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Biochemical effects of pollutant exposure in fish from urban creeks in Greenville, SC.

Peter van den Hurk, ~~Lindsay Gerzel, Peter Calomiris,~~ and Dennis C. Haney

Commented [DH1]: Peter – I just realized neither Peter or Lindsay worked on this study – you had a female grad student that worked on this, though I can't remember her name. Peter and Lindsay worked on the lab experiment and the Saluda study only.

Abstract.

The city of Greenville, SC is a rapidly expanding urban area located on the Reedy River in the upstate of South Carolina. Historical and current point-source pollutants and runoff from impermeable surfaces have resulted in a contaminated river environment, which through previous studies was demonstrated to be reflected in biological effects in fish species in the river. Because it was not known how much smaller tributaries in the urbanized area were contributing to the pollution of the main stem of the Reedy River, we collected fish (bluehead chub (*Nocomis leptocephalus*), creek chub (*Semotilus atromaculatus*), bluegill (*Lepomis macrochirus*) and redbreast sunfish (*Lepomis auritus*)) from five smaller urban creeks in the Greenville area and measured several biomarkers of exposure in these animals. Enzymatic activities of cytochrome P450-1A (CYP1A) and glutathione S-transferase (GST) were measured, and bile samples were analyzed for fluorescence caused by polynuclear aromatic hydrocarbons (PAHs), and for excreted estrogenic compounds. The results show that some creeks triggered significant biomarker responses in collected fish, while others were relatively clean. In particular, PAHs appear to be prevalent and caused biochemical effects, while estrogenic compounds were not significantly increased in the bile of fish from these urban creeks. A striking observation was the difference in enzyme activities in chub species compared to sunfish species; sunfish had up to 5 times higher CYP1A activities than chubs, while the chubs had significantly higher GST activity than sunfish. These species differences should be taken into account when they are incorporated in environmental risk assessments and biological effects monitoring programs.

Introduction.

The stress that anthropogenic influences puts on natural streams in urbanized areas has been well recognized, and is described as the "urban stream syndrome" (Paul & Meyer, 2001; Walsh et al., 2005). Symptoms of this syndrome are a flashier hydrograph, resulting in changes in channel morphology, elevated concentrations of contaminants, including nutrients, and a reduced biotic richness, with a dominance of stress-tolerant species (e.g., Siligato& Boehmer, 2002; Wallace et al., 2013). Furthermore, rapid urbanization around expanding cities has turned once rural streams and creeks into discharge waterways for runoff from impervious surfaces in newly developed suburbs, shopping malls and industrial areas (Paul & Meyer, 2001). While point sources in these newly developed areas are usually well-known and are regulated and monitored by environmental control agencies, non-point sources are much more difficult to identify, and their effects on living biota in receiving streams are therefore much

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less known (Walsh et al., 2005). Nonetheless, non-point sources are known to affect stream ecosystems in urbanized areas, where studies have shown that invertebrate and vertebrate communities are impoverished in species composition and density when compared to streams in less disturbed rural areas (Wallace et al., 2013).

Known sources for pollution of urban streams are runoff from roads, driveways and parking lots that contain polynuclear aromatic hydrocarbons (PAHs) from oil residues, metals (e.g. copper), and brake pads (Sekabira et al., 2010; Yang et al., 2010). In addition, runoff from lawns and gardens add fertilizers, herbicides and pesticides to local waterways (Yang et al., 2015). Furthermore, overflow from sewage systems during heavy rain events, and discharge from leaking septic tanks may add household chemicals, pharmaceuticals and personal care products to urban creeks and streams (Martinovic-Weigelt et al., 2013). Finally, an often overlooked factor are turbidity spikes as a result of silt runoff from building sites and deforested areas (Selbig et al., 2013).

While chemical analysis of water samples can pinpoint which of the above mentioned sources are potential problems in certain streams, they usually are limited to snap-shot identification of a temporary condition. More useful time-integrated results are obtained through sediment analyses or through the deployment of passive samplers with semi-permeable collection devices (Chandler et al., 2015). But a more biological relevant predictor for ecological effects is the analysis of biochemical responses in resident biota, ideally combined with bioaccumulation data (van der Oost et al., 2003). Because these resident, stress-tolerant species are exposed to environmental contaminants in polluted urban streams, it is likely they display biochemical changes as an adaptation to their contaminated environment. Furthermore, biomarker responses in these less sensitive species should correlate with the disappearance of more sensitive species from the species assemblage, and may thus be used as a management tool for water quality managers. Unfortunately, very few studies have been carried out that evaluate the use of biomarkers in management of urban stream water quality (Hanson & Larsson, 2009).

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~~Through this approach bioavailability and toxicological effects can be assessed and critical sources for relevant toxicants can be identified.~~

However, previous studies in the Reedy River have demonstrated that fish health was impacted by pollution sources that discharge on the river, especially in and below the Lake Conestee reservoir where significant effects of pollutants like PAHs and heavy metals were observed (Schreiber et al., 2006; Otter et al., 2012). In addition, in locations directly downstream of the main wastewater treatment facility for the city of Greenville (Mauldin Road WRRF), significant effects were observed in biomarkers for endocrine disruption (Truman et al., 2010). These studies triggered the question if, and in what magnitude, smaller tributaries originating in the urbanized area of the greater Greenville area would contribute to the earlier observed pollution effects in resident fish.

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As such, we used a biomarker approach to assess possible chemical contamination in a series of urban creeks in the greater Greenville area in the upstate of South Carolina. Greenville is situated on the Reedy River, which originates about 10 miles north of the city, near the town of Travelers Rest. ~~. In previous~~

studies we have demonstrated significant pollution effects in fish in the main stem of the river (Schreiber et al., 2006; Truman & Van den Hurk, 2010; Otter et al., 2012). However, the Reedy River also has a number of smaller tributaries that originate in the urbanized area of greater Greenville. The objective for this study was thus to investigate if, and how much, these smaller urban creeks contribute to the measured pollution effects in the main river.

Among the more common fish species in wadeable streams of the SC upstate are several sunfish species (*Lepomis auritus and L. macrochirus*) and two chub species (*Nocomis leptocephalus* and , *Semotilus atromaculatus*). Samples of these fish were collected from five urban creeks in the Greenville area, together with three locations in the main stem of the Reedy River: a location just upstream of, and one just downstream of the Mauldin Road WRRF, and a reference site upstream of the city. In the collected fish the following parameters were measured: bile fluorescence and induction of cytochrome P-450-1A (CYP1A) as biomarkers for PAH exposure, activity of glutathione-S-transferase as an indicator for oxidative stress, and the presence of estrogenic compounds in bile as an indicator for exposure to xenoestrogens. Special attention was paid to potential species differences in the biomarker responses between the sunfish and the chub.

Materials and methods

Sampling Sites and Collection

The urban creeks and the Reedy River were sampled in July and August 2009 (Fig. 1). We used WMS 10.0 to delineate watershed areas upstream of each sampling point, then determined percent development within each watershed using ArcMap 10.2 and land cover data for the Piedmont region of South Carolina provided by USDA CropScape (USDA 2012). The details of each site, and the species collected are presented in Table 1. Fish were collected with a backpack electrofisher (Smith-Root Corporation), a 10'x14'x 1/8" seine, and long-handled dip nets. The collected fish were identified in the field and the selected species were transported to the laboratory in 5-gal carboys filled with aerated water from the collection site. Fish were measured (standard length [mm] and weighed [g]), and gall bladders and livers were harvested from each fish. Gall bladders were placed on ice in dark microcentrifuge tubes; livers were wrapped in aluminum foil and frozen in liquid nitrogen before both were stored in a -80°C freezer for later analysis.

Preparing Liver Post-Mitochondrial (S9) Fractions

Livers were weighed and individually homogenized with a glass Potter-Elvehjem homogenizer in 2 mL of chilled homogenization buffer (van den Hurk, 2006). Liver homogenates were then centrifuged at 10,000 x g and 4° C for 20 min (Eppendorf 5810 R), after which the supernatant (S9 fraction) was divided into three aliquots for later determination of Ethoxyresorufin-O-Deethylase (EROD) activity, GST activity, and total protein concentration. The EROD and GST aliquots were stored in a -80°C freezer and the protein aliquot was stored in a -20°C freezer prior to analysis. Protein concentrations were measured

with a bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL), using bovine serum albumin (BSA) to prepare the standard curve.

Ethoxyresorufin-O-Deethylase (EROD) Assay

Liver S9 fractions were diluted to 1.0 mg/ml total protein concentration, and 100 µl of diluted S9 fractions (in duplicate) were added to a black 96-well plate. The reaction was started by adding 2.5 mM NADPH in 150 µl of reaction buffer (0.2% BSA, 5mM $MgCl_2$, 0.1 mM ethoxyresorufin) to the assay wells (Schreiber et al., 2006). The fluorescence was then recorded at Ex 530, Em 585 nm in 5-10 min intervals over 30 min (SpectraMAX Gemini, Molecular Devices Corporation, CA). One set of boiled S9 fractions was used in duplicate as a blank. A 7-step dilution series of resorufin in methanol was used to generate a standard curve ranging from 0-800 nM.

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Glutathione S-Transferase activity

Activity of GST was measured as the conjugation of glutathione with 1-chloro-2,4-dinitrobenzene (CDNB) by cytosolic protein (Mierzejewski et al., 2014). The total reaction mixture of 250 µl contained 0.1 M HEPES buffer (pH 7.6), 1 mM glutathione (GSH), and 25 µg of S9 protein. The reaction was started by adding CDNB (1 mM final concentration). Formation of the CDNB conjugate was measured by taking absorption readings on a SpectraMax 190 plate reader (Molecular Devices Corporation, CA) at 9 s intervals for 2 min at 344 nm, and was quantified by using the molar absorptivity of 9.6 mM^{-1} for the enzymatic product.

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Table 1. Collection sites and number of fish per species for each site.

Site ID	Watershed Area (km^2)	Percent Development	Chubs Collected	Sunfish Collected	Total Fish Collected
C1	3.25	68.55	*N. leptocehpalus* (4) *S. atromaculatus* (1)	*L. auritus* (5) *L. macrochirus* (5)	n = 15
C2	23.55	89.36	*N. leptocehpalus* (5)	*L. macrochirus* (13)	n = 18
C3	3.57	67.86	*N. leptocehpalus* (3) *S. atromaculatus* (7)	*L. auritus* (1) *L. macrochirus* (3)	n = 14
C4	2.13	52.13	*N. leptocehpalus* (2) *S. atromaculatus* (6)	----	n = 8
C5	3.98	60.71	*S. atromaculatus* (5)	*L. macrochirus* (5)	n = 10
RR1	20.15	38.08	*S. atromaculatus* (5)	*L. auritus* (5) *L. macrochirus* (2)	n = 12

RR2	124.62	68.54	*N. leptocehpalus* (3)	*L. auritus* (9) *L. macrochirus* (6)	n = 18
RR3	160.09	72.74	----	*L. auritus* (5) *L. macrochirus* (7)	n = 12

RR1 was a reference stream, upstream of the city of Greenville. Watershed areas and percent development were calculated using WMS 10.0, land cover data from USDA CropScape, and ArcMap 10.2.

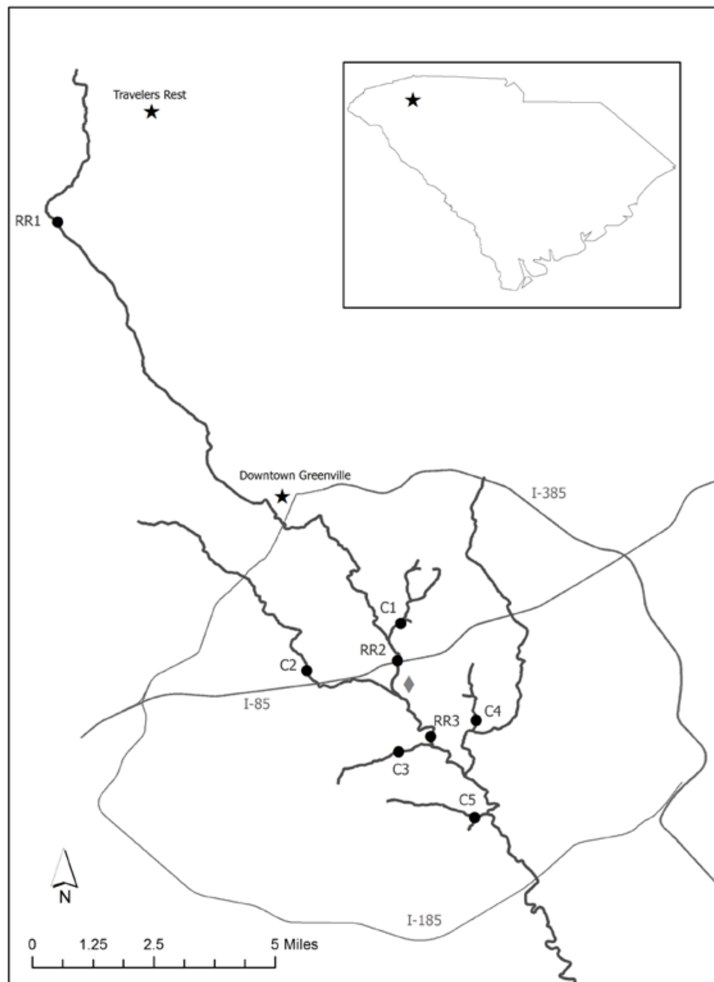

Figure 1. Map of the sampling locations in this study. Except for site RR1, a reference site, all other samples were made in urbanized sections of greater Greenville, SC. ♦ indicates the location of the Mauldin Road WRRF.

Bile Fluorescence

Gall bladders were thawed and bile was released into dark microcentrifuge tubes (1.5 ml). An aliquot of 140 µl deionized water was added to each tube; tubes were vortexed and centrifuged at 4000 x g and 4° C for 5 min (Eppendorf 5810 R) to separate diluted bile from gallbladder tissue. Total protein content in these bile dilutions was determined as described above. Next, three consecutive serial dilutions (1:250, 1:500, 1:1000) were prepared in dark microcentrifuge tubes using a 50% methanol solution. Fluorescence of aromatic compounds (FAC) was then measured in three replicate aliquots from each dilution at 290/335 nm Ex/Em for 2-ring PAHs and 380/430 ~~Ex 380, Em 430~~ nm Ex/Em for 5-ring PAHs on the SpectraMAX Gemini plate reader mentioned above. Fluorescence data were plotted against dilution, and the values of the highest dilution not showing inner filter effects were used for further calculations. The FAC values were corrected using a methanol:water blank and normalized to equivalent protein concentrations (van den Hurk, 2006).

Bile estrogens

The presence of estrogenic compounds in bile samples of male and juvenile fish was measured using an estrogen receptor competitive binding assay (Mierzejewski et al., 2014). Bile samples were incubated overnight at 37° C with 200 units β-glucuronidase/sulfatase to deconjugate all estrogenic metabolites. Deconjugated metabolites were extracted with ethyl acetate and dried under nitrogen gas. Extraction samples were then diluted 1:50 and 100 µl of each sample was added to 50 µl of binding buffer (10% glycerol, 2 mM dithiothreitol, 1 mg/ml BSA, 10 mM Tris buffer, pH 7.5). Next, 100 µl of radiolabeled ^{3}H-17β-estradiol (100 ng/ml, 0.8 µCi/ml) was added to every tube, immediately followed by 50 µl of the estrogen receptor (100 ng/ml). The tubes were incubated at room temperature for 2 hours, followed by an overnight incubation at 4˚C. The next day 1 ml of ice cold charcoal suspension (3 g charcoal/ml binding buffer) was added to each tube. The tubes were gently shaken to mix, incubated on ice for 5 minutes, then centrifuged at 1800 x g for 10 minutes. The supernatant was collected into scintillation vials and mixed with 4 ml of scintillation fluid (Ultima Gold™ Packard, Meriden, CT). Samples were counted for 10 min using a Beckman LS 1800 liquid scintillation counter (Irvine, CA). Concentration of test samples displacing approximately 50% ^{3}H-E2 binding from each receptor (IC50) was determined from a standard curve using samples with unlabeled 17β-estradiol. Results were calculated as a percentage of binding of the estrogen receptor and concentration of receptor-binding compound in ng/ml binding buffer, which was then assessed for dilution factors and calculated as ng estrogenically active compound per ml, and represents the relative measure of estrogen receptor binding potential (EDC) of compounds in 1 ng of bile.

Statistical Analyses

Statistical analyses were conducted using the Prism 4 software package (Graphpad Software, Inc.) and R version 3.0.1. Independent samples T-tests were used to test for significant differences among species of chub and sunfish; as there were none, both chub species and both sunfish species were lumped together for further analysis. The EROD, GST, and bile fluorescence data were $\log_{10}$ transformed to

meet test assumptions, then a factorial ANOVA followed by a TukeyHSD post hoc was performed to test for differences between sites and between species for each of these parameters. To further examine these parameters, a correlation analysis was also performed, comparing mean $\log_{10}$ transformed values for chubs with mean $\log_{10}$ transformed values for sunfish at all sites where both groups were collected. Because linearity was still questionable, Spearman's correlation tests were performed for these four parameters.

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I couldn't figure out what sort of correlation you did originally. It looks like you might have correlated each species mean values at each site with the sites themselves, but that is invalid. In a correlation, both columns need to be numeric, and sites is a category, not a number. Artificially calling them numbers (e.g., 1, 2, 3, 4, etc.) will allow the test to be performed but I believe to be unsupportable, since those sites are not all connected in an ordered way. You could do a regression analysis, theoretically, but that requires linearity, which we do not have. So, I did something else that I think makes the same point. I correlated the mean values of chubs and sunfish at each site where both exist with one another. This ends up showing that the 5ring and EROD data are highly correlated across sites whereas this is not true for GST or 2-ring data.

A limited number of bile samples (0-4 males/juveniles per site) was available for analysis of estrogenic compounds. Given the lack of significant differences between species for bile fluorescence (see Results below), it was assumed that combining data for bile estrogens from both species groups per site was justifiable so that a sufficient number of data points per site would be available for statistical analysis. After a $\log_{10}$ transformation the bile estrogen data met test assumptions, so the single factor ANOVA and TukeyHSD post hoc tests were performed to test for differences between sites.

In all cases, results were considered significantly different if $p < 0.05$.

Results

Fish were collected from three locations in the Reedy River and five locations in smaller tributary creeks of the Reedy River. In most locations a mix of chub and sunfish was found, except at site C4 where only chubs were collected and at RR3 where only sunfish were collected. The two chub species that were collected were bluehead chub (*Nocomis leptocephalus*) and creek chub (*Semotilus atromaculatus*). The two sunfish species used for analysis were bluegill (*Lepomis macrochirus*) and redbreast sunfish (*Lepomis auritus*). For the parameters discussed here, there were no statistically significant differences between the two chub species or the two sunfish species, therefore the data for both groups were pooled.

EROD induction

EROD activity showed large differences between the two species groups (Fig. 1). Factorial ANOVA analysis demonstrated a highly significant difference between chubs and sunfish ($p = 1 \times 10^{-15}$) and across sites ($p = 5 \times 10^{-15}$), but not for interactions. At every site where both chubs and sunfish were collected, EROD activity in sunfish were significantly higher than EROD activity in the chubs. When analyzed within species groups, EROD activity in chubs from sites C1 and C2 were significantly higher than in chubs collected from sites C3 or C4, and chubs collected at site RR2 had significantly greater EROD activity levels than those collected at site C3. EROD activity in sunfish from site C2 was significantly higher than in sunfish collected at sites RR3 or C5.

Despite the large differences in EROD activity between chubs and sunfish, the spatial patterns are comparable for the sites where both species groups were present. Correlation analysis showed that there was a significant positive correlation in EROD expression between chubs and sunfish across sites (Spearman's correlation; $p=0.05$, $\rho = 0.83$).

Bile fluorescence

Bile fluorescence was measured at 2~~3~~ excitation/emission wavelength pairs, which are characteristic for 2-ring, ~~4-ring~~ and 5-ring aromatic hydrocarbons. Neither the 2-ring or 5-ring fluorescence data showed any significant interaction or differences between species (Factorial ANOVA, $p > 0.05$). However, differences between sites were observed for both the 2-ring (Factorial ANOVA, $p = 0.004$) and 5-ring compounds (Factorial ANOVA, $p = 4 \times 10^{-12}$; Fig. 2) in bile. For the 2-ring compounds, chubs collected at site C4 had significantly higher fluorescence than chubs from site C1, and for sunfish, those collected at site C2 had significantly elevated bile fluorescence compared to fish collected at RR2. For the 5-ring compounds, there were no significant differences between the upstream reference site (RR1) and the downstream sites in the Reedy River (RR2 and RR3). However, 5-ring bile fluorescence activity in both chubs and sunfish collected from site C2 was three to four times higher than in fish from sites C3 and RR1. For sunfish, 5-ring bile fluorescence activity in fish collected from site C2 was also significantly greater than that observed in fish from sites C5 and RR2. Similarly, activity in fish from site C4 was also significantly higher than in fish from RR1, although only chubs were collected at site C4. Finally, chubs from sites C4 and C5 had significantly elevated 5-ring bile fluorescence compared to those collected at site C3.

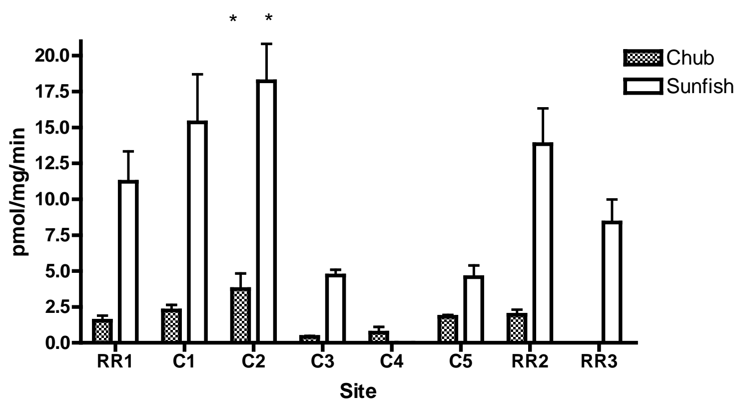

Figure 1. EROD activities for chub and sunfish.

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Correlation analyses between chubs and sunfish differed when comparing 2-ring to 5-ring bile fluorescence values. For the 2-ring fluorescence data, there was no significant correlation (Spearman's correlation, $p > 0.05$) between chubs and sunfish at sites where both groups were present. However, for the 5-ring fluorescence data of the averages per site, there was a significant positive correlation between the two groups (Spearman's correlation; $p=0.003$, $\rho = 0.90$).

Glutathione-S-transferase activity

Analysis of GST activity in chubs and sunfish revealed significant differences between species groups (Fig. 3). For this parameter the activity was 2 to 3 times higher in chubs than in sunfish at all sites where both groups were collected (Factorial ANOVA, $p = 4 \times 10^{-12}$). There were also significant differences across sampling sites (Factorial ANOVA, p= 0.0001), though only with chubs, where GST activity in chubs collected at site C3 was significantly lower than for those collected at sites C1. However, there was not a significant interaction between sites and species groups (Factorial ANOVA, $p > 0.05$). Similarly, there was no significant correlation of mean GST levels between chubs and sunfish across sites where both were found (Spearman's correlation, $p > 0.05$).

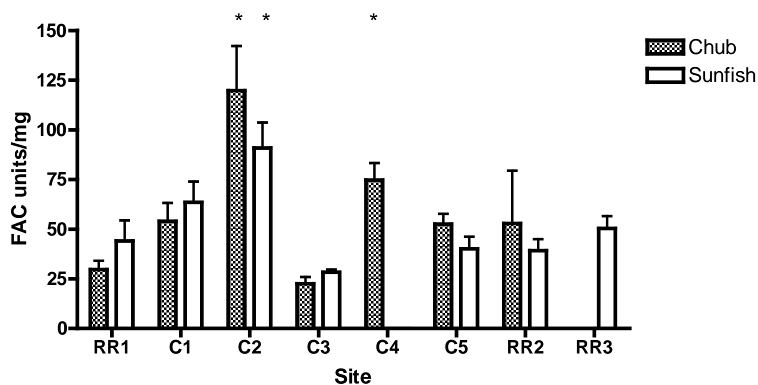

Figure 2. Bile fluorescence for chub and sunfish species.

Bile estrogens

Combined chub/sunfish bile estrogen values showed that there was a significant site effect (ANOVA, p= 6×10^{-5}). Fish from the upstream reference site (RR1) had the lowest amount of estrogenic compounds in their bile, being significantly lower than the bile samples from fish collected at sites site C5 C1, C2, C3, RR2 and RR3 (Fig. 4). As well, fish from site C5 had significantly lower amounts of estrogenic compounds than did fish collected at RR3.

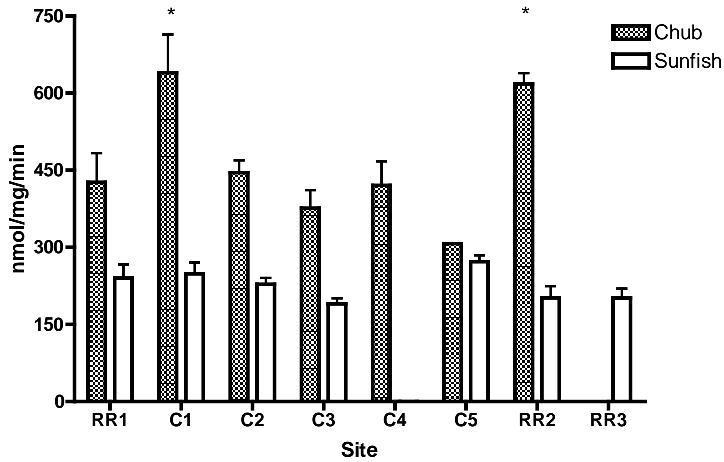

Figure 3. Activity of glutathione S-transferase in chub and sunfish.

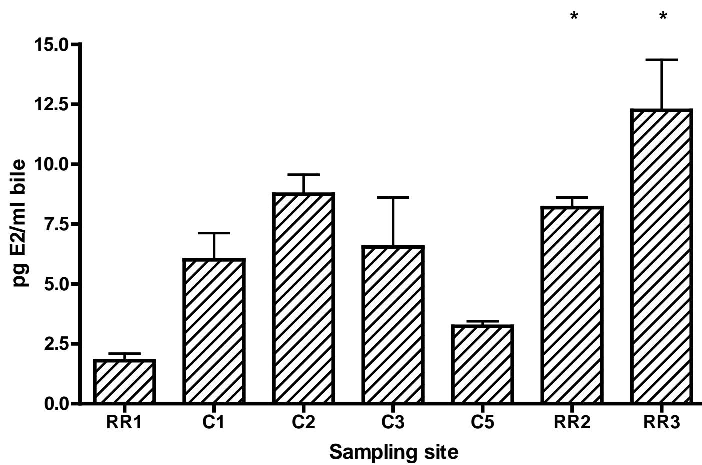

Figure 4. Concentrations of estrogenic compounds in male bile samples, expressed as pg 17β-estradiol equivalents/ml bile.

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Discussion

The results for the biomarkers measured in fish from the urban tributaries to the Reedy River show a varied response. Some of these urban creeks are significantly polluted with PAHs; site C2 (Brushy Creek) in particular showed a consistent response with induced levels of cytochrome P450-1A, as measured with the EROD assay, and high levels of fluorescent PAH metabolites in bile samples. Indeed, the fish collected at site C2 had significantly higher EROD and bile fluorescence activity than did fish collected at RR1 and R2 in the main channel of the Reedy River. This likely reflects the extremely high degree of development in this site's watershed, as this was the most highly developed site we sampled at (watershed nearly 90% developed land cover). In contrast, site C1 (Collins Creek) and C4 (Laurel Creek) exhibited intermediate and varied responses in sampled fish with respect to PAH exposure. Fish from site C4 had low EROD values compared to those collected at sites C1 and C2, but had relatively high bile fluorescence levels, being significantly greater than fish collected at site C1 (2-ring fluorescence) and RR1 and C3 (5-ring fluorescence). Fish collected at site C1, in contrast, had levels of EROD activity significantly higher than that seen in fish sampled at sites C3 and C4, but low 2-ring bile fluorescence activity and intermediate 5-ring bile fluorescence activity. Finally, sites C3 (Marrow Bone Creek) and C5 (Donaldson Creek) showed consistently low biomarker responses to PAH exposure. Indeed, even the upstream reference site was as high or higher than sites C3 and C5, indicating that there might be sources of CYP1A-inducing chemicals in the upper parts of the Reedy River watershed. Furthermore, our correlation results suggest that chubs and sunfish responded similarly across sites with respect to PAH exposure, as for both 5-ring fluorescence and EROD activity significant positive correlations were observed between chubs and sunfish at all sites where both were collected. Thus, our results show that small urban creeks can have high PAH exposure, but that this is also highly variable across the landscape, even in streams with similar levels of development.

In contrast, exposure to oxidative stress, as measured by GST activity, was far less variable across sites. Sunfish exhibited no significant differences across sites in GST activity, and for chubs, s~~S~~ignificantly increased GST activity was only observed in fish from site C1, where levels in these fish were higher than fish collected at site C3, similar to what was seen with respect to EROD activity. Once again, fish collected from site C3 had the lowest biomarker responses. ~~, indicating that this location induced oxidative stress in chubs, but this was not reflected in the biomarkers for PAH exposure in fish collected from this location.~~ As such, site C3 appears to be the least disturbed location we sampled, indicating that this would be an even better reference site than the RR1 site. ~~site upstream of the city of Greenville.~~

~~no significant responses in any of the fish from the urban creeks, but did show elevated levels in the downstream Reedy River sites. This is in concordance with a previous study done in the Reedy River (Truman & Van den Hurk, 2010), in which 3 different biomarkers for xenoestrogen exposure showed significant increases downstream of the Mauldin Road wastewater treatment plant. Some of the urban creeks (C1, 2, and 3) appear to induce elevated bile estrogen levels in the fish; but while these elevated levels are not significantly different from the reference site in the upstream Reedy River, it remains to~~

~~be investigated if with larger samples sizes persist it can be concluded that the urban creeks do contribute to the higher levels of estrogenic compounds in the main stem of the Reedy River.~~

Considering the sampling locations in the main stem of the Reedy River first, the results of the study presented here indicate that there are fewer significant differences between the Reedy River reference location upstream of the city of Greenville and the Reedy River locations downstream of the city than reported before. However, there was still a significant increase in endocrine disrupting estrogenic compounds in bile samples from fish collected below the Greenville wastewater treatment facility. As well, most of the previously reported effects of PAHs were obtained in fish from Lake Conestee and Boyd Mill pond, and the sediments in Lake Conestee in particular are known for very high metal and PAH concentrations, and these are most likely the source for the biological effects observed in fish from these locations (Otter et al., 2012).

In regards to the small urban creeks we sampled, t~~T~~he sources for the pollutants that induce biomarker responses in these ~~urban~~ creeks are difficult to identify. There are no NPDES regulated point sources in any of the creeks, so the sources must be considered as urban runoff and other non-regulated sources. For the PAHs, urban runoff has been investigated in numerous other places as a potential source. In recent years, especially runoff from roadways and parking lots has been of concern, not only because of leaked fossil fuels and engine oil and grease, but also because it has become fashionable to resurface driveways and parking lots with coal tar mixtures (Mahler et al., 2014), and it has been clearly established that these surface coatings are rich in PAHs, which wash off during rain storms and end up in surface waters and sediments (Witter et al., 2014). Luckily this source of PAHs has been recognized and local and state regulating offices are banning the use of the PAH-containing resurfacing materials. For example, the city of Greenville has recently banned the use of these resurfacing materials (Greenville News, 2013), which should lead to a reduced input of PAHs into the Greenville urban creeks and the Reedy River.

Relatively little is known about how these species respond to pollution. The two sunfish species studied here are generally considered very tolerant to environmental perturbations (e.g., Jenkins and Burkhead, 1994), and chubs, though also considered to be tolerant to anthropogenic influences, may be more sensitive to pollutants like PAH's. These sensitivity differences between sunfish and chub species can be derived from studies in which changes in species assemblages are evaluated as a result of pollution sources (Schorr & Backer, 2006; Meador & Carlisle, 2007). However, the underlying physiological and biochemical mechanisms for the sensitivity of chub and the tolerance of sunfish are poorly investigated.

In a comparative study, bluegill showed to have a relatively low EROD activity, the indicator for cytochrome P450-1A expression, compared to species such as Atlantic salmon, tilapia and channel catfish (Gonzalez et al., 2009). Considering that we measured even lower EROD activity in chubs, it can be concluded that the chubs have very low CYP1A expression, which may partly explain their sensitivity to environmental pollutants like PAHs, PCBs and dioxin-like chemicals. The same study showed that bluegill also had relatively low GST efficiency compared to the other species; channel catfish and tilapia had 3-4 times higher efficiency of GST towards the substrate CDNB, which had been observed before

Commented [DH11]: This is somewhat inconsistent with the results. Here you say sites C12 and 3 aren't different from RRR1 but in the results you say C2 is different from RR1. So, I changed this to match the results. BUT, if the results are wrong we'll need to redo this.

(Ankley & Agosin, 1987). Based on the results presented here that show that on average the GST activity in chubs is 2-3 times higher than in bluegill, makes the GST activity in chubs comparable to a number of other fish species (Gonzalez et al., 2009). In another study by Brammell et al., (2010), comparing two sunfish species (longear sunfish and bluegill), animals were dosed with BaP or PCB77, a dioxin-like planar halogenated aromatic hydrocarbon. Both species responded by significant increases of EROD activity after exposure to both chemicals, but did not show an increase in GST activity. This confirms our results that bluegill don't appear to have a well-developed GST system that is involved in detoxifying reactive metabolites that cause oxidative stress inside the organism.

Creek chubs, because of their high abundance in urban streams are often used as indicators for environmental stressors. For example, studies have been published on the effects of urbanization (Fitzgerald et al., 1999), mining effluents (Stair et al., 1984; Dubé et al., 2006), and PCB-contaminated streams (Henshel et al., 2006) on individual growth and population structure of this species. However, despite the ecological importance of the creek chub, very little work has been published on the biochemical responses of chubs to pollutants. In a study on the effects of perfluorooctane sulfonate (PFOS), a widely used surfactant, on several freshwater fish species, creek chub displayed significantly higher oxidative damage in liver than did rainbow trout or white sucker (Oakes et al., 2005). Unfortunately, none of the biomarkers measured in our study were reported in this paper, but the high levels of liver damage compared to other species does indicate that despite their prevalence in urban streams, chub have a less developed toolbox of detoxification pathways.

The observed species differences in biochemical responses to environmental pollutants triggers curiosity about the evolutionary history of the enzymatic pathways that are involved in detoxification of those pollutants. Vertebrates have an extensive system of detoxification enzymes, consisting of phase 1 enzymes, like the cytochrome P-450 isoforms, that create a functional group on the toxic chemical, and the phase 2 enzymes, like glutathione S-transferases, UDP-glucuronosyltransferases and sulfotransferases, that conjugate enzyme-specific side groups to the compounds with a functional group, thus reducing the toxicity of the chemicals, and usually making them easier to excrete (Schlenk et al., 2008). The diversity of isoforms of these enzymes is large, and can cover a wide variety of substrates. During early phylogeny of fish several genome multiplication events may have taken place, which would have opened the possibilities for the evolution of numerous enzyme isoforms (Vandepoele et al., 2004). Combined with the evolution of herbivory in different fish taxa this may have resulted in the wide variety of detoxification pathways that can be observed in current fish species (German et al., 2010). It is only in the last 100-200 years that fish have been exposed to a variety of anthropogenic chemicals, which would suggest that resistance to environmental pollutants is linked to much older mechanisms of dealing with natural toxicants in the aquatic environment. Based on our observations, a variety of detoxification strategies has evolved in fish species, which needs to be further investigated to explain different sensitivities in species, and which can then be used in environmental risk assessment strategies and biological effects monitoring programs.

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