparisons were made based upon site alone. Sites were significantly different ($p<0.001$), with activities in fish from site 178408 significantly greater than all other sites measured (Figure 6A). This site was approximately 4 to 5 times higher (65 pmol/mg protein/minute) than the second highest result at site 159553 (approximately 15 pmol/mg protein/minute). When all sites were combined according to ecobasin, the three ecobasins were not significantly different. Fish from the PDALSP had the highest mean EROD activity, followed by the Saluda Sandhills and the Savannah Sandhills (Figure 6B). Average activities for fish in all ecobasins ranged from 6 to 8 pmol/mg protein/minute.

Bile Fluorescence Assay

Fluorescence at all measured wavelengths was normalized to bile protein. Neither sex nor species significantly influenced the results at any site for any of the three wavelength pairs measured. Therefore, data were pooled at each site. For wavelength pair 290/330 nm (indicative of 2-ringed PAH metabolites) sites were statistically different ($p=0.005$), but multiple comparison procedures failed to discern any further significant differences between sites. The highest mean fluorescence value at this wavelength

occurred in the bile of fish from site 215668 (20,000 fluorescence units/mg bile protein), closely followed by 87719 and 205370 (Figure 7A).

Comparisons of bile fluorescence measured at 341/383 nm (indicative of 4-ringed PAH metabolites) resulted in significant differences between sites ($p<0.001$). Multiple comparison procedure showed that bile fluorescence in fish from sites 87719, 236192, and 142478 were significantly greater than the lowest fluorescence value measured (98871) (Figure 8A). All other sites were not statistically different. Highest sites averaged approximately 10,000 fluorescence units/mg bile protein, while the lowest was measured at less than 2,000 fluorescence units/mg bile protein.

Comparisons of bile fluorescence measured at wavelength pair 380/430nm (5-ringed PAH metabolites) resulted in significant differences in samples from each site ($p<0.001$). Multiple comparison procedures revealed that fluorescence values from fish at site 87719 (1500 fluorescence units/mg bile protein) were the only values that were significantly greater than the two lowest values [sites 287580 and 98871 (250 and 300 fluorescence units / mg bile protein, respectively) (Figure 9A)]. Otherwise, there were no other statistical differences.

Statistical comparisons of the ecobasins revealed significant differences between the three study areas. Fluorescence values were statistically different among ecobasins for 2-ringed PAH metabolites ($p=0.017$), but multiple comparison procedures failed to show any further significant differences between ecobasins at the $p=0.05$ level (Figure 7B). Comparisons for 4-ringed metabolites showed that fish from the PDALSP was significantly greater than those from the Savannah Sandhills ($p=0.012$) but not the Saluda

Sandhills (Figure 8B). Finally, for 5-ringed metabolites, fish from both the Saluda Sandhills and the PDALSP were significantly greater than the Savannah Sandhills ($p=0.017$), but were not significantly different from each other (Figure 9B). Overall trends indicate that average fluorescence values for all three metabolite sizes were highest in the PDALSP sites, followed by the Saluda Sandhills and the Savannah Sandhills (Figures 7-9).

GST Assay

Gender was found to significantly affect GST activities. Therefore, all data were separated at each site on the basis of sex. Sites were statistically different ($p=0.003$) for females, with GST activities in fish at site 145650 significantly greater than those at the lowest site 236192 (Figure 10A). Overall, average values for females ranged from approximately 80 nmol/mg protein/minute to 350 nmol/mg protein/minute. For males and juveniles, despite the fact that sites were found to be significantly different ($p<0.001$), no further differences were detected using multiple comparison tests (Figure 11A). Average values were higher than those measured in female samples and ranged from 125 nmol/mg protein/minute to 350 nmol/mg protein/minute. When data were combined according to ecobasin, the differences observed were not statistically significant for either sex. For female fish GST activities were highest in samples from the PDALSP, followed by the Savannah Sandhills and the Saluda Sandhills (Figure 10B). For male fish GST activities were highest in samples from the Saluda Sandhills (260 nmol/mg protein/ minute),

followed by the PDALSP (240 nmol/ mg protein/minute) and the Savannah Sandhills (210 nmol/mg protein/minute) (Figure 11B).

Estrogen Binding Assay

Unfortunately, bile extracts from the bile fluorescence assays were too dilute for appropriate comparisons, as all of the samples fell below the lowest standard (0.31 ng) of the standard curve of the assay. A few undiluted bile samples remained from the bile fluorescence assay and were used to test the validity of the extraction techniques. Only a few sites (216167 and 159553) had enough undiluted bile to perform the assay, so there were not enough samples to make statistical comparisons or draw conclusions about the potential exposure of these fish to estrogenic compounds. However, the assays that were performed did show that reproductively active female fish were distinguishable from other fish, as they were the only samples that showed any activity in the estrogen binding assay. As expected, all other samples, including male and juvenile fish, had activities that were below the detection limit of the assay.

Somatic Indices

Condition Factor (K)

Neither gender nor species significantly influenced the results. Therefore, data were pooled at each site. Sites were statistically different from each other ($p<0.001$). Multiple comparison procedures revealed that one site (178408) was significantly greater than the two lowest sites (205370 and 177553) (Figure 12A). All other sites were

statistically similar. Average condition factors ranged from 1300 g/cm^3 to 2200 g/cm^3 for fish at individual sites. Significant differences did not exist among ecobasins ($p=0.741$), but there was a general trend toward lower condition factors in both the Saluda Sandhills and PDALSP when compared to the Savannah Sandhills ecobasin (Figure 12B).

HSI Measurements

Neither gender nor species significantly influenced the results. Therefore, data were pooled at each site. Sites were statistically different ($p<0.001$). Multiple comparison procedures revealed that HSIs in fish at site 87719 were significantly greater than those at the three lowest sites (145650, 231143, and 216167), while a large number of sites were significantly greater than fish from site 145650 (205370, 100467, 98871, 236192, 155269, 177553, 215668, and 142478), where the lowest mean HSI value was measured (Figure 13A). Mean HSIs ranged from a low of 0.6 to a high of 2.0 (Figure 13A). Significant differences did not exist among ecobasins, and samples from each ecobasin had a mean of approximately 1.0 to 1.1 ($p=0.103$). However, the Saluda Sandhills and PDALSP were each slightly higher than the Savannah Sandhills (Figure 13B).

SSI Measurements

Neither gender nor species significantly influenced the results. Therefore, data were pooled at each site. Sites were statistically different from each other ($p<0.001$), but no further differences between sites were discerned using Dunn's multiple comparison procedure as sample sizes were too small. Highest SSI values were seen in fish at sites

98871, 87719, 236192, and 215668 (Figure 14A). Overall results ranged from means of less than 0.1 to 0.5. SSIs were statistically different among ecobasins ($p<0.001$), with fish in the PDALSP significantly higher than those in both the Saluda Sandhills and Savannah Sandhills (Figure 14B).

GSI Measurements

Significant differences existed at each site based on gender but not species, so samples were compared based on gender alone. There were not enough juveniles sampled for comparisons among ecobasins, so comparisons of ecobasins were made for males and females only. Female fish demonstrated had a general trend toward decreasing GSIs throughout the sampling season. Female fish from site 216167 had significantly greater GSIs than those at sites 145650 and 98871 ($p<0.05$) (Figure 15A). Female GSIs ranged from a high of less than 0.5 to over 10. Male and juvenile GSIs were not significantly different at any site and ranged from a low of approximately 0.2 to 1.0 (Figure 16A). Male GSIs did not significantly differ by ecobasin, but female GSIs were significantly smaller in fish from the PDALSP when compared to the Saluda Sandhills but not the Savannah Sandhills (Figures 15B and 16B).

Relationships between Biomarkers

There were not enough samples of juvenile tissues for regression analyses, so GST-J and GSI-J values were not included in comparisons. Strongest correlations were found between measurements of bile fluorescence (Table 4). Fluorescence values for 4-

ringed metabolites (341/383 nm) and 5-ringed metabolites (380/430 nm) had statistically significant correlations (r^2 of 0.64). Relationships between bile fluorescence for 2-ringed (290/330 nm) and other fluorescence measurements were statistically significant but showed weaker correlations, with r^2 values ranging from 0.36 when compared to 4-ringed, and 0.31 for 5-ringed metabolites (Table 4). Condition factor was also found to be positively correlated with EROD activities (r^2=0.38). Other biomarker correlations had r^2 values of less than 0.25 and were not determined to be biologically significant (Table 4).

Relationships between Biomarkers and Community Structure

No significant relationships were found between any biomarker measurement and species richness (number of species captured at each site) (Table 4).

Relationships between Biomarkers and Land Use

Neither watershed area nor buffer zone area were found to significantly correlate biomarker measurements (Tables 5 and 6). Human population densities in the watershed area at each site did not significantly correlate with any results (Table 5). The only land use parameters that significantly influenced biomarker results was percentage of impervious surface cover in watershed area and in the buffer zone (Tables 5 and 6). Developed land was positively correlated with bile fluorescence measurements and the HSI ($p<0.05$). Other land use correlations were weaker and not statistically significant.

Ecobasins were significantly different on the basis of land use, specifically developed land, impervious surface cover, forested land, and wetland coverage in each ecobasin. Ecobasins were statistically different as far as percent of watershed area that is developed or covered by impervious surface cover ($p<0.05$), but further differences were not discerned using multiple comparison tests. Overall, the Saluda Sandhills and PDALSP had a greater percentage of developed land than the Savannah Sandhills, while the Savannah Sandhills had a significantly higher percentage of forested land than the other ecobasins tested (Figures 2-5) The PDALSP was the only ecobasin that was significantly higher concerning wetland coverage. All other land uses were statistically similar between ecobasins.

DISCUSSION

EROD Assay

EROD induction has been observed in sunfish in both controlled laboratory studies and in contaminated field settings. Our samples were found to range from approximately 0 to 65 pmol/mg/min, with an average of approximately 6 pmol/mg/min (Figure 4A). EROD activities for the fish in this study are consistent with previous CYP1A activities published for sunfish and Centrarchid fish, though our findings are slightly lower than most published results. For example, Adams et al. (1996), in a July 1990 study of redbreast at sites on the Pigeon River in Tennessee, found EROD activities to range from 15.9 to 28.9 pmol/mg/min for male redbreast at reference sites and 3.1 to 7.7 pmol/mg/minute for reproductively active female fish at the same sites. Schreiber et al. (2006) also found similar results for largemouth bass (*Micropetrus salmoides*), a Centrarchid fish from the same family as sunfish, sampled in August from reference sites along the Reedy River in the piedmont of South Carolina. EROD activities for these fish ranged from approximately 2.5 to 9 pmol/mg/min. Our overall activities were markedly lower than those measured by Jimenez et al. (1988), in their study of feral bluegill sunfish, who observed that summer EROD activities in this species averaged 75 pmol/mg/min. These activities are nearly ten times the basal activities seen in our study. Only fish collected at one site in the present study (178408, which averaged 65 pmol/mg/min) showed activities similar to those measured by Jimenez et al (1988).

It was initially hypothesized that gender would account for the low EROD

activities observed in our study. However, no significant differences were seen detected between male and female fish. Reproductively active female fish tend to have decreased CYP1A activities when compared to reproductively active males due to the effects of estradiol on detoxification abilities and the reallocation of energy resources during reproduction (Stegeman and Hahn 1994). Since part of the sampling season overlapped with the reproductive season for sunfish, it was expected that gender differences would influence EROD activities, but differences were not observed in this study. Our results were particularly surprising because sex differences were observed for GST activities (Figures 9 and 10). While our results were surprising, ours is not the first to fail to observe sex-based differences in CYP1A activities during reproductive seasons. Kirby et al. (1999) found no significant gender differences in EROD activities in feral flounder (*Platichthys flesus*) during spawning and pooled male and female EROD results at sampling sites accordingly. One explanation for the lack of differences observed is that a majority of our sites were sampled in August and September as reproduction was nearing its end (Marcy et al. 2005). It is possible that CYP1A activities in female fish had returned levels comparable to males during the sampling period, and that any gender differences were present at the time of sampling were not large enough to be discerned with the statistical tests performed.

Other studies have noted low EROD activities in sunfish unrelated to gender or spawning season and have formulated several explanations for these results. Schlenk et al. (1996) were unable to find a significant correlation between CYP1A protein content and EROD induction in bluegill, observing large amounts of CYP1A protein in fish

samples without corresponding EROD inductions. They attributed this discrepancy to the possibility of CYP1A enzymatic activity being susceptible to heat inactivation, whereas the actual protein content would be less influenced by heat degradation. It is unlikely that heat inactivation would have played a role in the EROD activities seen in this study, as samples were immediately flash-frozen in liquid nitrogen in the field and other biomarker assays (such as the GST assay) were performed on the same S9 fractions without any sign of decreased activity or degradation. Schlenk et al. (1996) also hypothesized that sunfish contain endogenous inhibitors of CYP1A activity. The presence of endogenous inhibitors has thus far not been examined, so it is unknown if these contributed to the lower EROD activities observed in this study. However, as there are other published reports of CYP1A and EROD activities in sunfish (Adams et al. 1996, Jiminez et al. 1988), it is unlikely that these fish contain endogenous inhibitors for this enzyme. It is also unlikely that exogenous inhibitors contributed to the measured EROD activities, as environmental CYP1A inhibitors generally have to be present in extremely high environmental concentrations to exert an effect on EROD activity (Whyte et al. 2003). Instead, it is very likely that the values measured for sunfish in this study are indicative of basal levels of metabolism for these species.

Strongest relationships were found between the condition factor (K) of the fish and EROD activities, with significant positive correlations found between these two biomarkers (Table 4). Condition factor is a measure of the overall health of a fish and a indication of the free energy storage within the fish (Neff and Cargnelli 2004).This stored energy can be used to perform a number of functions, including reproduction, predator

avoidance, and foraging. In most studies, the CYP1A induction corresponds to a reduced condition factor. For example, Foster et al. (2001) found that increased EROD activities in sturgeon (*Acipenser transmontannus*) from organochlorine pesticides corresponded to a decrease in the condition factor of fish. They observed that decreased condition factor is evidence of overall bodily stress as fish use energy for other biological functions besides storage. Reduced condition factors can also result from toxicant stress due to anorexia or poor feeding (Sorrensen and Bouer 1984). EROD activities observed in the present study are not believed to be indicative of severe toxicant stress and assumed to be representative basal CYP1A activities. Perhaps the extra bodily lipids that would elevate the condition factor also could provide energy for increased basal detoxification abilities to the fish, which would explain the positive correlations between EROD and K.

No other significant correlations were found between EROD activity and any other biomarker (Table 4). It is somewhat surprising that EROD induction was not correlated with bile fluorescence measured in this study, as both are measures of PAH exposure. Aas et al. (2000), in a laboratory experiment with Atlantic cod (*Gadus morhua*), observed significant correlations between EROD data and fixed wavelength fluorescence of bile. Collier and Varanasi (1991) also found significant correlations between bile fluorescence and MFO activities in a study of English sole from the Puget Sound. Contrary to our study, which was entirely field-based, these studies were performed in laboratory settings. Most field studies find correlations of EROD data with bile fluorescence results to be highly variable, as potential confounding variables cannot be controlled. For example, Otter (2006) compared EROD results with fluorescence data

for largemouth bass from the Reedy River of South Carolina and found no relationship between EROD data and any measurements of bile fluorescence. It is possible that the elevated bile fluorescence observed in sampled fish from this study is due to transient PAH exposure that was not sufficient to induce CYP1A. Arcand-Hoy and Metcalfe (1998) found that the presence of fluorescent aromatic compounds in feral brown bullhead catfish (*Ameiurus nebulosus*) was indicative of recent or transient PAH exposures. Additionally, other studies have noted the high inherent variability associated with measurements of fluorescent aromatic hydrocarbons in fish bile (Leadly et al. 1998), making discerning relationships between EROD activities and bile fluorescence more difficult.

Another difficulty specific to the present study is that many of the fish sampled were smaller and classified as juveniles (Table A-1). At sites with the highest measurements of bile fluorescence (for example, site 205370), we were unable to take EROD measurements because there was not enough liver tissue remaining to perform the assay. The inclusion of these EROD values could potentially have strengthened any correlation tests performed.

Although significant differences did not exist between ecobasins on the basis of EROD induction, EROD activities in fish from the Saluda Sandhills and PDALSP, along with bile fluorescence and HSI, were elevated above fish from the Savannah Sandhills. The results suggest PAH exposure and are consistent with a greater proportion of disturbed (developed and agricultural) land in these ecobasins when compared to the Savannah Sandhills. These types of land disturbance are typically associated with

increases in concentrations of PAHs and other CYP1A inducers in the aquatic environment. For example, the combustion of coals, oil, fossil fuels, and wood for household and automobile uses produces PAHs, and urban sprawl has significantly increased the concentrations of these compounds in the environment in the past decades (Van Metre et al. 2003). PAHs tend to be deposited in aquatic systems, particularly in areas that have high percentages of impervious surfaces (such as cities or residences), which increases the chances of runoff or atmospheric deposition (Takada 1990). There were no significant correlations between percentage of developed land and EROD induction in any ecobasin. However, it is possible that the levels of PAHs and associated compounds occurring from urbanization were insufficient (either in duration or concentration) to produce significant differences between ecobasins or sampling sites but were still able to increase basal CYP1A activities in fish from developed watersheds. More research investigating the effects of non-point source pollution from urbanization and the relationships between land use measurements and hepatic biomarkers are needed to fully determine the effects that theses have on CYP1A induction.

Bile Fluorescence Assay

Overall, there appears to be a dominance of fluorescence of 2-ringed PAH metabolites in bile fluorescence samples, as normalized fluorescence values for the 290/330 wavelength pair are 2 to10 times higher than either of the other wavelength pairs measured. Other studies have observed this same trend (for example, Otter 2006) even at reference sites, indicating that fluorescence at this wavelength pair is intrinsically higher

than the other two pairs measured. A second possible reason for the dominance observed in this study is the physical structure of this type of PAH. These compounds are smaller and therefore more water soluble than larger PAHs, making them more readily absorbed, metabolized, and excreted by fish than bulkier pyrene-type (4-ringed) or benzo(a)pyrene-type (5-ringed) compounds (Schwartzenbach et al. 2001). Leadly et al. (1998) found that a prime route of PAH exposure in the channel catfish and other benthic species is water uptake via their gills. This supports results similar results in bluegill exposed to PAH-contaminated sediment reported by Clements et al. (1994). Smaller-ringed PAHs are typically poor substrates for CYP1A and generally do not lead to EROD induction. This could explain their prevalence in the bile of fish from most of the streams sampled without the corresponding EROD induction.

Bile fluorescence at each wavelength pair was correlated with fluorescence values at other wavelengths as well as with land development (Tables 4-6). Correlations ranged from 0.56 to 0.6 between fluorescence measurements and were the strongest correlations among all biomarker measurements. Additionally, bile fluorescence at all wavelengths was well correlated with measurements of land development in both the buffer zone around the stream and the entire watershed area (Tables 5 and 6). The correlations between bile fluorescence measurements would appear to indicate that there were multiple sources of both pyrogenic and petrogenic PAHs coming from developed areas. It is possible that activities associated with development could explain the prevalence of both of these types of PAHs in sampled fish.

A common consequence of rapid development is urban sprawl (Hess et al. 2001). Construction of homes and other structures frequently corresponds to increases in impervious surfaces from tree removal, parking lots, roofs, patios, sidewalks, and other materials for housing (US EPA 2006). Additionally, as humans continue to move to more rural areas, they have increasingly become more dependent upon automobiles for travel, which is also a major source of contamination. Van Metre et al (2003) found that tire wear, oil leaks and spillage, roadway wear, soot, exhaust, and asphalt wear each generated PAHs of varying sizes. Pollution from roads has been shown to spread as far as 600 meters from the source, with some contaminants even more widespread and dispersed (Litman 2001). Transportation not only increases PAHs in the environment but also the associated structures required for increased transportation, such as parking lots and roads. Parking lots and roadways are typically the single largest category of impervious surface in developed areas, covering up to 60% of commercial lands (Litman 2001). It was observed that most of the developed land in the in this study was devoted to road-like surfaces. The combination of PAH output and impervious surface cover in urbanized areas results in increased PAH runoff to streams from cities and developed lands as the compounds are washed from the roads during storm events and transported to bodies of water such as rivers and streams (Takada 1990).

It is likely that development is the cause of for the significant differences observed in bile fluorescence values between ecobasins as well. Trends in bile fluorescence were similar for all three wavelengths, with fish from the Saluda Sandhills and the PDALSP higher than the Savannah Sandhills for each measurement (Figures 6-

8). This corresponded to greater percentages of developed land in these ecobasins as compared to the Savannah Sandhills. Additionally, the Savannah Sandhills had a higher prevalence of forested areas other ecobasins studied, which have been shown to mitigate the effects of urban non-point pollution by the trapping of toxicants in leaf litter and soil, limiting the deposition of anthropogenic contaminants into surface waters (Correll 1997). The hydrophobic nature of PAHs encourages sorption to soils as well as the riparian zone. The trapping of toxicants at the riparian area helps to improve water quality by removing particulates, pesticides, and other contaminants (Correll 1997). It is impossible to directly attribute bile fluorescence to differences in land use. However, significant relationships between land use parameters and fluorescence measurements do exist.

GST Assay

Relatively little research has been performed investigating the activities of piscine GSTs, particularly for sunfish. Donnarumma et al. (1988), in comparative studies of hepatic teleost GSTs, estimated the activity of bluegill GSTs to CDNB to be approximately 350 nmol/mg/minute. The results of our study show that mean sunfish GST activities toward CDNB range from 75 to 350 nmol/mg/minute, which is the basal level activity seen in most fish species (George 1994) (Figures 10A and 11A). There were little differences between sites. Only female fish from site 145650 showed significant differences from fish at lowest site 236192. Otherwise, sites were statistically similar and within published basal ranges of sunfish GST activities.

In the present study GST activities were significantly influenced by gender. It is commonly observed that reproduction will increase GST activities. Hughes and Gallagher (2004) found that GST activities in largemouth bass were increased following exposure to 17-β-estradiol. When the bass were injected with very high levels of estradiol (2 mg/kg), GST activities increased to 1.5 times those of controls after 48 hours. Surprisingly, we saw the opposite in our samples. While both male and female GST activities were significantly correlated, female fish showed proportionally lower GST activities than males. It is possible that elevated estradiol levels present during breeding affect GST induction in the same manner as it does EROD induction, as both are potentially susceptible to a differential allocation of resources during reproductive months (Stegeman and Hahn 1994). Reproductive differences could also account for the only significant differences observed between samples. Female fish show seasonal effects in GST activity that could be associated with reproductive status. It was evident that GST activities, particularly in female fish, increased throughout the sampling season, further suggesting that reproductive status may have had a significant effect on the results (Figure 10A). Fish were sampled earlier in the season from the Saluda Sandhills than from the PDALSP, and female fish from the Saluda Sandhills also had lower GST levels (Figure 10A and 10B). While reproductive status is the most likely explanation for the results observed, particularly in female fish, further research regarding sexual differences in GST activities in sunfish is needed to confirm the effects of estradiol on GST activities.

Temperature can also influence GST activities in fish. Figueiredo-Fernandes et al (2005), found that Nile tilapia (*Oreochromis niloticus*) exposed to paraquat, had greater GST activities at 17°C than 27°C, although this difference was not significant. George et al. (1990) observed that both phase I and phase II metabolic enzymes in the plaice (*Pleuronectes platessa*) were significantly influenced by temperature, with enzymatic activities steadily increasing throughout the summer, fall, and winter to peak in the early spring. It is likely that the trends in ecobasin GST-F activities are more strongly influenced by season and reproductive status rather than xenobiotic exposure.

Interestingly, male fish showed the same patterns as other markers of PAH metabolism such as bile fluorescence and EROD activities, with the Saluda Sandhills and PDALSP higher than the Savannah Sandhills (Figure 10B). While no significant correlations were observed between any biomarker of PAH metabolism and GST-M activities, it is nonetheless noteworthy that GST activities demonstrated the same trends as other biomarkers of xenobiotic exposure. GSTs are typically less responsive to contaminants than other enzymes, and most studies find it difficult to relate elevated GST activities to other biomarkers of contaminant exposure. GST is commonly induced only 2 to 5 fold in the presence of contaminants such as EROD inducers, which further complicates discerning relationships between biomarkers (George 1994). Andersson et al. (1985), in a study with rainbow trout, found that exposure to CYP1A inducers increased activities of EROD by 50-70 fold, whereas GST activities in the same fish were increased by only 1.5 to 3-fold. McFarland et al. (1999) were unable to find a relationship between GST activities in brown bullhead catfish and pollution from a coking plant in the Black

River of Ohio, despite the correlation of other biomarkers to PAH exposure. In the present study, it is possible that the PAH exposure that resulted in increased bile fluorescence may have been insufficient to induce phase I or II enzyme activities beyond basal levels, but still slightly increased the enzymatic activities in more developed areas.

Because female GST activities were found to be strongly affected by season and reproductive status, it was difficult to find any relationship between female GST activities and land use parameters. However, male GST activities, which were less affected by reproductive status, were positively correlated with land development and impervious surface area (Tables 5 and 6). GST activities can be induced to combat the oxidative stress associated with phase I detoxification reactions that occur during PAH metabolism, including reactive oxygen species (Nebert et al. 1993). PAHs are commonly associated with urban development and can lead to some GST induction, a potential explanation for the positive (though statistically insignificant) correlations between land development and GST activity. Few studies have been performed examining the relationship between land use and GST activity, but it is possible that the increase in human activities and urban development leads increases in contaminants. Further research investigating the effects of land use and the non-point pollution associated with development is needed to discern the relationship between these two variables.

Condition Factor (K)

Most condition factors in sampled fish were similar and ranged from 1300 g/cm^3 to 2200 g/cm^3. Only one site was found to be significantly higher than the others. Fish

from site 178408 was significantly higher than fish from sites 205370 and 177553, while all other sites were statistically similar (Figures 12A and 12B). Generally, condition factor reflects the size of the fish and the energy reserves available for various functions including growth, survival, and reproduction (Barnham and Alexander 1998). It is possible that the age of the fish likely had some influence over the differences observed between these sites. Age has been shown to influence the value of K due to the different energetic needs of fish at different ages (Barnham and Alexander 1998). Juvenile fish are still developing and expending much energy in growth, so these fish would be unlikely to be storing additional energy in the form of lipids in their bodies. Field notes that all fish from sites 205370 and 177553 were classified as juveniles because of their sizes, with the smallest lengths and weights of any fish captured during the study (Table A-1). They also lacked discernable gonads, further confirming their status as juveniles. Sunfish with the largest bodily masses were captured at site 178408, a potential reason that the condition factors for these fish were significantly different from those of site 205370 and 177553.

When samples were combined according to ecobasin, condition factor showed an opposite pattern to EROD, HSI, bile fluorescence, and male GST activities (Figure 12B). Highest condition factors were observed in the Savannah Sandhills, while lower Ks were found in the Saluda Sandhills and PDALSP. This can be the result of the higher percentage of forested land in the Savannah Sandhills. Studies of urbanization have found that urban runoff is a significant source of metals and PAHs that are deposited in the water and sediments, with levels of PAHs higher at highways and for industrial uses than for residential areas (van Metre et al. 2003). Each of these is generally associated with

reduced condition factor in fish. For example, studies of the yellow perch (*Perca flavescens*) from sites contaminated by organic contaminants (PAHs and PCBs) and metals (mercury, cadmium, arsenic, and zinc) showed that fish from contaminated sites exhibited significantly smaller condition factors than fish from a reference site (Hontella et al. 1995). These effects are generally lessened by an increase in forested cover near the water, which not only provides shade and higher quality habitat, but also helps to prevent contaminants from reaching the stream (Wang et al. 2001). It would be beneficial to further determine the effects of riparian zones on surface runoff as well as determine the effects of both land uses on the condition factor of fish.

HSI Measurements

The HSI is influenced by many factors, including age, sex, nutritional status, toxicant stress, and diet (Adams and McLean 1985). Although this makes interpreting the results fairly difficult, the HSI is often used to support other biomarker measurements and (Leadly et al. 1998). In the present study, mean HSIs ranged from 0.5 to 1.8, which are consistent with the findings of previously reported results. For example, redbreast sunfish in eastern Tennessee had HSIs ranging from 0.62 to 0.87 for males and 0.8 to 1.7 for females at control sites in July samples (Adams et al. 1992). It was apparent in this study that there was considerable variation between fish from the same sample sites as well as between different sites.

The variation that was observed between sampling sites can be attributed to several factors. As with all other somatic measurements, reproductive status likely

affected the observed results. The HSI can increase prior to reproduction in both males and females. It has been observed that fish, particularly females, can store energy in the liver for reproduction (Arnott et al. 2000). Most of this energy in females is due to the presence of increased estrogens in circulation at the time of reproduction. Vitellogenin synthesis will occur in the liver of female fish following estrogen stimulation, and this will generally increase the size of the liver to sizes that are several times larger than males during the breeding season (Orlando et al. 1999). Our results support this, as we observed a trend toward higher HSIs over the sampling period as reproduction ceased in September although no gender differences were observed between males with females. As with other measurements, the HSI likely peaks in sunfish in the spring before reproduction, declines immediately following spawning, then gradually increases as the fish recover from the depletion of energy for gonad development. Bullow et al. (1978) found that HSIs will generally decrease in bluegill in the late spring and in the summer, as fish use their liver storage for gonad development, and then increases during recovery from reproduction.

It is also possible that the overall increase in HSIs during the sampling period is due to a second factor unrelated to reproduction. As noted before, parasites were present at many sites in the PDALSP. Though the liver is typically less affected by parasitic infection than other organs (Esch and Huffnies 1973), even when numerous parasites exist on it, progressive hepatomegaly is still commonly observed when fish are under parasitic stress (Lowe-Jinde 1980). This may partially explain the higher HSIs in fish from the PDALSP.

Despite the observed differences in reproduction and parasitism, the HSI still appears to be somewhat related to land development and shows the same patterns as other biomarkers of xenobiotic metabolism (bile fluorescence, EROD and GST activity). The HSIs in fish from the Saluda Sandhills and PDALSP were slightly elevated above the Savannah Sandhills, a trend that is positively correlated to land development and impervious surface cover. As stated above, impervious surface cover and development is associated with increased PAHs and surface runoff, both of which have been shown to lead to increases in the HSI. Sites at which fish had the greatest HSIs (205370 and 100467) were consistent with HSI values for sunfish exposed to PAHs and PCBs (Theodorakis et al. 1992). Mean values in that study ranged from 0.9 to 1.4 for control fish, while those exposed to PAHs ranged as high as 2.25. Similarly, Terreault et al. (2003) observed an increase in HSIs concurrent with increased EROD activities in slimy sculpin (*Coltus cognatus*) and pearl dace (*Semotilus margarita*) exposed to oil sands, attributing this to either fatty liver from extensive xenobiotic metabolism or an increase in phase I enzyme production in the liver to combat xenobiotic stress.

The HSI was also significantly correlated to certain bile fluorescence measurements, strengthening the possible relationship between PAH metabolism and liver size (Table 4). Few studies have been performed linking increased HSIs with increased bile fluorescence, but generally there is a positive correlation between the two variables. Pinkney et al. (2000) found the HSI to be positively correlated with biliary PAH concentrations in a river with known PAH contamination. The correlation of HSIs with bile fluorescence and with land development would appear to indicate that

significant differences in HSIs between ecobasins is due to urban runoff combined with both reproductive and parasitic influences.

SSI Measurements

Spleen size increases in response to a number of factors, both biotic and abiotic (Selgrade 2004). As a result, it is frequently difficult to determine a single causal agent when using the SSI to assess the immunological fitness of an organism (Schwager et al. 2001). Indeed, in this study it is apparent that there are several factors that contributed to the difficulty of interpreting the SSIs observed for these fish.

The reproductive status of the fish sampled likely affected the results. Fish sampled in August and September had markedly higher SSIs than fish sampled in May and June. This is one of the reasons that the samples from the PDALSP had higher average SSIs than fish from the other two ecobasins. These fish were sampled when they were reaching the end of the reproductive season. Reproduction may significantly decrease SSIs in fish, particularly in female fish. For example, Kortet et al. (2003) found that spleen size is decreased in both male and female roach (*Rutilus rutilus*) during the breeding seasons due to a re-allocation of resources during the reproductive season. More energy is needed for reproduction and spawning than during the non-breeding season. In the present study, both male and female fishes show the similar trends of increasing SSI throughout the sampling season.

A second reason for the increase in spleen size observed in PDALSP sites may be due to parasitic infection. Parasitic infection can induce lesions and cause a proliferation

of reticuloendotheila cells and eosinophilic granulocytes, resulting in an increased SSI observed in parasitized fish (Esch and Huffines 1973). Field notes from many sites in the PDALSP ecobasin indicate that parasites were visible on fish obtained from many sites, including sites 87719, 236192, and 98871, where the largest SSIs were observed in sampled fish. These parasites were tentatively identified as *Posthodiplostomum minimum*, a white liver grub that penetrates the fish between scales and attaches to the liver, kidney, spleen, and mesenteries through the circulatory system (Reed 1955, Miller 1954). This is among the most common of the many parasites that infects sunfish and has been found to enlarge the sunfish spleen due to the spleen's role in hematopoetic and immunological functioning (Adams et al. 1998). *P. minimum* were not noted in any samples from the Savannah or Saluda Sandhills. It was difficult to determine the extent of parasitism in fish or determine exactly which parasites were present as no histological examination or exact quantification was performed on these samples. However, visual inspection of fish would suggest that fish in the PDALSP showed more signs of parasitic infection than fish from other ecobasins.

Parasitic infection could affect fish in several ways, and evidence for both beneficial and detrimental effects has been found. Some have found that parasitism is not detrimental to population structure as long as it does not impact reproduction. For example, smallmouth bass were able to grow and reproduce at rates similar to non-parasitized fish even when infected with up to fifty parasites per fish (Esch and Huffines 1973). Studies of stickleback fish have shown that parasitism by plerocercoids (the larva of tapeworm) caused the fish to have spleens that were approximately three times larger

than uninfected fish, but that fish grew faster and in some cases actually had better body conditions than uninfected fish (Arnott et al. 2000). This growth enhancement is either a response of the fish to infection or a response of the parasite to enhance survival.

Conversely, parasitism can be an energetic drain in fish, causing them to be more vulnerable to the effects of abiotic and biotic stressors. Lemly and Esch (1984), in a study of artificially parasitized bluegill, found that fish with large numbers of *Uvulifer ambloplitis* (a flattened worm also known as a fluke) did not survive increasingly colder water temperatures, whereas lesser parasitized and non-parasitized fish were better able to adapt to the changes in water temperature. There appears to be a reciprocal relationship between parasitism and toxicant stress, as parasitism makes fish more vulnerable to the effects of toxicants, while the presence of toxicants may make the fish more vulnerable to parasitic infection.

In this study parasitic infections are present without significant identifiable toxicant stress. However, parasitized fish are still more vulnerable to anthropogenic disturbance that can have implications for community structure in the future. Interestingly there appears to be a slight negative relationship between the SSI and species richness at each site in the PDALSP. However, community measurements of assemblage structure are dependent upon many factors including habitat and stream size, so it is unlikely that parasitic infection is contributing to assemblage structure changes. However, long-term studies assessing the impact of these parasites on the overall community structure would be helpful to determine the effects of these parasites in South Carolina streams.

GSI Measurements

The GSIs measured in this study are typical of both reproductively active and inactive fish. Male sunfish typically have GSIs of 1.5 to 2 during the breeding season, which then recede to 0.41 to 0.88 after reproduction (Kaya 1973). Female fish are more variable, with ranges of 4 to 6 for reproductively active female fish and 0.47 to 0.82 during gonad regression (Kaya 1973). While no significant trends were observed for male and juvenile fishes, female fish from sites in the PDALSP were found to have much smaller GSIs than in other ecobasisns (Figure 15B). These fish were sampled in August and September, well after the typical breeding season for sunfish. As a result, the GSIs are correspondingly smaller for these fish, and female GSIs return to values that are approximately equal to those seen for males and juveniles of the same time period.

Gonad regression following the breeding season is common in sunfish. Studies with the green sunfish, *Lepomis cyanellus*, have shown that water temperature is a key factor in gonad size (Kaya 1973). Long photoperiods and elevated water temperature typically lead to increased gametogenesis and gonad development as fish prepare for breeding. Accordingly, gonads in sunfish will regress in a process called gonad involution, which also occurs more rapidly at higher temperatures (Kaya 1973). Pumpkinseed sunfish also showed temperature dependent responses in respect to GSIs, with both male and female GSIs increasing with increasing temperature (Burns 1976).

Female fish typically utilize many nutrient resources for spawning and reproduction, so it is to be expected that there would be negative relationships between the GSI and other somatic indices. Vitellogenesis and the production of eggs typically

requires for female fish to obtain energy from other sources and divert energy use toward reproduction (Orlando et al. 1999). This is reflected in the negative relationships between the female GSI and other somatic indices (Arnott et al. 2001). We observed negative relationships between were seen between the GSI and the SSI and HSI in female fish, again likely because of the differential allocation of resources during the reproductive season.

Relationships between Biomarkers and Community Structure

Fish in South Carolina freshwater streams have been shown to respond to disturbances that are anthropogenic, biotic, and abiotic (Paller et al. 1996, Adams et al. 1995). However, most studies of the effects of xenobiotics have focused on a single species without determining the effects that this exposure may have on higher levels of biological organization, such as populations and communities. Generally, few studies have been able to definitively tie changes at the molecular level to changes at the community level (Forbes et al. 2005). Most find that it is risky to attempt to use molecular results to explain population or community structure, as numerous factors affect population dynamics, including natural heterogeneity, unpredictability of the natural environment, and physiological compensations developed by individuals to combat xenobiotic exposure (Lagadic 2002).

Links between specific biomarkers and community level effects have been found in a few instances. Previous studies have used redbreast sunfish as a sentinel species and

measured a suite of biomarkers to determine if biomarker responses on the tissue level mimicked responses at the community level. Adams et al. (2000) sampled a downstream gradient of contamination from industrial sources in a freshwater stream in Tennessee to discern if the changes in redbreast at the molecular level (phase I and II enzyme induction, somatic changes, hormonal changes) corresponded with changes at the population and community level, observing increases in species richness as sample sites increased in distance from contaminant sources. EROD induction was observed to be among the best predictor of effects at the community level because of the sensitivity of MFO enzymes. It has been suggested that the molecular changes from xenobiotic exposure may change the behavior of individual fish and fish interactions in populations and communities. Changes in population function and structure can drastically change community and ecosystem functioning, leading to detrimental effects over time.

Relationships between any biomarker measurement and community structure (as measured by species richness) were not observed in this study. Many of the biomarker measurements of xenobiotics exposure were at basal levels. This may be attributed to transient exposures that are unlikely to affect the overall health of the fish. It is therefore not to be expected that any of the biomarker measurements would be indicative of any kind of population-level or community-level effects. Weak relationships between all data were found to exist between species richness, EROD induction, and bile fluorescence, as correlation coefficient ranged from as small as 0.02 to 0.22 (Table 4). The lack of relationship between any of these variables seems to point to other factors unrelated to

contaminant exposure, including habitat structure, that shape the communities at these individual sites.

It is noteworthy that parasites are known to have a controlling effect on community structure in animals, along with competition and predation, and can have a wide range of effects on populations (Minchella and Scott 1991). The modification of fish behavior and habitat selection, the effects on reproductive success, and the changes in competition between fish resulting from parasitic infection work to alter population dynamics and community structure, which can affect fish abundance, species richness, and diversity over longer periods of time (Mouritsen and Poulin 2002). It would be worthwhile to continue to study specific sites in the PDALSP to see if future changes occur in community structure over time as a result of decreased fitness from parasitic infection

SUMMARY AND CONCLUSIONS

This study is part of the first state-wide assessment of wadeable streams in South Carolina and is the first to estimate the ecological health of fish in the smaller streams of the Saluda Sandhills, Savannah Sandhills, and Pee Dee Atlantic Southern Loam Plains. It is also among the first to use GIS-based land use parameters to estimate the effects that land use may have on biomarkers of xenobiotic exposure. The present study summarizes the first year of data and provides information about the health of fish in three ecobasins.

The suite of biomarkers used in the present study is indicative of several types of contaminants that are commonly found in urban environments. The overall condition of the fish was measured through the condition factor, which is a crude estimate of the fitness of individuals. Bodily condition was further determined using common somatic indices such as the hepatosomatic index (HSI), spleen-somatic index (SSI), and gonadosomatic index (GSI). It has been noted that several of these types of biomarkers are non-specific for a particular type of contaminant but can be used to support other, more specific biomarkers.

To further assess the exposure of fish to xenobiotics, several biomarkers of specific contaminant types were used. Phase I and phase II detoxification enzymes were measured to determine PAH exposure and oxidative stress. EROD activity was used to determine the extent of CYP1A induction by larger PAHs, and GST activity were used to further support measurements of EROD induction and provide a measure of oxidative stress within the fish. EROD is a classical biomarker of PAH metabolism and is among

the most sensitive enzymatic biomarkers used to determine PAH exposure. GST is generally less sensitive to xenobiotic exposure than CYP1A but can be further used to support measured EROD activities. Bile fluorescence was also measured to determine the metabolism of PAHs in sunfish and provide an estimate of the exposure of fish to 2-ringed, 4-ringed, and 5-ringed PAHs. As bile fluorescence is less susceptible to degradation than CYP1A, it is frequently a strong compliment to measurements of phase I and phase II enzymatic measurements.

From the data, it was evident that several factors affected the biomarker expression observed in fish in the studied ecobasins.

- First, it was apparent that there were significant seasonal and reproductive impacts on certain biomarkers. It was evident that reproductive effects were more pronounced in female fish, a potentially confounding factor in the interpretation of biomarker results. Sex was found to significantly influence GSI measurements and GST activities, but seasonal trends were apparent in the results observed for the HSI and SSI as well. Gender and season have been shown to affect all of the biomarkers measured for this study, and our results further confirm the importance of controlling these variables for field studies.
- Secondly, biotic influences such as parasitic infection potentially influenced the results as well. Parasitic infection can cause a differential allocation of energy resources so that a fish can mount an immune response to the parasite. Indeed, in our samples, negative correlations were observed between the SSI and the HSI and GSI, particularly in fish that were observed to have parasites present on the

liver in the PDALSP. It is unfortunate that a more detailed analysis of the liver and spleen was not performed in sampled fish to identify the parasites that were present in the PDALSP. Perhaps future analyses can perform histological examinations to determine specific effects of parasites on tissues.

- Finally, land use was found to significantly influence many biomarkers. Very few studies have been performed relating biomarker results to land use parameters, and ours is the first to relate these measurements in South Carolina. As very little research has been performed regarding land use and biomarkers, it is unknown to what extent development can progress before significantly deleterious effects begin to occur. At the present time, both biomarkers of exposure (bile fluorescence) and effect (HSI) were related to developed land, and it is believed that these effects will continue to increase as development progresses in the watersheds. It is unknown how development affects biomarker responses or how development affects fish on the molecular scale. In the present study, it is likely that increased land development is causing non-point source pollution in the streams studied. The significant differences in biomarker measurements in fish from different ecobasins may be related to differences in land use within each ecobasin.

Species	Common Name	Habitat	Spawning Season	Diet
Lepomis auritus	Redbreast	Variety of substrate types and deeper pools with undercut banks, stumps, woody debris and cover.	Late May through the end of July	Aquatic and terrestrial insects, microcrustaceans, crustaceans, and mollusks
Lepomis macrochirus	Bluegill	Versatile and can inhabit lakes, reservoirs, and ponds with a broad range of pH, salinities, and temperatures	May through August, with a peak in June.	Insects that live on aquatic vegetation zooplankton
Lepomis gulosus	Warmouth	Frequently associated with aquatic vegetation, brush piles, submerged roots, and rock rubble in slow, still waters such as ponds and reservoirs	April through August, with a peak in June	Aquatic insects, crustaceans such as amphipods and shrimp, fish
Lepomis marginatus	Dollar	Generally shallower waters than other Centrarchids such as small headwater streams, side channels, and backwaters	May through August	Aquatic insects, terrestrial insects, snails, oligochates
Lepomis microlophus	Redear	Lowland swamps, reservoirs, ponds, and sluggish waters	April through June	Adults feed primarily on sediment-dwelling prey such as mollusks while juveniles eat invertebrates and zooplankton
Lepomis gibbosus	Pumpkinseed	Sluggish waters and quiet lakes with heavy vegetation	May though August	Juveniles feed on soft invertebrate while adults feed on vegetation-dwelling gastropods, mollusks, snails, and plankton

Table 1. Individual characteristics of sunfish used in this study. From Marcy et al. (2004).

Classification	Definition
Water	All areas of open water or permanent ice/snow cover
Developed	Areas characterized by a high percentage (30 percent or greater) or constructed materials (e.g. asphalt, concrete, buildings, etc.) and can be further subdivided into: • Developed Open Space (<20% impervious cover) • Developed Low Intensity (20-49% impervious cover) • Developed Medium Intensity (50-79% impervious cover) • Developed High Intensity (80-100% impervious cover).
Barren	Areas characterized by bare rock, gravel, sand, silt clay, or other earthen materials with little or no "green" vegetation present
Forested	Areas characterized by tree cover (natural or semi-natural woody vegetation, generally greater than 6 meters tall); tree canopy accounts for 25-100% of cover
Shrubland	Areas characterized by natural or semi-natural woody vegetation with aerial stems, generally less than 6 meters tall, with individuals or clumps not touching or interlocking
Grassland/Herbaceous	Areas dominated by grammanoid or herbaceous vegetation, generally greater than 80% of total vegetation
Agricultural	Areas characterized by herbaceous vegetation that has been planted or is intensely managed for the production of food, feed, or fiber. Herbaceous vegetation accounts for 75 to 100% of cover
Wetlands	Areas where the soil or substrate is periodically saturated with or covered with water

Table 2. Land use classifications as defined by the United States Environmental Protection Agency's Multi-Resolution Land Characteristics Consortium (US EPA 2007).

Ecobasin	Site Number	Sample Date	Latitude	Longitude	Watershed Area (km^2)
Saluda Sandhills	207511	5.24.2006	33.99107	81.26844	5.29
Saluda Sandhills	216167	5.24.2006	33.94643	81.2934	59.74
Saluda Sandhills	205370	5.30.2006	34.00502	81.09119	6.23
Savannah Sandhills	287580	6.06.2006	33.56462	81.87298	63.01
Savannah Sandhills	346456	6.13.2006	33.55402	81.80125	5.07
PDALSP	231143	8.01.2006	33.87917	80.41662	129.74
PDALSP	132724	8.02.2006	34.35708	79.51189	26.65
PDALSP	236192	8.03.2006	33.86467	80.39501	59.38
PDALSP	153122	8.08.2006	34.24792	79.76696	92.94
PDALSP	178408	8.09.2006	34.12706	79.8215	6.52
PDALSP	177553	8.09.2006	34.13659	79.67572	5.35
PDALSP	100467	8.15.2006	34.51595	79.74564	72.09
PDALSP	159553	8.16.2006	34.22031	79.48608	30.76
PDALSP	155269	8.17.2006	34.26095	79.94437	16.78
PDALSP	215668	8.22.2006	33.94879	80.41985	32.79
PDALSP	142478	8.23.2006	34.30009	79.24415	5.17
PDALSP	87719	9.06.2006	34.58005	79.69834	5.12
PDALSP	145650	9.20.2006	34.28546	79.68657	78.56
PDALSP	98871	9.20.2006	34.52654	79.38184	4.62

Table 3. Site and sampling dates and locations.

	EROD	290/330	341/383	380/430	GST-M	GST-F	K	HSI	SSI	GSI-M	GSI-F	Species Richness
EROD		-0.08	-0.01	0.00	-0.01	0.11	**0.38**	-0.18	0.12	-0.03	0.00	0.00
290/330	-0.08		**0.36**	**0.31**	0.04	-0.10	**-0.36**	0.14	0.01	0.12	-0.04	0.00
341/383	-0.01	**0.36**		**0.64**	-0.02	-0.30	0.08	0.19	0.08	0.15	-0.09	0.02
380/430	0.00	**0.31**	**0.64**		0.02	0.25	0.13	**0.21**	0.09	0.00	-0.13	-0.05
GST-M	-0.01	0.04	-0.02	-0.02		0.41	-0.09	**-0.31**	0.04	-0.06	-0.07	-0.01
GST-F	0.11	0.10	0.30	0.25	0.41		0.13	0.07	0.01	0.10	-0.10	0.02
K	**0.38**	**-0.36**	0.08	0.13	-0.07	0.13		-0.01	0.22	0.02	0.00	0.00
HSI	-0.18	0.14	0.19	**0.21**	**-0.31**	0.07	-0.01		0.05	**0.31**	-0.03	-0.09
SSI	0.12	0.01	0.08	0.09	0.04	0.01	0.22	0.05		0.10	-0.08	-0.07
GSI-M	-0.03	0.12	0.15	0.09	-0.06	0.10	0.02	0.31	0.10		-0.05	0.01
GSI-F	0.00	-0.04	-0.09	-0.13	-0.07	-0.10	0.00	0.03	-0.08	-0.05		0.00
Species Richness	0.00	0.00	0.02	-0.05	-0.01	0.02	-0.01	-0.08	-0.07	0.01	0.00	

Table 4. Correlations (r^2 values) between biomarker measurements. Significant correlations are in bold.

	EROD	290/330 (2-ringed PAHs)	341/383 (4-ringed PAHs)	380/430 (5-ringed PAHs)	GST-M	GST-F	K	HSI	SSI	GSI-M	GSI-F
Impervious Surface	0.05	**0.21**	**0.37**	**0.26**	0.01	-0.06	-0.13	**0.28**	0.00	-0.04	-0.00
Forested	0.00	-0.10	-0.18	-0.04	0.06	0.00	0.03	-0.16	-0.22	-0.30	0.19
Shrub	0.08	0.00	-0.02	-0.03	0.00	0.14	0.01	0.00	0.09	0.23	0.16
Grass	0.00	-0.05	-0.29	-0.22	-0.08	0.00	0.03	-0.07	-0.04	-0.16	0.61
Agriculture	-0.02	-0.02	-0.01	-0.06	-0.02	0.04	0.04	-0.06	0.24	0.18	-0.24
Wetland	0.10	-0.01	-0.71	-0.02	-0.15	0.00	0.01	0.02	0.00	0.35	0.01
Total Surface Area	-0.04	0.00	-0.04	-0.02	-0.01	0.14	-0.05	-0.17	-0.14	-0.02	0.00
Population	-0.03	0.01	0.00	0.00	0.00	0.14	0.06	-0.08	-0.07	-0.10	0.00

Table 5. Correlations (r^2 values) between biomarker measurements and watershed land use percentages. Significant correlations are in bold. Buffer zone land usages are described in Table 1.

	EROD	290/330 (2-ringed PAHs)	341/383 (4-ringed PAHs)	380/430 (5-ringed PAHs)	GST-M	GST-F	K	HSI	SSI	GSI-M	GSI-F
Impervious surface	0.00	0.16	**0.27**	0.17	0.10	-0.02	-0.14	0.20	-0.02	-0.95	0.03
Forested	0.00	-0.03	-0.02	0.01	-0.01	-0.02	0.00	-0.01	-0.10	-0.22	-0.17
Shrub	0.01	0.07	-0.03	0.01	0.02	-0.12	-0.13	0.00	-0.02	-0.01	0.10
Grass	-0.01	0.06	-0.05	0.00	0.01	-0.05	-0.01	-0.01	0.00	-0.03	-0.38
Agriculture	0.01	0.00	0.00	-0.01	0.04	0.00	0.00	-0.02	0.05	0.06	0.00
Wetland	0.00	-0.04	-0.02	-0.04	-0.05	0.05	0.12	0.00	0.06	0.22	-0.38
Total Surface Area	-0.03	-0.08	-0.03	-0.01	-0.01	-0.08	0.00	-0.11	-0.04	0.00	0.32

Table 6. Correlations (r^2 values) between biomarker measurements and buffer zone land use percentages. Significant correlations are in bold. Buffer zone land usages are described in Table 1.

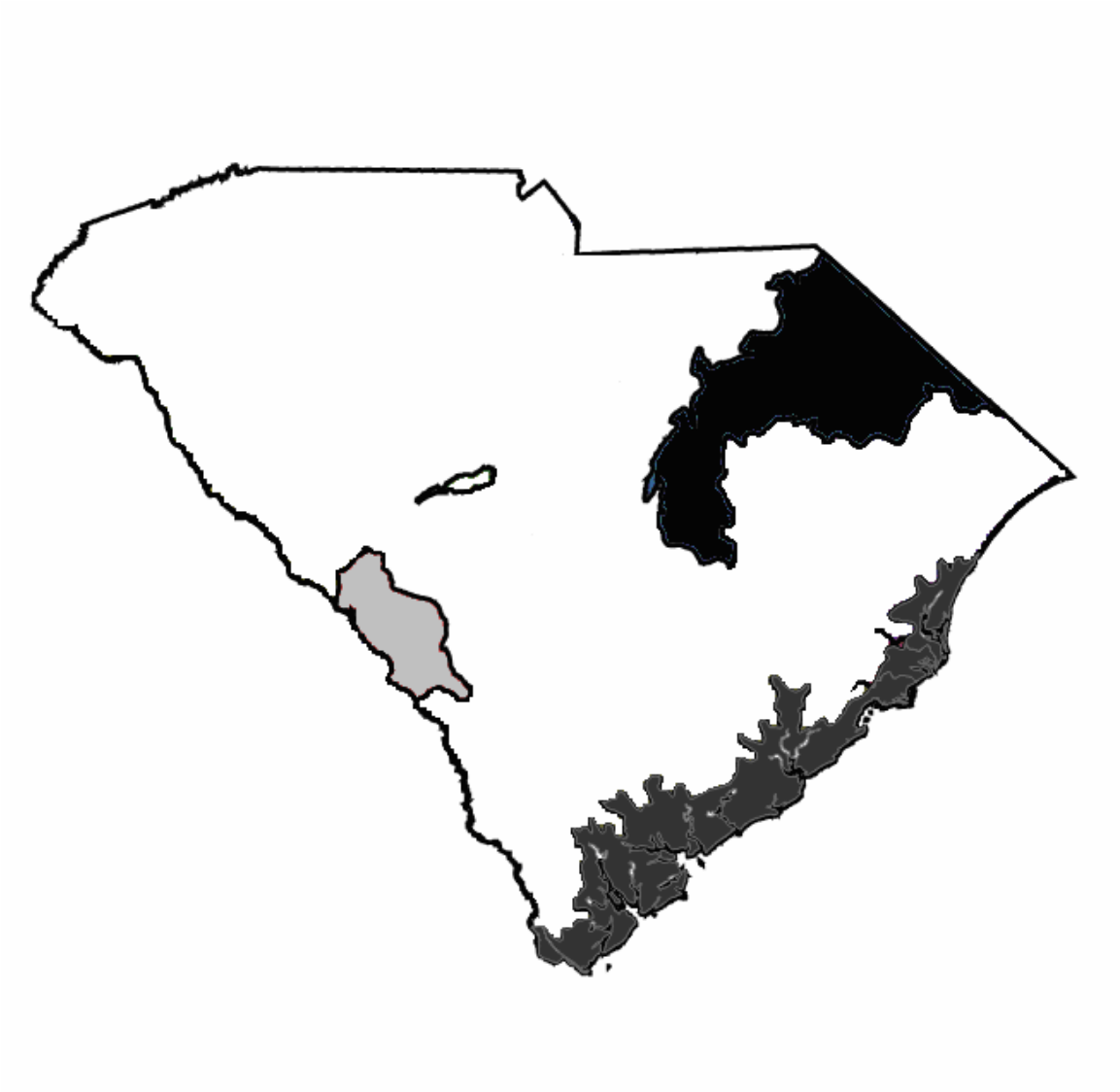
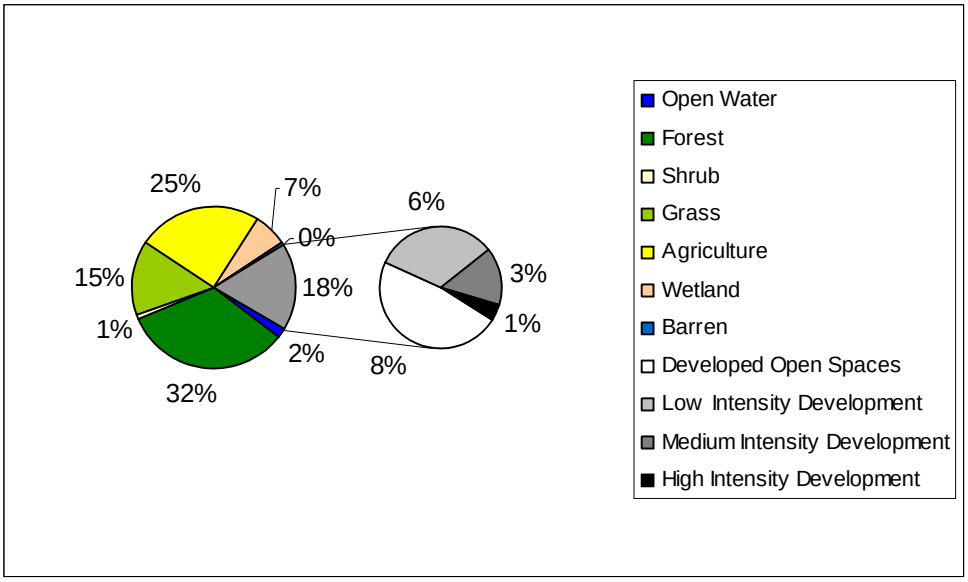


Figure 1. Map of sample locations. White area indicates Saluda Sandhills. Gray area indicates Savannah Sandhills. Black area indicates PDALSP. Individual site locations are given in Table 3.

A.



B.

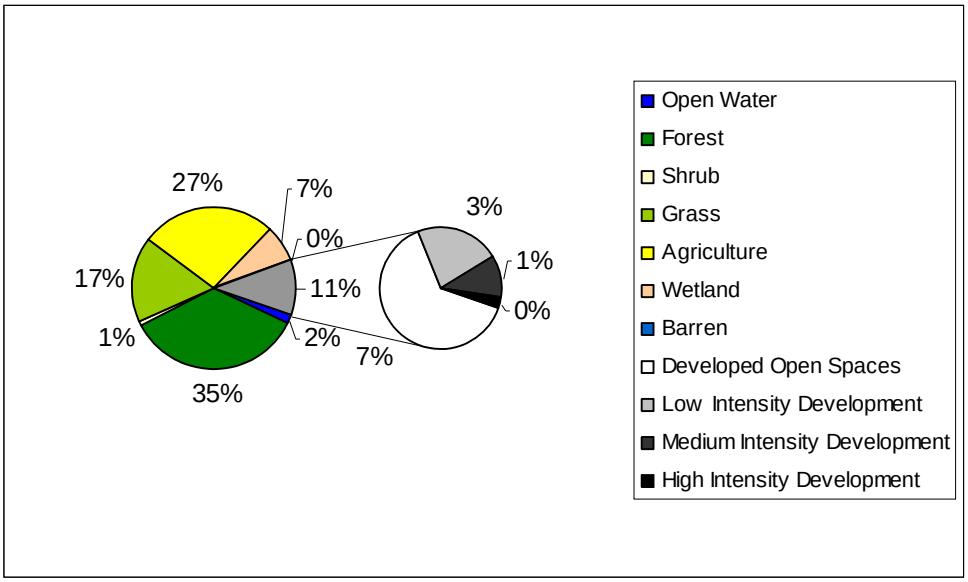
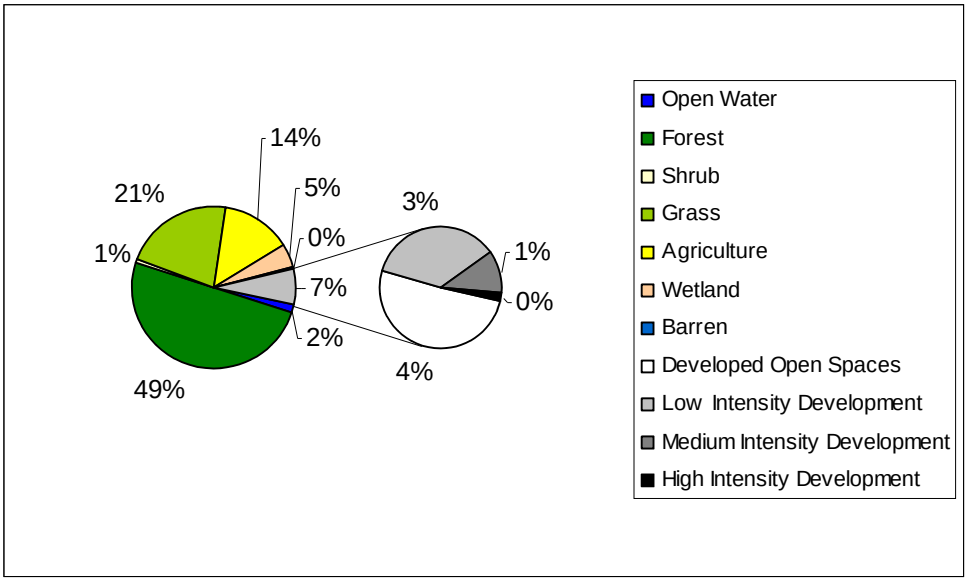


Figure 2. Land use for sample sites in the Saluda Sandhills ecobasin. Graph A represents the land use percentages for the watershed areas around all sample sites in the ecobasin. Graph B represents land use percentages for the 100 meter buffer zone around all sample sites in the ecobasin. Land uses are described in Table 2.

A.



B.

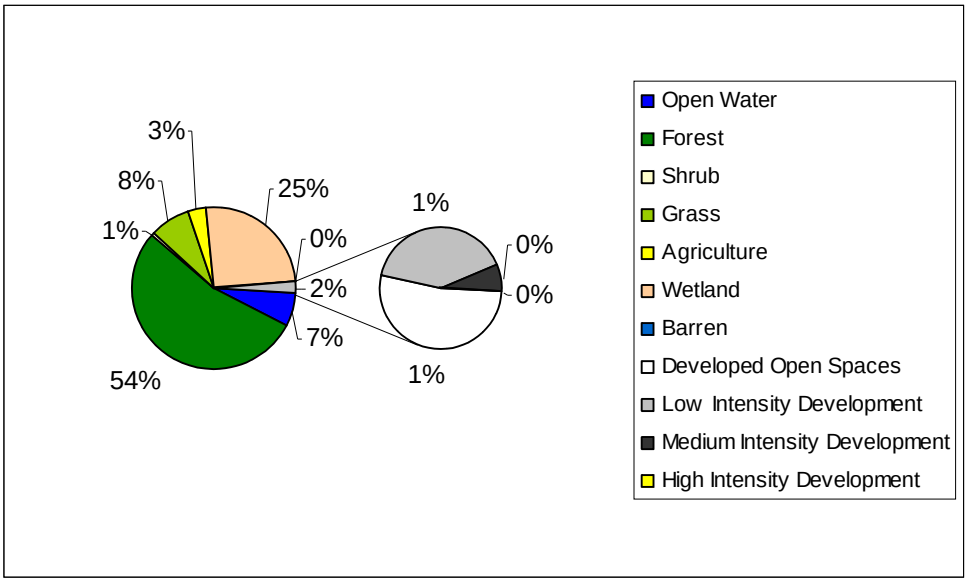
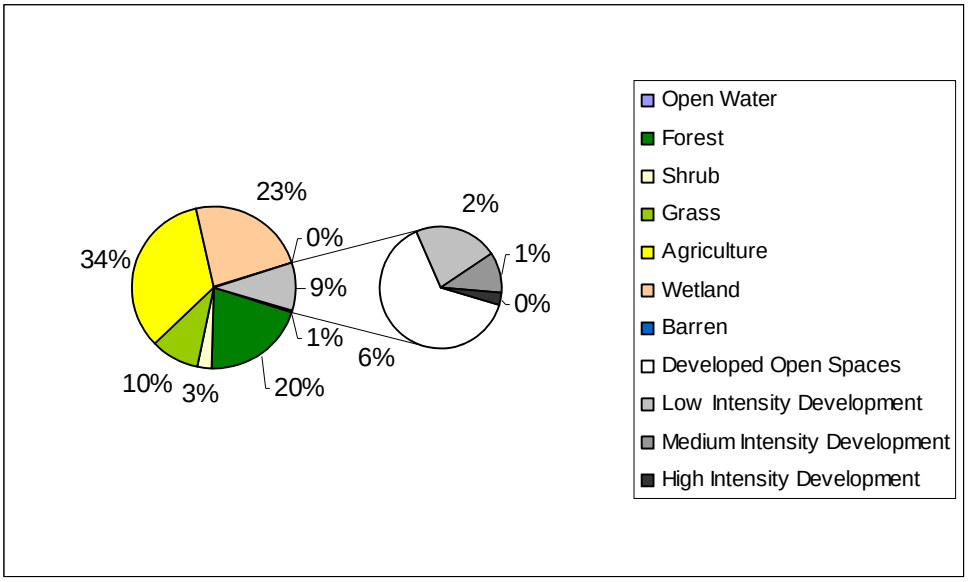


Figure 3. Land use for sample sites in the Savannah Sandhills ecobasin. Graph A represents the land use percentages for the watershed areas around all sample sites in the ecobasin. Graph B represents land use percentages for the 100 meter buffer zone around all sample sites in the ecobasin. Land uses are described in Table 2.

A.



B.

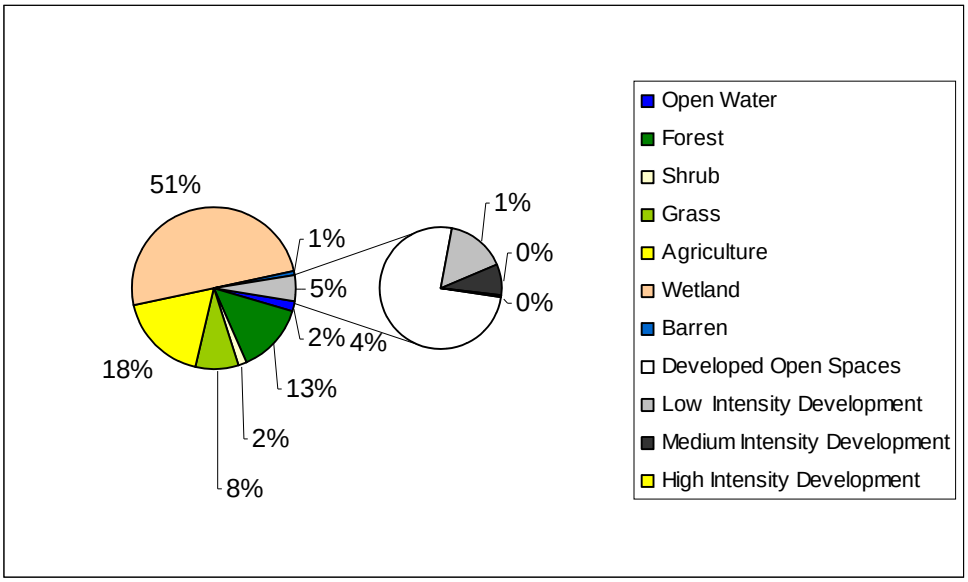
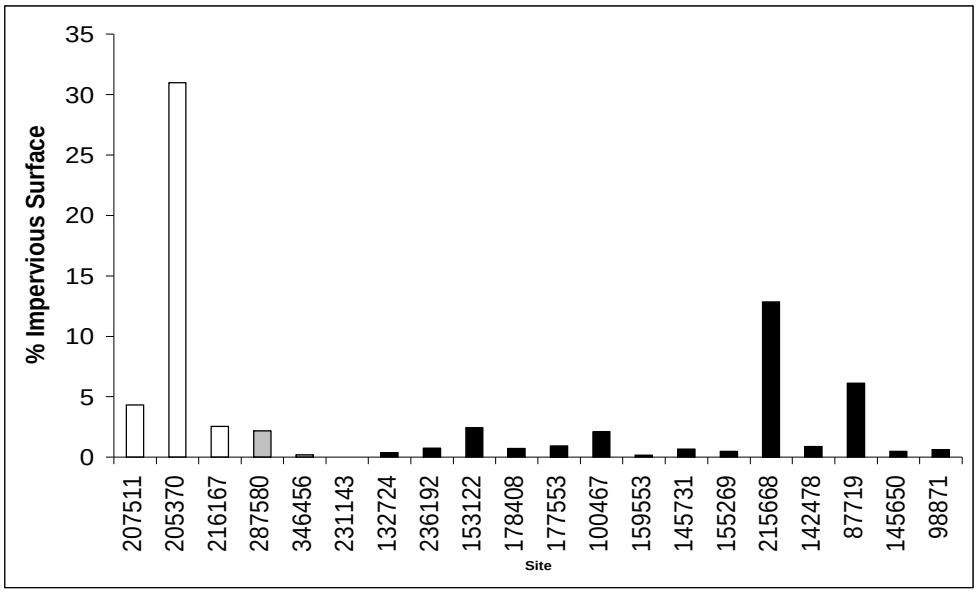


Figure 4. Land use for sample sites in the Saluda Sandhills ecobasin. Graph A represents the land use percentages for the watershed areas around all sample sites in the ecobasin. Graph B represents land use percentages for the 100 meter buffer zone around all sample sites in the ecobasin. Land uses are described in Table 2.

A.



B.

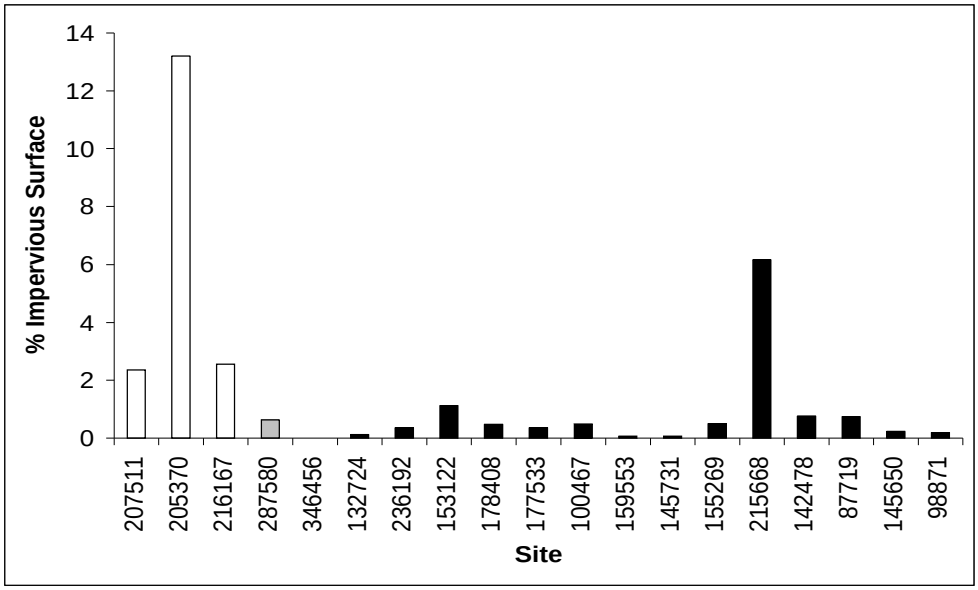
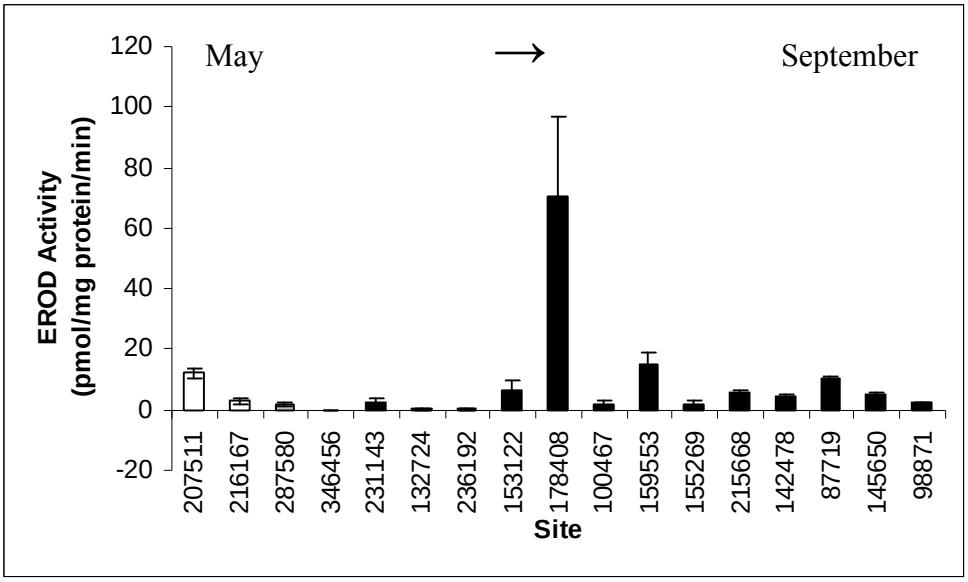


Figure 5. Impervious surface cover by site. Graph A represents impervious surface cover in the watershed area. Graph B represents impervious surface cover in the buffer zone.

A.



B.

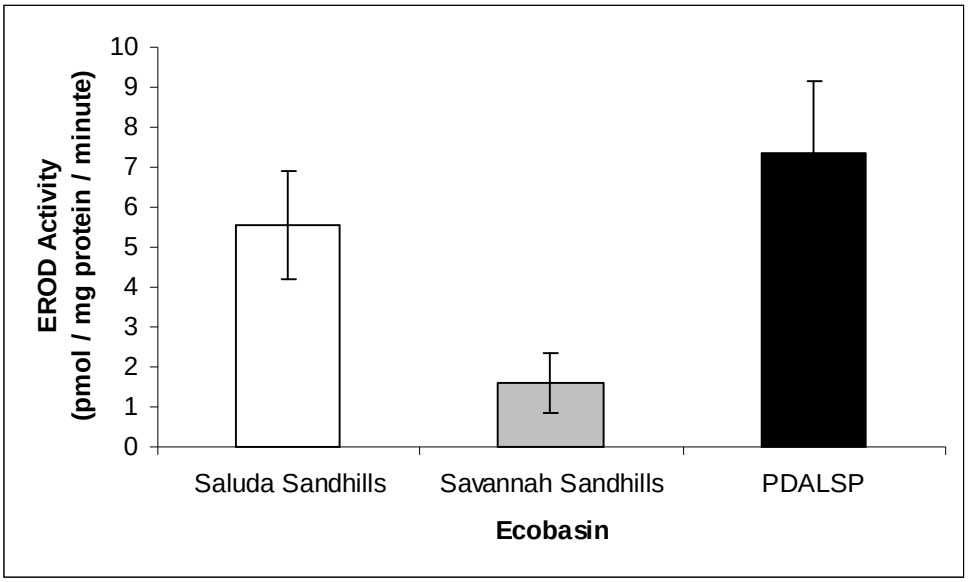
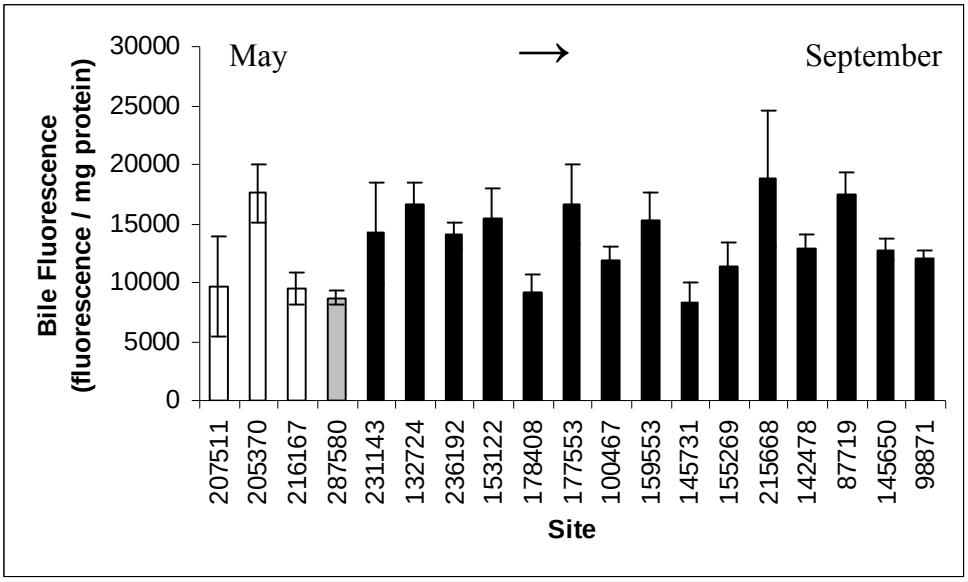


Figure 6. EROD activities in S9 fractions in *Lepomis* sp. from May through September 2006. Graph A represents individual sites. Graph B represents mean results for all sites within specified ecobasin. All results are mean ± SEM. Individual fish characteristics are presented in Appendix A-1.

A.



B.

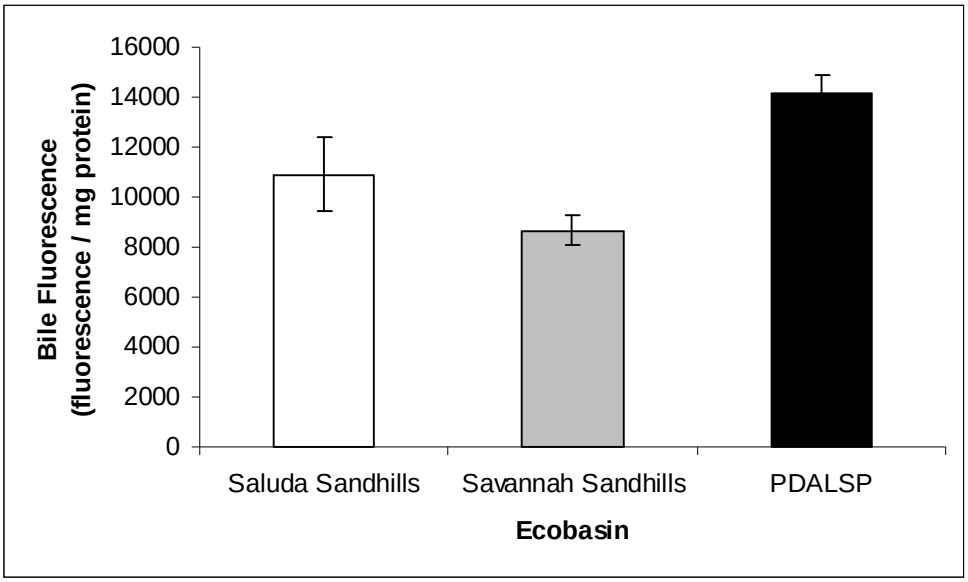
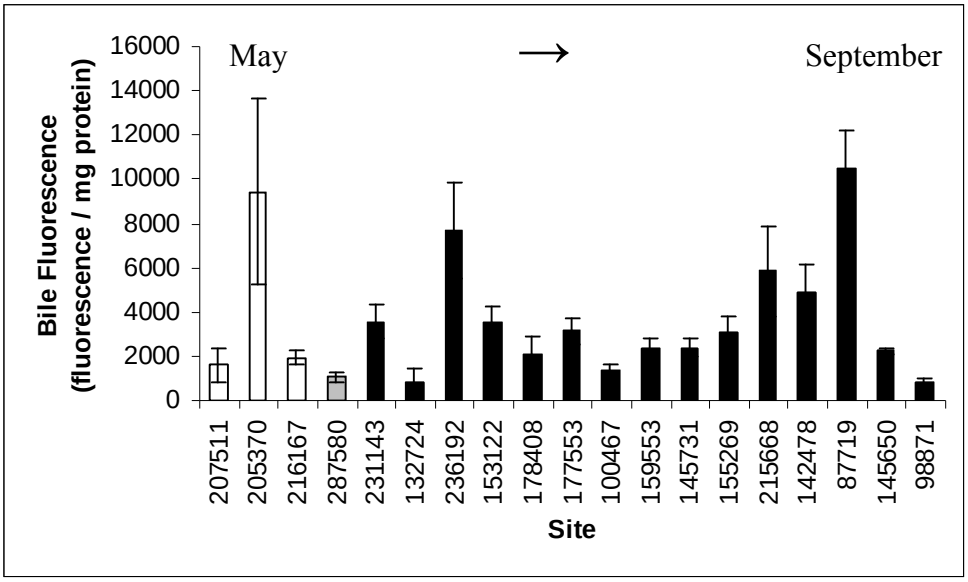


Figure 7. Fluorescence of 2-ringed PAHs in the bile of *Lepomis* sp. from May through September 2006. Graph A represents individual sites. Graph B represents mean results for all sites within specified ecobasin. All results are mean ± SEM. Individual fish characteristics are presented in Appendix A-1.

A.



B.

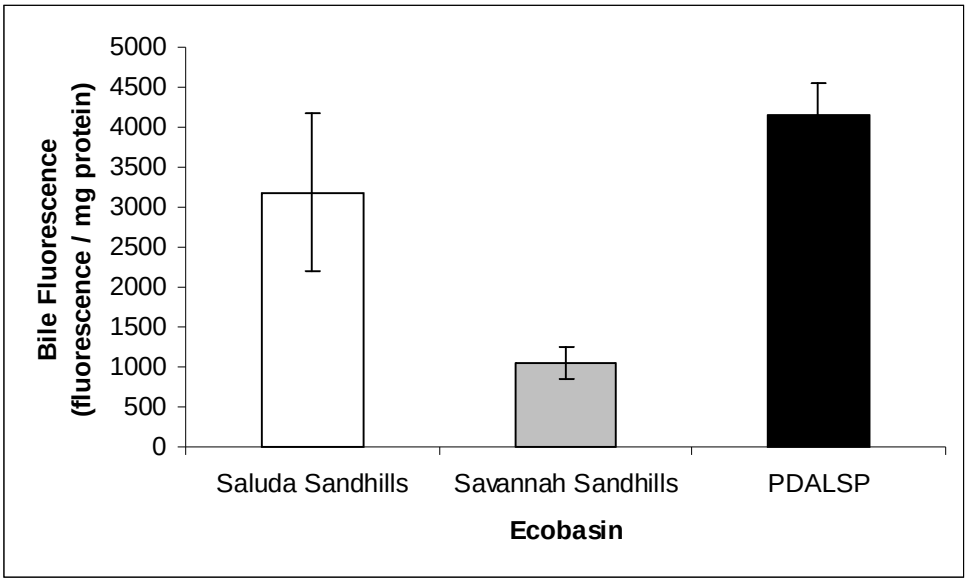
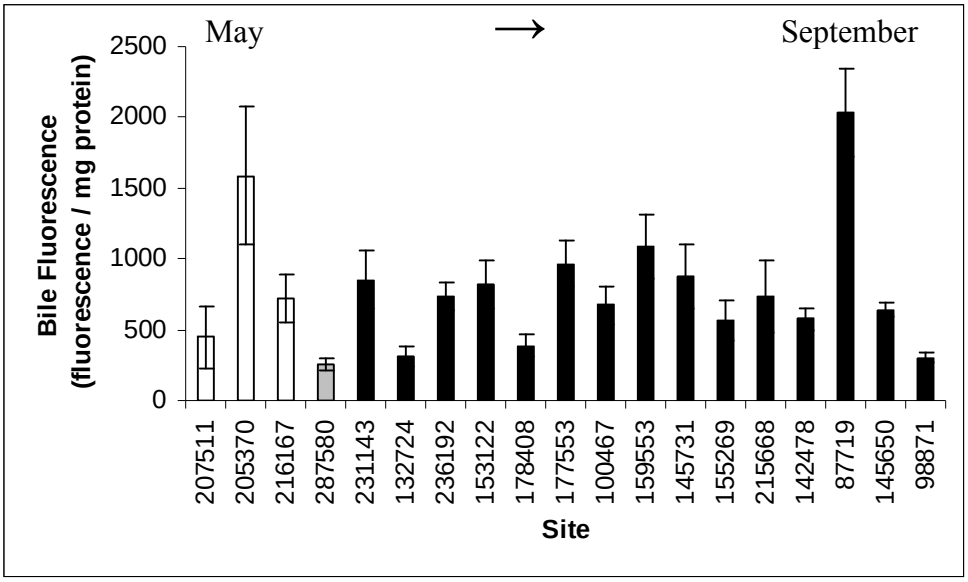


Figure 8. Fluorescence of 4-ringed PAHs in the bile of *Lepomis* sp. from May through September 2006. Graph A represents individual sites. Graph B represents mean results for all sites within specified ecobasin. All results are mean ± SEM. Individual fish characteristics are presented in Appendix A-1.

A.



B.

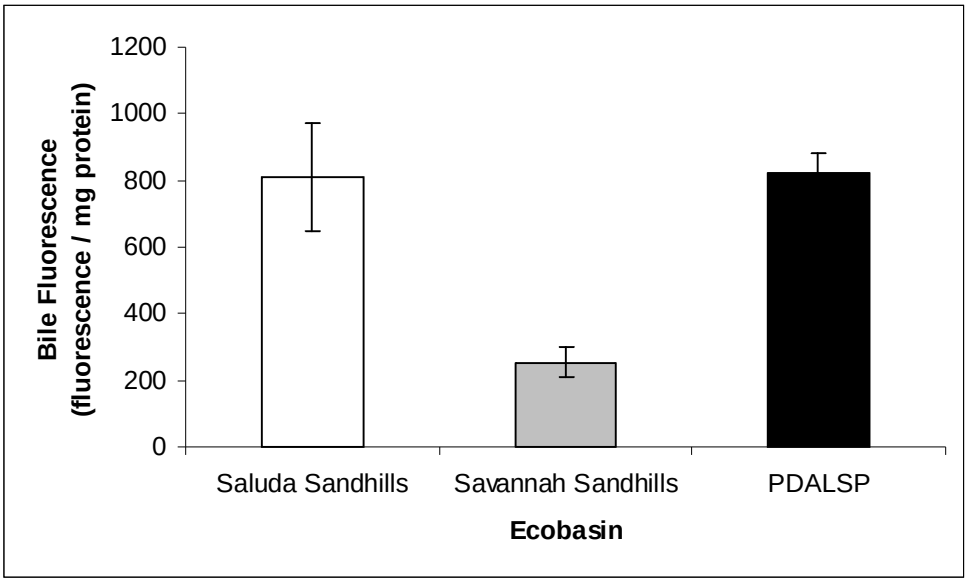
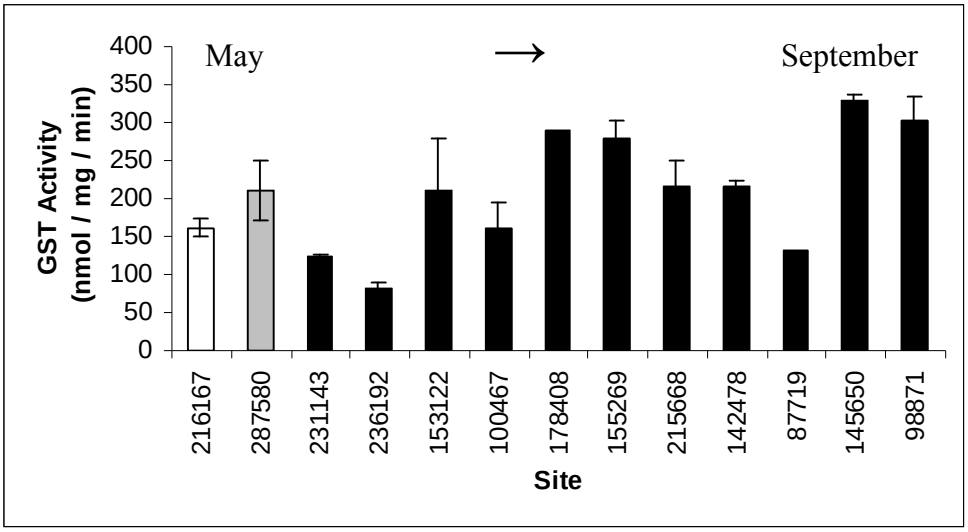


Figure 9. Fluorescence of 5-ringed PAHs in the bile of *Lepomis* sp. from May through September 2006. Graph A represents individual sites. Graph B represents mean results for all sites within specified ecobasin. All results are mean ± SEM. Individual fish characteristics are presented in Appendix A-1.

A.



B.

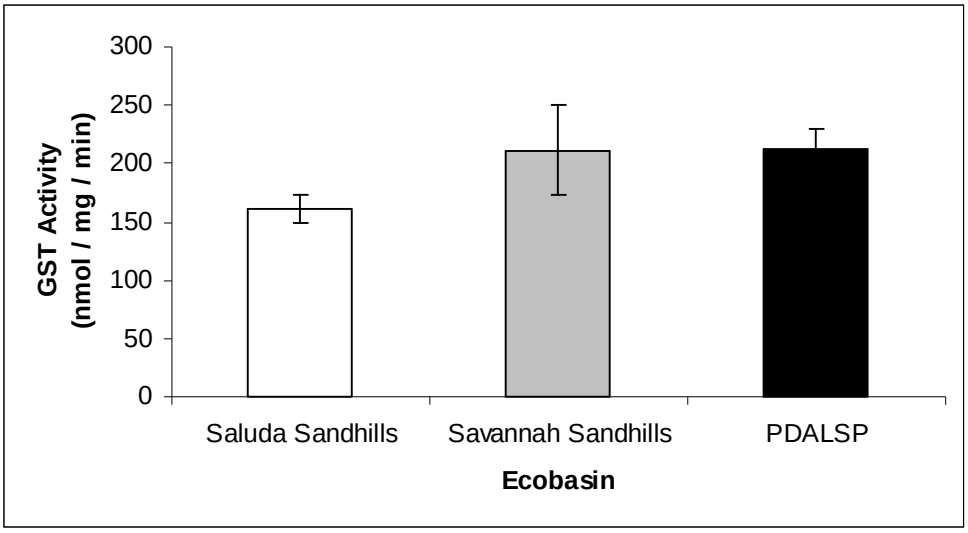
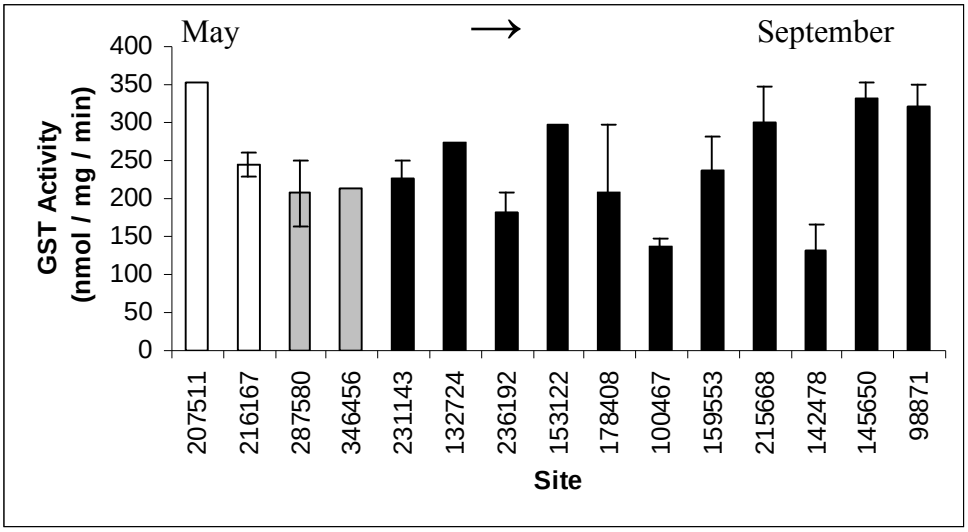


Figure 10. GST activities in the S9 fractions of female *Lepomis* sp. from May through September 2006. Graph A represents individual sites. Graph B represents mean results for all sites within specified ecobasin. All results are mean ± SEM. Individual fish characteristics are presented in Appendix A-1.

A.



B.

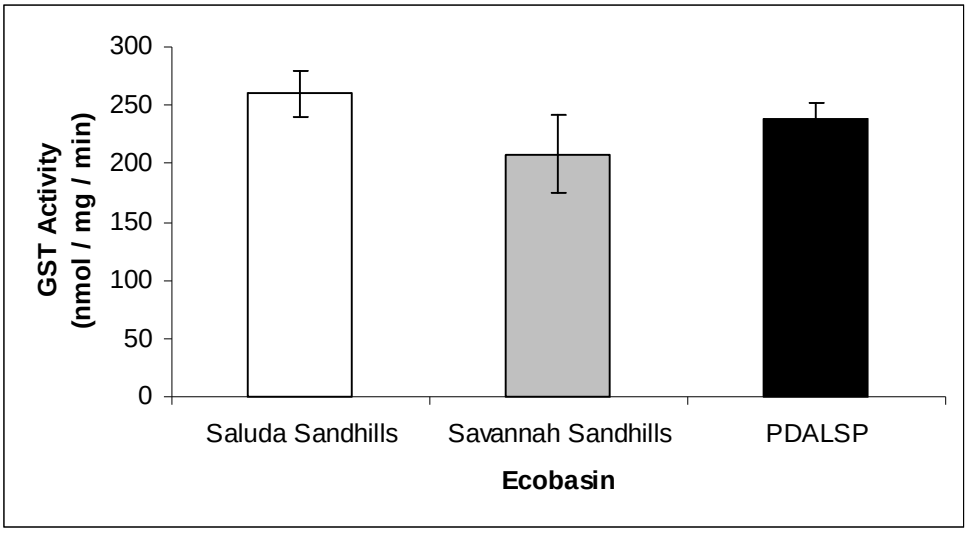
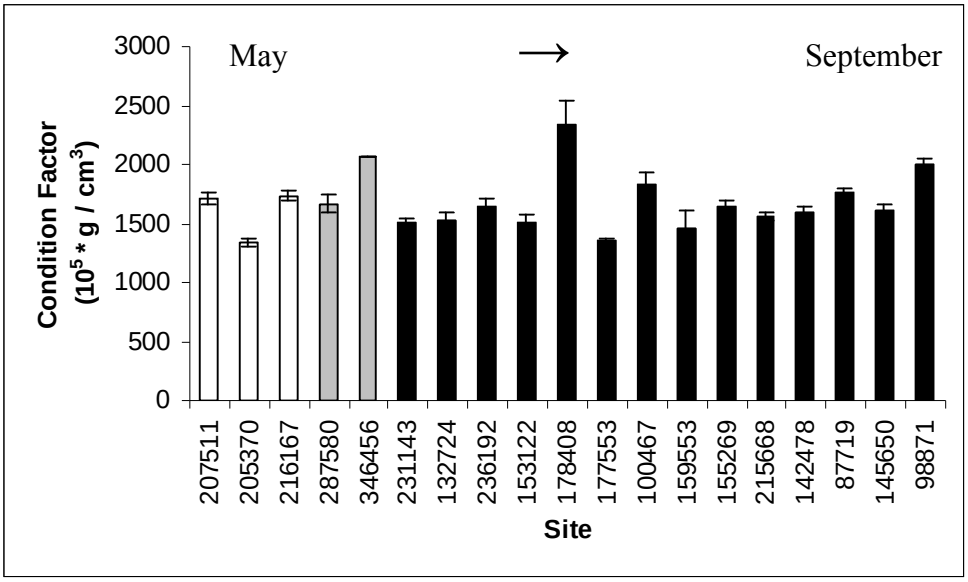


Figure 11. GST activities in the S9 fractions of male *Lepomis* sp. from May through September 2006. Graph A represents individual sites. Graph B represents mean results for all sites within specified ecobasin. All results are mean ± SEM. Individual fish characteristics are presented in Appendix A-1.

A.



B.

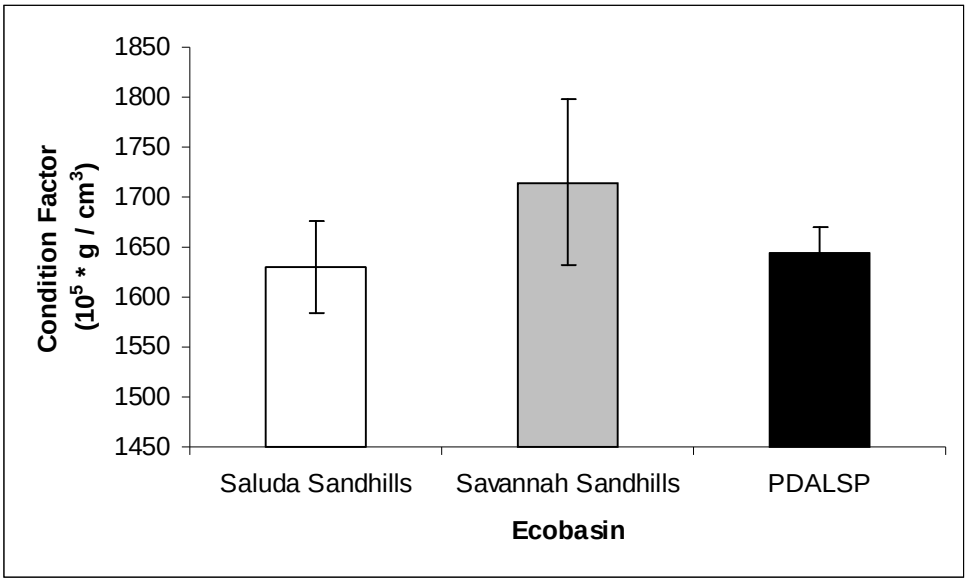
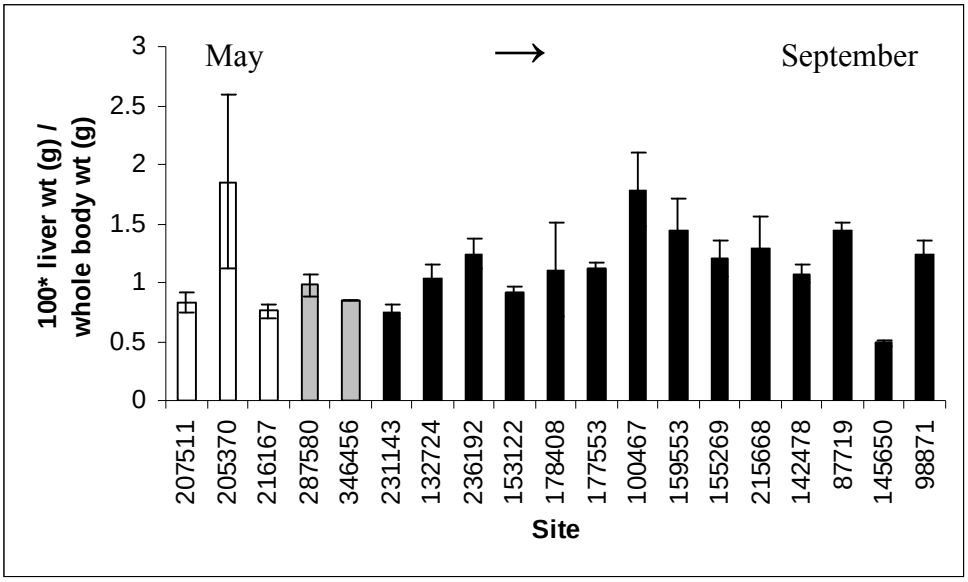


Figure 12. Condition factors (K) of *Lepomis* sp. from May through September 2006. Graph A represents individual sites. Graph B represents mean results for all sites within specified ecobasin. All results are mean ± SEM. Individual fish characteristics are presented in Appendix A-1.

A.



B.

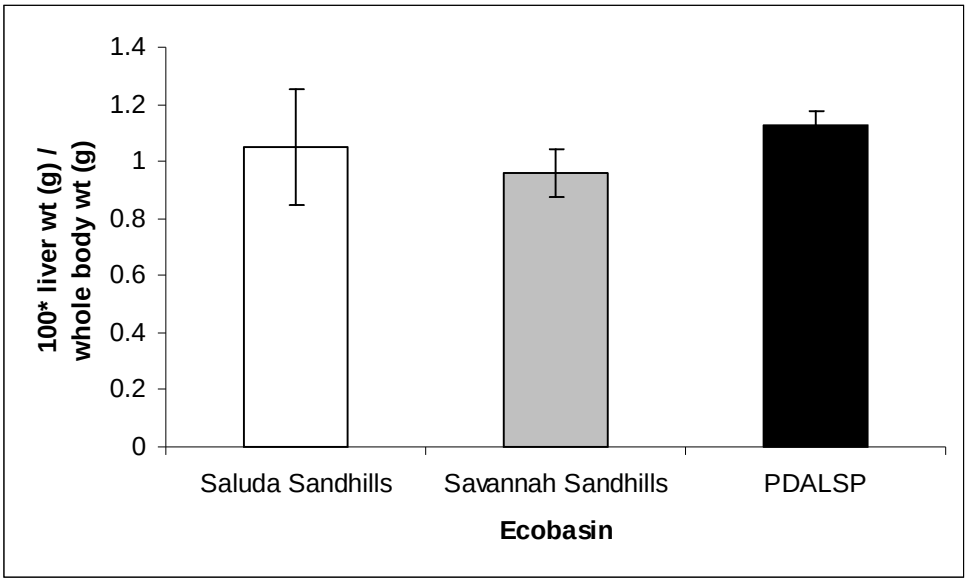
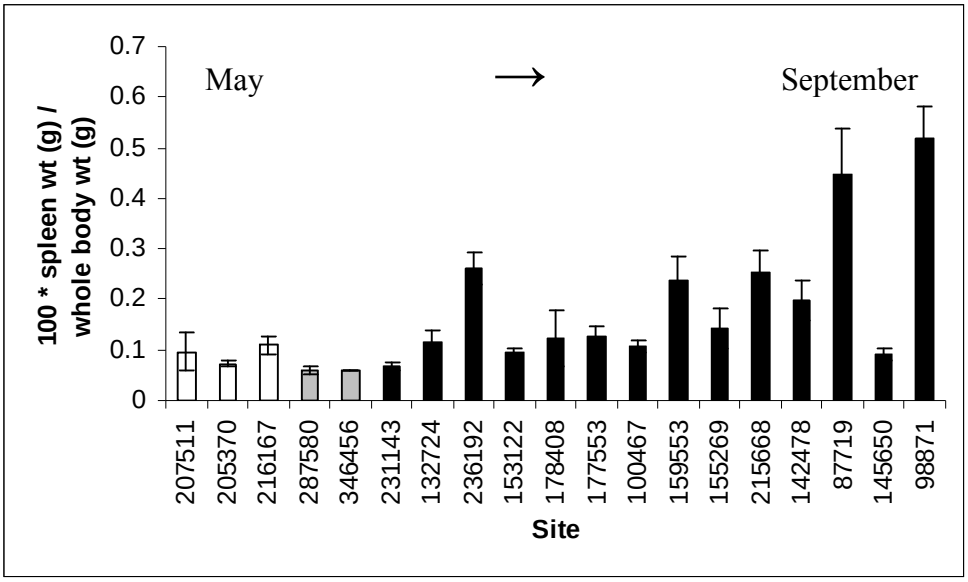


Figure 13. Hepatosomatic indices (HSI) of *Lepomis* sp. from May through September 2006. Graph A represents individual sites. Graph B represents mean results for all sites within specified ecobasin. All results are mean $\pm$ SEM. Individual fish characteristics are presented in Appendix A-1.

A.



B.

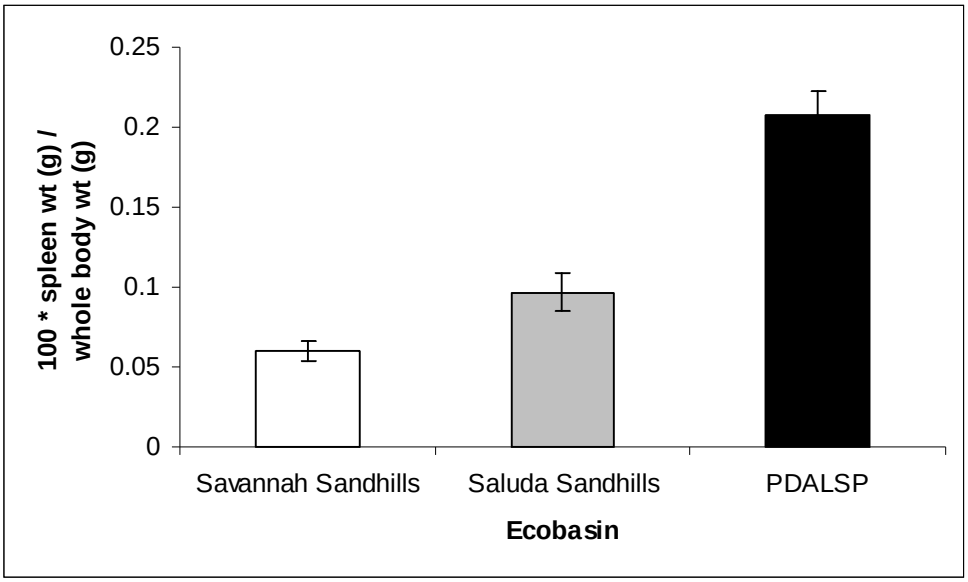
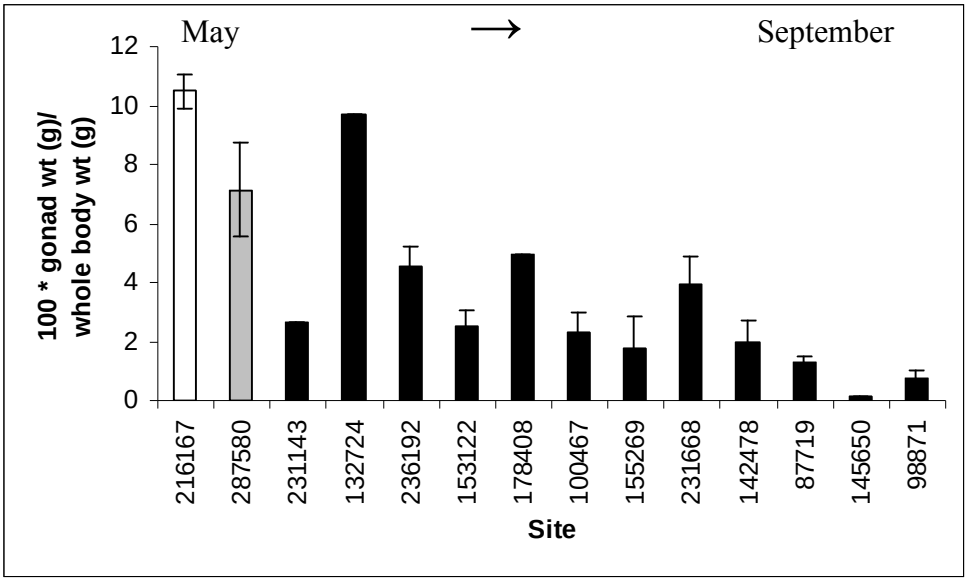


Figure 14. Spleen somatic indices (SSI) of *Lepomis* sp. from May through September 2006. Graph A represents individual sites. Graph B represents mean results for all sites within specified ecobasin. All results are mean ± SEM. Individual fish characteristics are presented in Appendix A-1.

A.



B.

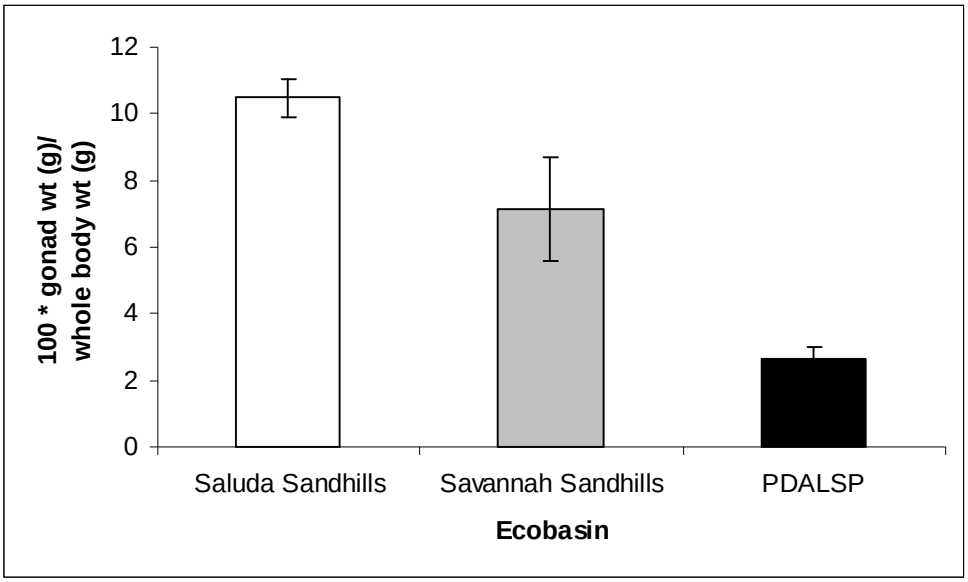
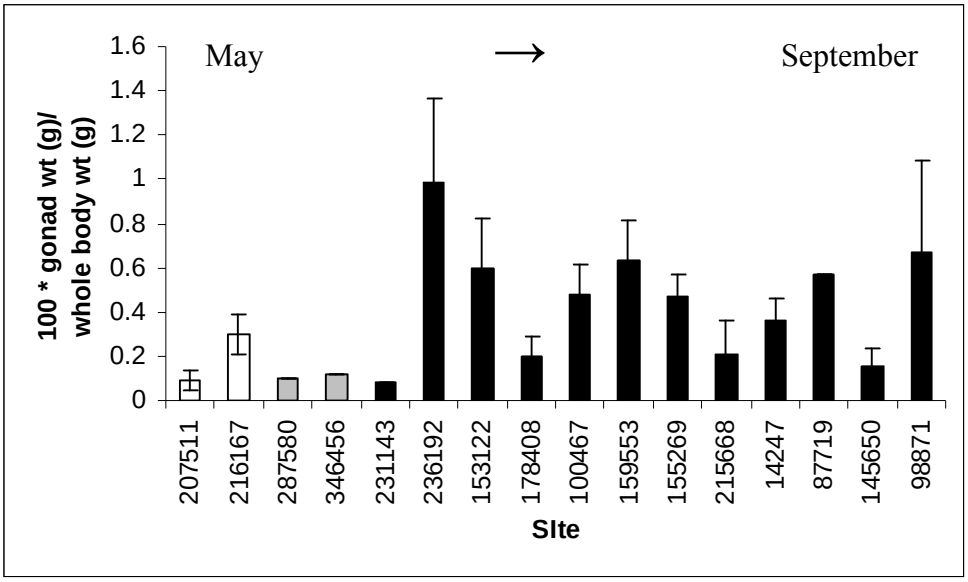


Figure 15. Gonadosomatic indices of female *Lepomis* sp. from May through September 2006. Graph A represents individual sites. Graph B represents mean results for all sites within specified ecobasin. All results are mean ± SEM. Individual fish characteristics are presented in Appendix A-1.

A.



B,

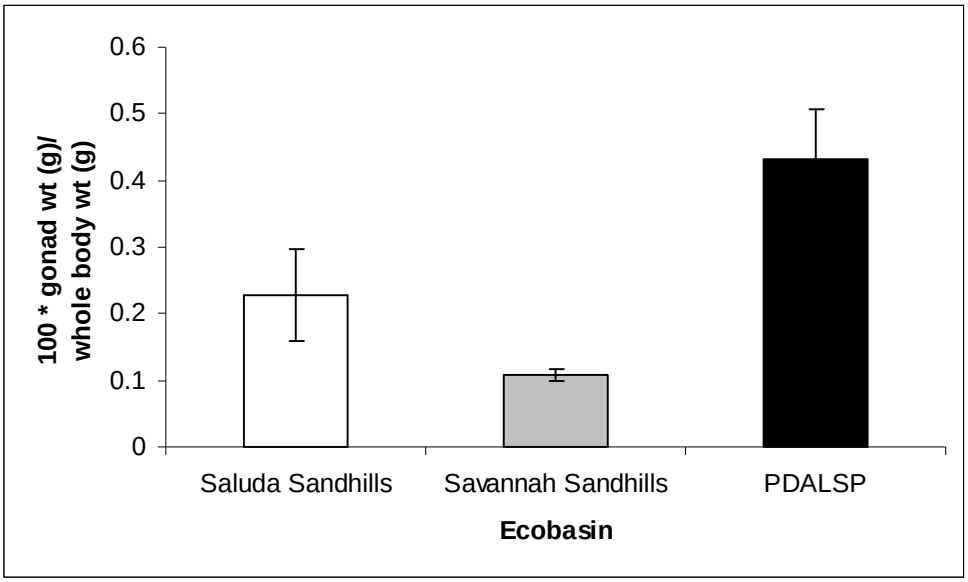


Figure 16. Gonadosomatic indices (GSI-M) of male *Lepomis* sp. from May through September 2006. Graph A represents individual sites. Graph B represents mean results for all sites within specified ecobasin. All results are mean ± SEM. Individual fish characteristics are presented in Appendix A-1.

APPENDIX

Table A-1

Physical Characteristics of Fish

Site	Date Sampled	Fish	Sex	Weight (g)	Length (cm)	Species
207511	5.24.2006	1	M	48.3	14.2	Bluegill
207511	5.24.2006	2	M	26.6	11.5	Bluegill
207511	5.24.2006	3	J	29.6	12	Bluegill
207511	5.24.2006	4	J	19.2	10.7	Bluegill
207511	5.24.2006	5	J	20.7	10.4	Bluegill
205370	5.24.2006	1	J	2.1	5.5	Bluegill
205370	5.24.2006	2	J	2.7	6	Bluegill
205370	5.24.2006	3	J	4.4	7	Bluegill
205370	5.24.2006	4	J	5.1	7	Bluegill
205370	5.24.2006	5	J	2.6	5.8	Bluegill
216167	5.24.2006	1	M	75.1	16.5	Redbreast
216167	5.24.2006	2	M	82.3	15.8	Redbreast
216167	5.24.2006	3	F	49.2	14.5	Redbreast
216167	5.24.2006	4	F	55.1	15	Redbreast
216167	5.24.2006	5	M	88.2	16.9	Redbreast
216167	5.24.2006	6	M	30.1	12	Redbreast
216167	5.24.2006	7	M	45.5	13.9	Redbreast
216167	5.24.2006	8	M	34.7	12.5	Redbreast
216167	5.24.2006	9	F	29.5	12.3	Redbreast
216167	5.24.2006	10	F	27.4	11.7	Redbreast
287580	6.06.2006	1	F	33.9	12.8	Redbreast
287580	6.06.2006	2	F	31.9	12.3	Redbreast
287580	6.06.2006	3	M	40.8	13.7	Redbreast
287580	6.06.2006	4	M	42.5	13.6	Redbreast
287580	6.06.2006	5	F	31.1	12.5	Redbreast
287580	6.06.2006	6	F	27.0	12.5	Redbreast
346456	6.13.2006	1	M	33.8	11.8	Redbreast
231143	8.01.2006	1	J	6	7.6	Redbreast
231143	8.01.2006	2	J	9	8.5	Redbreast
231143	8.01.2006	3	M	30	12.5	Redbreast
231143	8.01.2006	4	J	8	8	Redbreast
231143	8.01.2006	5	M	30	12.5	Redbreast
231143	8.01.2006	6	M	151	20.9	Redbreast

Site	Date Sampled	Fish	Sex	Weight (g)	Length (cm)	Species
231143	8.01.2006	7	F	23	11.5	Redbreast
231143	8.01.2006	8	M	31	12.8	Redbreast
132724	8.02.2006	1	M	11	9.1	Redbreast
132724	8.02.2006	2	F	12	8.9	Dollar
132724	8.02.2006	3	M	7	7.8	Bluegill
132724	8.02.2006	4	M	7	7.8	Bluegill
236192	8.03.2006	1	M	28	11.4	Dollar
236192	8.03.2006	2	F	22	10.5	Dollar
236192	8.03.2006	3	M	18	11.1	Dollar
236192	8.03.2006	4	M	24	11.3	Dollar
236192	8.03.2006	5	M	20	10.2	Dollar
236192	8.03.2006	6	F	14	9.9	Dollar
236192	8.03.2006	7	F	15	10	Dollar
236192	8.03.2006	8	F	13	9.2	Dollar
236192	8.03.2006	9	M	19	10.4	Dollar
236192	8.03.2006	10	M	8	8.8	Bluegill
236192	8.03.2006	11	F	14	9.3	Dollar
236192	8.03.2006	12	F	17	9.8	Dollar
153122	8.08.2006	1	F	62	16.1	Redbreast
153122	8.08.2006	2	F	83	17	Redbreast
153122	8.08.2006	3	F	58	16.4	Redbreast
153122	8.08.2006	4	F	50	15.4	Redbreast
153122	8.08.2006	5	M	115	18.4	Redbreast
153122	8.08.2006	6	F	42	15.5	Redbreast
153122	8.08.2006	7	F	38	12.9	Bluegill
153122	8.08.2006	8	M	30	12.8	Bluegill
153122	8.08.2006	9	M	25	11.9	Bluegill
153122	8.08.2006	10	M	NA	10.5	Red Breast
178408	8.09.2006	1	M	50	13.9	Pumpkinseed
178408	8.09.2006	2	F	71	14.4	Warmouth
178408	8.09.2006	3	M	167	19.6	Warmouth
178408	8.09.2006	4	M	97	15	Warmouth
177553	8.09.2006	1	J	6	7.4	Warmouth
177553	8.09.2006	2	J	6	7.6	Warmouth
177553	8.09.2006	3	J	8	8.2	Warmouth
177553	8.09.2006	4	J	9	8.5	Bluegill
177553	8.09.2006	5	J	5	7.4	Warmouth
177553	8.09.2006	6	J	3	6.1	Warmouth
177553	8.09.2006	7	J	3	6	Warmouth
177553	8.09.2006	8	J	6	7.7	Warmouth
177553	8.09.2006	9	J	3	6.3	Warmouth
177553	8.09.2006	10	J	4	6.8	Warmouth
100467	8.15.2006	1	F	19	9.4	Dollar
100467	8.15.2006	2	F	28	11.8	Warmouth

Site	Date Sampled	Fish	Sex	Weight (g)	Length (cm)	Species
100467	8.15.2006	3	F	7	7	Dollar
100467	8.15.2006	4	M	6	6.9	Dollar
100467	8.15.2006	5	M	42	13.8	Redbreast
100467	8.15.2006	6	M	26	11.4	Pumpkinseed
100467	8.15.2006	7	M	21	11.3	Bluegill
100467	8.15.2006	8	F	55	NA	Redbreast
100467	8.15.2006	9	M	7	7	Dollar
159553	8.16.2006	1	M	20	10.3	Pumpkinseed
159553	8.16.2006	2	M	31	13	Pumpkinseed
159553	8.16.2006	3	J	18	10.6	Pumpkinseed
159553	8.16.2006	4	J	15	10.2	Pumpkinseed
159553	8.16.2006	5	M	6	9.9	Pumpkinseed
159553	8.16.2006	6	J	5	6.5	Red Ear
159553	8.16.2006	7	J	5	6.8	Red Ear
145731	8.16.2006	1	M	NA	16.8	Warmouth
145731	8.16.2006	2	F	NA	13	Warmouth
145731	8.16.2006	3	J	NA	6.5	Dollar
155269	8.17.2006	1	F	54	15.4	Redbreast
155269	8.17.2006	2	F	97	17.7	Redbreast
155269	8.17.2006	3	J	8	8.2	Redbreast
155269	8.17.2006	4	J	7	7.4	Redbreast
155269	8.17.2006	5	J	5	7.1	Redbreast
155269	8.17.2006	6	M	67	15.8	Warmouth
155269	8.17.2006	7	F	26	11.3	Dollar
155269	8.17.2006	8	M	17	10	Dollar
155269	8.17.2006	9	M	17	9.9	Dollar
155269	8.17.2006	10	J	9	7.9	Dollar
215668	8.22.2006	1	M	43	13.9	Bluegill
215668	8.22.2006	2	M	49	14.5	Bluegill
215668	8.22.2006	3	M	36	13.1	Bluegill
215668	8.22.2006	4	J	22	11.1	Bluegill
215668	8.22.2006	5	F	15	9.6	Bluegill
215668	8.22.2006	6	F	37	13	Bluegill
215668	8.22.2006	7	J	21	11.2	Bluegill
215668	8.22.2006	8	F	16	10.3	Bluegill
215668	8.22.2006	9	M	14	9.8	Bluegill
215668	8.22.2006	10	M	12	9.5	Bluegill
142478	8.23.2006	1	M	30	12.4	Redbreast
142478	8.23.2006	2	M	32	13.5	Redbreast
142478	8.23.2006	3	F	18	10.2	Redbreast
142478	8.23.2006	4	M	20	10.7	Redbreast
142478	8.23.2006	5	F	22	11.4	Redbreast
142478	8.23.2006	6	F	18	10	Redbreast
142478	8.23.2006	7	F	16	10.4	Redbreast

Site	Date Sampled	Fish	Sex	Weight (g)	Length (cm)	Species
142478	8.23.2006	8	M	11	8.9	Pumpkinseed
142478	8.23.2006	9	M	5	6.9	Redbreast
142478	8.23.2006	10	M	6	7.5	Redbreast
142478	8.23.2006	11	M	6	6.9	Dollar
142478	8.23.2006	12	M	6	6.9	Redbreast
87719	9.06.2006	1	J	6	6.9	Dollar
87719	9.06.2006	2	J	7	7.5	Dollar
87719	9.06.2006	3	F	6	7	Dollar
87719	9.06.2006	4	M	5	6.5	Dollar
87719	9.06.2006	5	J	6	7	Dollar
87719	9.06.2006	6	J	9	8.4	Dollar
87719	9.06.2006	7	J	10	8	Dollar
87719	9.06.2006	8	J	8	8	Dollar
87719	9.06.2006	9	F	5	6.5	Dollar
87719	9.06.2006	10	J	5	6.4	Dollar
87719	9.06.2006	11	J	NA	7	Dollar
145650	9.27.2006	1	M	96	18.4	Redbreast
145650	9.27.2006	2	M	82	17.3	Redbreast
145650	9.27.2006	3	F	80	18.1	Redbreast
145650	9.27.2006	4	M	66	16.1	Bluegill
145650	9.27.2006	5	F	82	17.4	Redbreast
145650	9.27.2006	6	F	97	18.2	Bluegill
145650	9.27.2006	7	F	87	17.5	Redbreast
145650	9.27.2006	8	M	74	16	Redbreast
145650	9.27.2006	9	M	74	16.4	Red Ear
145650	9.27.2006	10	M	64	15	Redbreast
145650	9.27.2006	11	M	59	15	Redbreast
145650	9.27.2006	12	M	20	10.9	Pumpkinseed
145650	9.27.2006	13	M	44	14.4	Bluegill
98871	9.27.2006	1	M	23	10.1	Dollar
98871	9.27.2006	2	M	22	10.1	Dollar
98871	9.27.2006	3	F	19	9.7	Dollar
98871	9.27.2006	4	F	19	10.1	Dollar
98871	9.27.2006	5	M	17	9.9	Dollar
98871	9.27.2006	6	F	19	9.6	Dollar
98871	9.27.2006	7	F	17	9.5	Dollar
98871	9.27.2006	8	M	13	8.9	Dollar
98871	9.27.2006	9	F	17	9.5	Dollar
98871	9.27.2006	10	M	10	7.9	Dollar

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