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Running title: BaP metabolism in chub and sunfish

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Abstract

Observations in a previous study on biomarker responses in fish collected from urban creeks in Greenville, SC, indicated that there might be considerable differences in the expression of biotransformation enzymes in chub and sunfish species. To further investigate these species differences a dosing experiment was performed in which bluehead and creek chub (*Nocomis leptocephalus* and *Semotilus atromaculatus)*, and redbreast sunfish, pumpkinseed, and bluegill (*Lepomis auritus, L. gibbosus,* and *L. macrochirus)* were injected with benzo[a]pyrene (BaP) as a model compound for common pollutants in urban creeks. Fish were injected with BaP doses of 0, 25 and 50 mg/kg, and after 3 days BaP metabolites in bile, and enzymatic activities of cytochrome P450-1A (CYP1A), UDP-glucuronosyltransferase (UGT) and glutathione S-transferase (GST) were measured. CYP1A activity was significantly increased after BaP dosing in both species groups, but chubs had significantly lower levels than were observed in the dosed sunfish. The UGT activity in unexposed animals was comparable in both species groups, and significantly increased in both groups as a result of BaP dosage. Finally, GST activity was significantly higher in chubs, but did not change in either species group as a result of BaP exposure. There were no significant differences between species within each species group, and the results confirmed that unexposed chubs have much lower CYP1A activity, but a much higher GST activity than unexposed sunfish. The metabolized BaP was excreted in both species groups, but at the time of sampling there were no differences in the amount of BaP metabolites in the bile of dosed animals. The differences in baseline enzyme activity and induction capacity between both species groups are an

example of phylogenetically determined differences between fish families, and may explain why chubs are in general more sensitive to exposure to environmental pollutants than sunfish. This conclusion was corroborated by the observation that the highest BaP dose of 50 mg/kg was close to the apparent LC_{50} for chub, while no mortality was observed in the sunfish at this dose.

Key Words: pollutants, species differences, biomarkers, detoxification, benzo[a]pyrene

Introduction

When animals are exposed to environmental pollutants that have the potential to induce harmful effects to the animal's physiology, a variety of detoxification pathways can be activated to reduce the toxic effects of the chemical pollutant and enhance the excretion of the toxicant. Upregulation or activation of these detoxification pathways can be measured through a variety of parameters, which are collectively known as biomarkers. The use of biomarkers has found widespread acceptance as indicators for exposure in target organisms, and in the establishment of bioavailability of environmental toxicants (Van der Oost et al., 2003).

In a recent study we used a biomarker approach to establish if small urban tributary creeks to the main stem of the Reedy River contributed to overall river pollution in and downstream of the city of Greenville, SC (Van den Hurk and Haney, 2016). Although initially the goal was to use only sunfish *(Lepomis sp.)* species for that study, several sampling locations did not yield any sunfish during sampling, but had enough chub (*Nocomis leptocephalus* and *Semotilus atromaculatus)* to get a good sample size. Because there were also sampling sites that yielded both sunfish and chub, we decided to analyze the selected biomarkers in both species groups, and test for species differences in their biomarker responses. The surprising result of this study was that sunfish and chubs had very different levels of activity of enzymes involved in biotransformation of environmental toxicants. The sunfish species had a much higher activity of cytochrome P450-1A (CYP1A) than chubs, both in animals from uncontaminated sites as in those from contaminated sites. On the other hand, chubs always had a higher glutathione-S-transferase (GST) enzymatic activity than the sunfish.

The observed differences in field collected chub and sunfish, which belong to two different orders (*Cyprinidae* and *Centrarchidae* resp.) within the class of the ray-find fishes (*Actinopterygii*) could indicate different phylogenetic signals in their respective genomes with respect to the expression and regulation of detoxification enzymes (Harvey and Pagel, 1991; Chiari et al., 2015). To further investigate if these species differences in biotransformation enzyme activities could be reproduced in a controlled dosing experiment, we designed a study in which chub and sunfish were collected from an unpolluted reference site and were dosed with several concentrations of benzo[a]pyrene (BaP), a model compound for the group of polynuclear aromatic hydrocarbons, which are commonly found in urban streams, and are known to induce several biotransformation enzymes that are commonly used as biomarkers for exposure to environmental pollution (Schlenk et al., 2008). Our hypothesis was that the differences in biotransformation enzyme activities between cyprinids and centrarchids, as observed in field collected

and exposed animals, would also be observed in a controlled dosing experiment. In addition to CYP1A and GST activity, phenol-type UDP-glucuronosyltransferase activity was also measured, as was the amount of BaP metabolites in bile samples from the dosed fish.

Materials and methods

Materials

Benzo[a]pyrene (≥ 97%) was purchased from Sigma-Aldrich; 9-hydroxy-benzo[a]pyrene was obtained from the National Cancer Institute Repository at the Midwest Research Institute, Kansas City, MO; tricaine methanesulfonate was purchased from Research Organics, Cleveland OH; all other chemicals were obtained from either Sigma-Aldrich or Fischer Scientific at the highest research quality available.

Sample Collection

For the dosing experiment, bluehead chub and creek chub (*N. leptocephalus* and *S. atromaculatus*), and readbreast sunfish, pumpkinseed, and bluegill (*L. auritus, L. gibbissus,* and *L. macrochirus*) were collected from rural sites along the Enoree River near Greenville, SC in June 2010. Weight range for the chub was 1.1 – 31.7 g (avg 7.3 g ± 0.99 SE); weight range for sunfish was 0.3 -43.1 g (avg 11.1 g ± 1.5 SE). 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. The fish used for dosing were acclimated in 10-gal aquaria with aerated, filtered water from the collection site for 3-14 d prior to injection. Animals were maintained on a 16:8 light:dark cycle at room temperature (21±2 °C) and were fed once daily.

Experimental Protocol

Both species groups were divided into four treatment groups: wild, vehicle control, a low-dose group and a high-dose group. The wild-captured, untreated group received no injection, and were sacrificed on the day of collection (n=14 chubs, n=11 sunfish). The control group was injected with the vehicle solution of a small amount of dimethyl sulfoxide (DMSO) in canola oil (n=10 chubs, n=8 sunfish), the low dose group was injected with 25 mg/kg (0.1 mmol/kg) benzo[a]pyrene (BaP) (n=12 chubs, n=9 sunfish), and the high-dose group was injected with 50 mg/kg (0.2 mmol/kg) BaP (n=12 chubs, n=8 sunfish). Before injection, the fish were anesthetized with 0.25 g/L (0.957 mM) tricaine methanesulfonate (MS-222) and weighed to calculate the volume of the BaP stock solution that needed to be injected to obtain the desired BaP dose per kg bodyweight. The fish were sacrificed 3 d after injection by submersing them in 1 g/L (3.8 mM) MS-222. Fish were then measured (standard length [mm] and weighed [g]), and gall bladders and livers were harvested from each fish. Gall bladders were stored in amber microcentrifuge tubes and placed on ice and livers were wrapped in aluminum foil and frozen in liquid nitrogen before both were stored in a -80°C freezer prior to later analyses.

Preparing Liver Post-Mitochondrial (S9) Fractions

Livers were weighed, thawed and individually homogenized with a glass Potter-Elvehjem homogenizer in approximately 5 volumes of cold 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, or S9 fraction was divided into three aliquots for determination of enzymatic activities, and an additional small aliquot for total protein concentration. The enzyme 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

Cytochrome P4501A activity was measured through the EROD assay (Schreiber et al., 2006). 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 reaction buffer (0.2% BSA, 5mM MgCl2, 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.

Glutathione S-Transferase Activity

GST activity 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.

UDP-glucuronosyltransferase Activity

Phenol-type glucuronidation through UDP-glucuronosyltransferase activity was measured using 9-hydroxy-benzo[a]pyrene as a substrate in a reaction volume of 500 μl containing 1 μM 9-OH-BaP, 0.1 M Tris-HCl buffer (pH 7.8), 5 mM MgCl$_2$, 100 μg S9 protein (mixed 5:1 (w:w) with Brij 58) and 200 μM UDP-glucuronic acid (Gaworecki et al., 2004). Samples were incubated at 27° C for 30 min, after which the reaction was stopped with 2 ml methanol. The pH of these samples was increased by adding 1 N NaOH, after which the glucuronidated product was measured by fluorescence spectrophotometry at Ex 295, Em 415 nm (James et al., 1997).

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 50:50 methanol:water solution. Fluorescence of aromatic compounds (FACs) was then measured in three replicate aliquots from each dilution at Ex 380, Em 430 nm on the SpectraMAX Gemini plate reader mentioned above. Raw 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).

Statistical Analyses

Statistical analyses were conducted using the Prism 4 software package (Graphpad Software, Inc.) and R version 3.0.1. Independent samples T-test was used on log-transformed measured endpoints to test for significant differences between the two chub species; a nonparametric Kruskal-Wallis test was used with the Wilcoxon Each Pair nonparametric comparisons test to identify if there were species differences within the sunfish species group. Because there were no significant differences between species within each species group, for further analysis of differences between Cyprinids and Centrarchids data were pooled within each species group. For the analysis of GST and UDP-glucuronosyltransferase activity, data were log transformed to meet criteria for normality and homogeneity of variances, then a factorial ANOVA followed by a Tukey's HSD post hoc was performed to test for differences between experimental groups and between species for each of these parameters. For bile fluorescence and EROD activity, transformations failed to achieve homogeneity of variance for the factorial ANOVA. As such, we compared activity of these parameters across experimental groups within each species separately. For the sunfish we were able to meet assumptions of the single factor ANOVA (followed by a Tukey's HSD post hoc) for both EROD and bile fluorescence activity after a log transformation. However, for chubs we could only do the parametric analysis for bile fluorescence activity, again on log transformed data. Equality of variance could not be achieved for EROD activity, so a Kruskal-Wallis test followed by the Kruskal MC post hoc was performed. Following these tests on bile fluorescence and EROD activity, Independent samples T-tests were then performed on log transformed data for the two parameters comparing chubs to sunfish within each experimental group. Because a second analysis was made on the same data, a Bonferroni correction was applied to all T-test results. All results were considered significantly different if p≤ 0.05.

Results

Mortality

The injected BaP dosages were based on previous experiences with other fish species (Gaworecki et al.,2004; Van den Hurk, 2006) , and were selected to be in the sub-lethal range, but high enough to

induce significant effects. However, soon after injection it became clear that the high dose of 50 mg/kg was too high for the chubs, as by day 3 of exposure 42% of the chubs had died in the high dose treatment. Chubs administered the low dose of 25 mg/kg also suffered substantial mortality, with 25% of the chubs dead by the end of the exposure time. No abnormal mortality was observed in the wild-caught chubs or the vehicle control treatment. No mortality was observed in the low or high dosed sunfish, or in the wild-caught fish; only in the vehicle control group did an unexplained 18% mortality occur.

EROD induction

For both chubs (Kruskal-Wallis, $p > 0.05$) and sunfish (ANOVA, $p > 0.05$) no significant differences were found in EROD activity between the animals that were freshly collected in the wild (0.5 and 1.9 pmol/mg/min, respectively) and the control animals that were injected with the vehicle control (0.35 and 2.2 pmol/mg/min), respectively, indicating that the vehicle solution had no effect on the outcome of the experiment (Fig. 1). Significant differences (Independent Samples T-test followed by a Bonferroni correction, $p < 0.05$ after correction) were measured between the species groups for all four experimental groups, with EROD activity at least three times higher in the sunfish than in the chub for all experimental groups. The sunfish data show the crude outline of a typical dose-response curve, but because of the limited dosing concentrations a complete dose-response curve could not be generated. Such a pattern was not observed with the chubs. Both dosing concentrations in the sunfish were significantly different from the vehicle control or the wild-caught animals (ANOVA followed by TukeyHSD post hoc; $p < 0.05$), with 4 - 5 times induction observed, but these groups were not significantly different from each other (ANOVA followed by TukeyHSD post hoc; $p > 0.05$). In the chub a 10 times induction was observed in the 25 mg/kg dosage (3.3 pmol/mg/min) compared to the control or wild caught fish (ANOVA followed by TukeyHSD post hoc; $p < 0.05$), but in the higher dosage of 50 mg/kg an unexpected low activity was observed (1.0 pmol/mg/min), that was not significantly different from the control or wild caught fish (ANOVA followed by TukeyHSD post hoc; $p > 0.05$).

Bile fluorescence

When fish are exposed to PAHs like benzo[a]pyrene, metabolites of these PAHs are produced by liver enzymes, which are then excreted into the bile. Because PAHs like BaP are highly fluorescent, the parent compound and its metabolites can be detected in bile through fluorescence spectrophotometry. In both the chub and the sunfish no significant differences (ANOVA, $p > 0.05$) in BaP metabolite fluorescence were seen between the wild caught fish and the vehicle control, indicating that there were no sources of PAHs near the collection site of the fish, and that the canola oil used in the vehicle solution did not contain PAH contamination (Fig. 2). However, for both species bile fluorescence levels were significantly elevated (ANOVA followed by TukeyHSD post hoc; $p < 0.05$) in both the 25 mg/kg and 50 mg/kg dosed groups compared to either the wild caught or control fish groups. For both species no significant difference was observed in BaP metabolite fluorescence between the 25 mg/kg and 50 mg/kg dosed groups (ANOVA, $p > 0.05$). Within the four experimental groups, sunfish bile fluorescence activity was

not significantly different than for chubs in the control, 25 mg/kg, or 50 mg/kg dosed groups (Independent Samples T-test with Bonferroni correction, p > 0.05). Though values in the wild caught fish were extremely low for both species, the sunfish fluorescence activity was significantly lower than was measured in the wild caught chub species (Independent Samples T-Test with Bonferroni correction, p< 0.05 after correction).

Glutathione-S-transferase activity

Within either the chubs or the sunfish there were no significant differences (Factorial ANOVA, p > 0.05) between the controls and the dosed fish for GST activity (Fig. 3), and no significant interaction was observed. However, the GST activity in both the wild caught and control sunfish were significantly (Factorial ANOVA and TukeyHSD post hoc, p < 0.05 for both) lower than in the wild caught or control chubs, respectively. With 427.3 nmol/mg/min the activity in the wild caught chubs was almost twice as high as the average activity of 236.4 nmol/mg/min in the wild caught sunfish. Although there appears to be a dose-response relationship in the chub, the lower average GST activity in the highest dose group should probably be attributed to the morbidity of the test animals, as mentioned above for the other measured endpoints.

UDP-glucuronosyltransferase activity

The activity of the phenol-type UDP-glucuronosyltransferase activity showed a significant (Factorial ANOVA followed by TukeyHSD post hoc; p < 0.05) increase in both species groups as a result of BaP exposure. In the chub the lower dosage of 25 mg/kg caused a significant increase compared to the wild caught animals, but this increase was not extended in the higher dose group, probably because of the aforementioned morbidity observed in these animals. No other comparisons were significantly different (Factorial ANOVA, p> 0.05). In the sunfish a clear dose-response relation was observed, with a significant doubling of the activity in the highest dose group compared to the control and wild caught fish (from 34.5 to 74.8 pmol/mg/min; Factorial ANOVA followed by TukeyHSD post hoc; p < 0.05). When comparing chubs to sunfish, the UGT activity in the 50 mg/kg BaP sunfish was significantly higher (Factorial ANOVA followed by TukeyHSD post hoc; p < 0.05) than in the high dose chub group. No other comparisons were significantly different (Factorial ANOVA, p> 0.05), and no significant interaction was observed.

Discussion

The experiments described in this study were inspired by observations made in a field study in which significant differences were demonstrated in the activity of biotransformation enzymes in chub and sunfish species exposed to mixtures of environmental toxicants (Van den Hurk and Haney, 2017). In that

study, chub and sunfish were collected from urban creeks in Greenville, SC. When these fish were analyzed for a set of biomarkers, striking differences were found, especially for a cytochrome P-450 isoform (CYP1A) and glutathione-S-transferases (GSTs). This triggered the hypothesis that differences in the expression of these enzymes might explain species differences in sensitivity to environmental pollutants. To confirm that these observed differences are indeed a result of exposure to toxicants, the study reported here was conducted, using BaP as a model compound for the larger group of PAHs that can be expected in environments like the urban creeks in Greenville (Yang et al., 2010).

The experiments reported here confirm the results obtained in the field study; wild caught chub have a significantly lower activity of CYP1A than sunfish, but chub have a significantly higher activity of GST than sunfish. Despite these differences in constitutively expressed levels of biotransformation enzymes, both species groups did increase CYP1A and phenol-type UGT activity as a result of exposure to BaP. Upregulation of these two enzyme isoforms are regulated by the aryl hydrocarbon receptor (Ah-receptor), and thus both chub and sunfish are expected to have this regulatory pathway (Bock et al., 1990). However, multiple forms of the Ah receptor are found in fish species, which should be considered when interpreting enzyme activity studies (Hahn et al., 2017).

While both chubs and sunfish did increase CYP1A activity in response to BaP exposure, their patterns did differ, with chubs reaching maximum activity in the lower dosage group, then declining at the high dose group. In contrast, sunfish CYP1A activity more closely resembles a classic dosing response, with highest activity in the 50 mg/kg group. Because there was considerable mortality in the chub high dosage group, it is possible that incapacitating toxicity levels were reached in the fish in this dose group before the expression of detoxification mechanisms could be effectively upregulated. Acute toxicity of BaP can be attributed to non-genotoxic effects on neural and cardiorespiratory systems (Gerger and Weber, 2015).

As a result of the biotransformation of BaP, more water-soluble metabolites are formed, which are then excreted in the bile (van den Hurk, 2006). The results presented here show that both chub and sunfish have high levels of these fluorescent metabolites in their bile, but interestingly, the species differences that are observed in the activity of the biotransformation enzymes are not reflected in the amount of BaP metabolites in the bile. For the chubs the pattern of bile fluorescence follows the activity pattern of the CYP1A, with the highest fluorescence in the 25 mg/kg dosage group, and lower amounts in the higher dosage group. In sunfish, comparable levels of bile fluorescence were observed as in the chub, which indicates that the amount of CYP1A activity does not necessarily correspond with the amount of BaP metabolites in the bile. This can be explained by rate-limiting steps in the biotransformation and excretion pathways (Bouchard & Viau, 1996). Which one of the detoxification enzymes forms the rate limiting step is not known, but it is possible that higher levels of these enzymes resulted in a faster excretion of the single dose of BaP in sunfish. Because we only collected samples three days after dosage, the bulk of the dosed BaP could have been excreted already in these fish with higher enzyme activity. An experiment with the goal of building a toxicokinetic model for the time resolved induction of CYP1A and UGT in these species, and the excretion of BaP metabolites could find an answer to this question (Heredia-Ortiz et al., 2011).

During the biotransformation of BaP by CYP1A several metabolites can be formed, like epoxides, diols, triols and tetrols; most of these are phenolic compounds which are subsequently conjugated by UGT or sulfotransferase (SULT) isoforms (Miller & Ramos, 2001). However, the epoxide metabolites that can be formed are electrophilic, and as such are good substrates for GST. While UGT and SULT are the predominant enzymes that conjugate BaP metabolites, the role of GST can be important, and therefore a sufficient activity of GST will help reduce the toxicity of BaP (Upadhyaya et al., 2010). The results presented here show that both chub and sunfish have ample activity of GST, which was not further upregulated by BaP dosing. Isoforms of GST can be upregulated by receptors like CAR and PXR (Higgins & Hayes, 2011), but are not known to be responsive to the Ah-receptor, which is in concordance with our results. The GST assay that we applied used CDNB as a substrate, which is a general substrate for all GST isoforms, and therefore upregulation of one of the individual GST isoforms as a result of the BaP exposure could have been masked by the activity of the other isoforms. Other techniques, like gene expression measurements, are better suited to identify upregulation of individual GST isoforms (Schlenk et al., 2008).

What is remarkable is the much higher overall GST activity in chub than in sunfish. Isoforms of GST can use a variety of electrophilic substrates, among which includes strong oxidizing compounds (Schlenk et al., 2008). High activity of GST can therefore be seen as an adaptation to oxidative stress. However, research in which changes in species assemblages were evaluated as a result of pollution sources (Schorr & Backer, 2006; Meador & Carlisle, 2007) do list chubs as a more sensitive species than sunfish, and our observed differences in biotransformation enzyme activity may contribute to the differences in sensitivity to environmental stressors. The chub had much lower constitutive CYP1A and UGT activity, and even though the proportional induction of CYP1A was much higher in chub after BaP dosage, the actual concentration of enzymes in the liver was still lower than in the sunfish. This would make the chub more sensitive to PAHs because they would not have enough enzyme to detoxify and excrete the compounds.

The higher PAH sensitivity of chubs compared to sunfish is reflected in the unintended mortality results of the experiment. Previous studies had shown that a dose of 50 mg/kg BaP is not acutely lethal for a variety of commonly used fish species in dosing experiments (Van den Hurk et al., 1998; Van den Hurk, 2006; Brammell et al., 2010). For this study 25 and 50 mg/kg were chosen as doses to induce enzyme activity. For the sunfish these doses worked satisfactorily, causing no mortality in the highest dose group. However, for the chubs the highest dose was close to the 3 day LC50 value, as a mortality of 42% was observed. Furthermore, even at 25 mg/kg a quarter of the test chubs had died. These data support that chub are indeed much more sensitive than sunfish to PAHs like BaP.

In another comparative study, bluegill had a relatively low CYP1A activity compared to species like Atlantic salmon, tilapia and channel catfish (Gonzalez et al., 2009). Considering that we measured significantly lower CYP1A activity in chubs than is bluegill, it can be concluded that the chubs have even lower CYP1A activity compared to other fish species. Just as in our study, this study also showed that bluegill had relatively low GST activity compared to the other species; channel catfish and tilapia had 3-4 times higher efficiency of GST towards the substrate CDNB, which has also been reported by Ankley & Agosin (1987).

Based on the results presented here that show that on average the GST activity in chubs is almost two times higher than in bluegill, it can be concluded that constitutive GST activity in chubs is comparable to a number of other fish species (Gonzalez et al., 2009). For example, in another study comparing two sunfish species (long ear sunfish and bluegill), animals were dosed with BaP (10 and 5 mg/kg) or PCB77 (0.1 and 1.0 mg/kg), a dioxin-like planar halogenated aromatic hydrocarbon which is also a good ligand for the Ah-receptor. Both sunfish species responded by exhibiting significant dose-dependent increases of CYP1A activity after exposure to BaP, but, as in our experiments, did not show a dose-dependent change in GST activity (Brammell et al., 2010). Unfortunately, little is known about UGT activity in chubs or sunfish. Ankley and Agosin (1987) report that UGT activity in bluegill is comparable to channel catfish, and Brammel et al. (2010) investigated the effect of BaP exposure on UGT in long ear sunfish, which was, contrary to our results, not upregulated after dosage of 10 or 50 mg/kg.

While chubs are typically considered to be tolerant to anthropogenic stress, being highly abundant in many urban streams, this tolerance seems to extend only to their ability to deal well with physical stressors such as highly fluctuating river flows, channelization, stream incision, etc., as our results suggest that they are very sensitive to chemical stressors. This observation is supported by other studies as well, in which creek chubs were classified as sensitive to anthropogenic stressors; 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 creek chub. 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 rainbow trout or white sucker (Oakes et al., 2005). Unfortunately, none of the biochemical parameters used in our study were measured in the study by Oakes et al., but the high levels of liver damage compared to other species does indicate that chub have a less developed suite of detoxification pathways. Based on these observations by Oakes et al. (2005), and the experimental results presented here, we can conclude that chub have relatively low overall biotransformation activity. That is even more pronounced considering that González et al. (2009) rank bluegill as "low metabolizers" in a comparison with seven other fish species. There are probably too many other physiological factors involved to directly relate overall biotransformation capacity to general sensitivity to environmental stressors, but the very low biotransformation activity in chub may be part of this environmental sensitivity.

The observed species differences in biochemical responses to environmental pollutants raises questions about the evolutionary history of the enzymatic pathways that are involved in detoxification of those pollutants. Most vertebrates have an extensive array 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 cofactors onto the phase 1 metabolites with a functional group, thus reducing the toxicity of the chemicals, and usually making them easier to excrete (Schlenk et al., 2008). There are a large variety of isoforms within these biotransformation enzymes, each with a group of preferred substrates. It is assumed that during early phylogeny of fish several

genome multiplication events took place (Iwabe et al., 1996), which would have opened possibilities for the evolution of a variety of enzyme isoforms (Vandepoele et al., 2004). The availability of a variety of potential detoxification enzymes could have facilitated the evolution of herbivory in different fish taxa, as is observed in current fish species (German et al., 2010). However, it needs to be kept in mind that it is only in the last 100-200 years that fish have frequently become exposed to a diversity of anthropogenic chemicals. This suggests that sensitivity to environmental pollutants is dependent on much older mechanisms of coping with natural toxicants, like PAHs, in the environment. Based on our observations and published work cited above, a variety of detoxification strategies has evolved in fish species, which needs to be further investigated to explain differing sensitivities in species. New knowledge about species sensitivities can then be used in environmental risk assessment strategies and biological effects monitoring programs that will protect sensitive species in a changing environment.

Acknowledgements

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Figures.
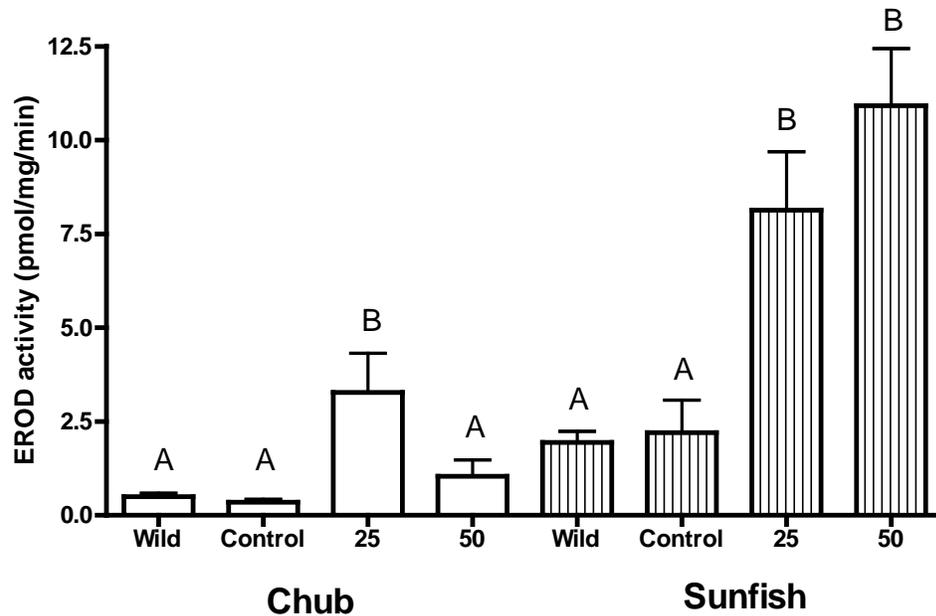
Figure 1. Mean ($\pm$ se) CYP1A activity, as measured by the EROD assay, in control and dosed chub and sunfish. Wild animals were sacrificed immediately after collection, control and dosed animals after 3 days of exposure. Control animals were injected with just the vehicle solution while dosed animals received 25 or 50 mg/kg BaP. Groups with different letter designations were significantly different from one another (ANOVA followed by TukeyHSD post hoc for sunfish, Kruskal-Wallis followed by a KruskalMC post hoc for the chubs; $p < 0.05$ for both). Within all 4 experimental groups, sunfish EROD activities were significantly higher than chub EROD activities (Independent Samples T-Test with Bonferroni correction, $p < 0.05$ after correction for all). Sample sizes per experimental group were (chubs, sunfish): Wild (14, 11), Control (8, 5), 25 mg/kg BaP (9, 9), 50 mg/kg BaP (7, 7).
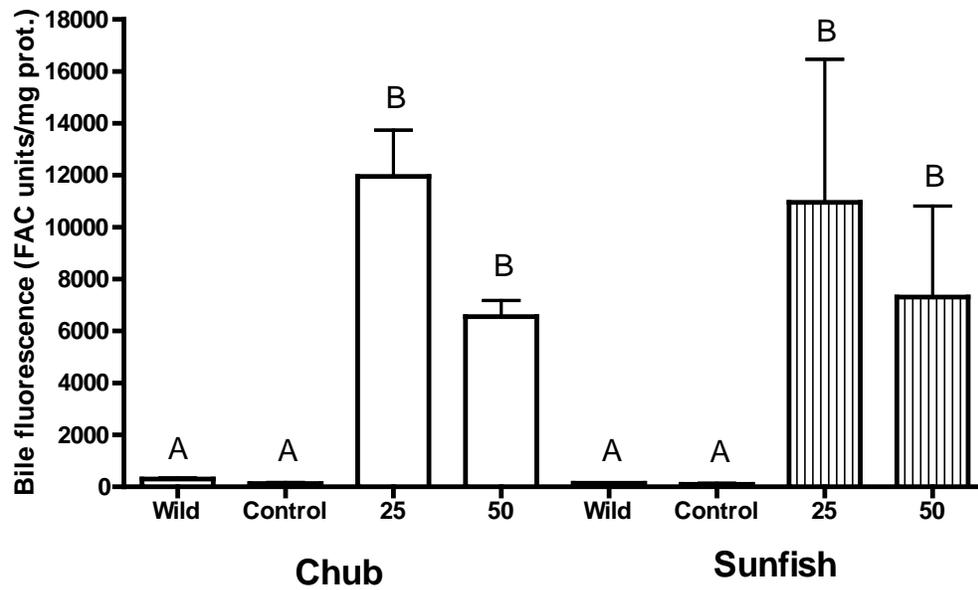
Figure 2. Mean (± se) total BaP metabolites in bile, as measured by bile fluorescence and normalized to total bile protein, in control and dosed chub and sunfish. Wild animals were sacrificed immediately after collection, control and dosed animals 3 days after dosing. Control animals were injected with just the vehicle solution while dosed animals received 25 or 50 mg/kg BaP. Groups with different letter designations were significantly different from one another within each species group (ANOVA followed by TukeyHSD post hoc; $p < 0.05$; species analyzed separately). Within the 4 experimental groups, chub bile fluorescence activity was only significantly higher than sunfish bile fluorescence activity (Independent Samples T-Test with Bonferroni correction, $p < 0.05$ after correction) in the wild caught animals. All other comparisons were not significantly different (Independent Samples T-Test with Bonferroni correction, $p > 0.05$). Sample sizes per experimental group were (chubs, sunfish): Wild (16, 10), Control (7, 4), 25 mg/kg BaP (8, 7), 50 mg/kg BaP (5, 8).
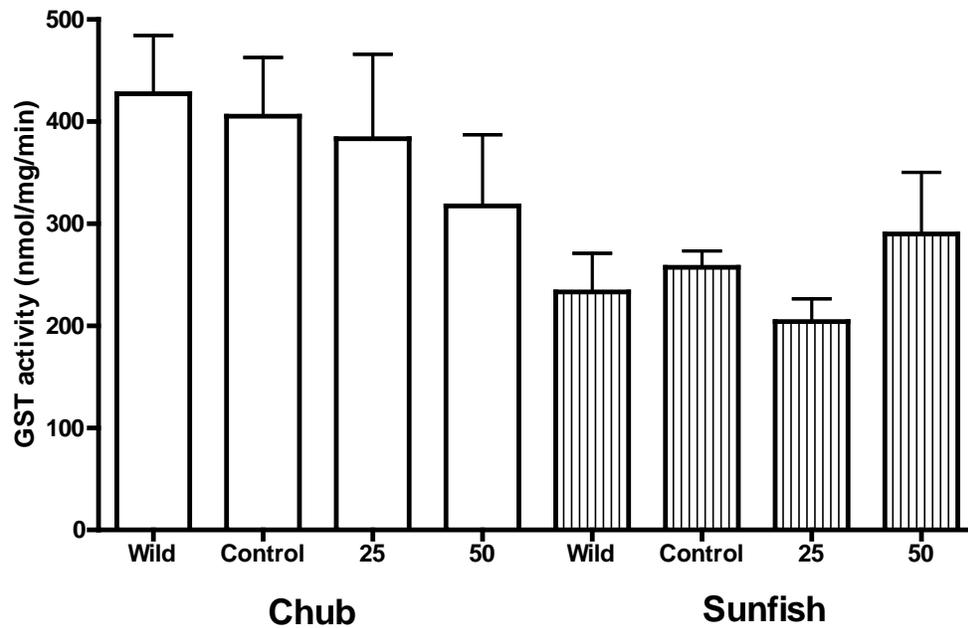
Figure 3. Mean (± se) total GST activity, as measured by conjugation of 1-chloro-2-nitrobenzene , in control and dosed chub and sunfish. Wild animals were sacrificed immediately after collection, control and dosed animals after 3 days of exposure. Control animals were injected with just the vehicle solution while dosed animals received 25 or 50 mg/kg BaP. No significant differences were seen between experimental groups for either species, nor was there a significant experimental group x species interaction (Factorial ANOVA, p > 0.05 for both). However, significant differences were seen across species within some experimental groups (Factorial ANOVA and TukeyHSD post hoc, p < 0.05 for both), where control and wild caught chubs had higher GST activities than did wild caught and control sunfish, respectively. Sample sizes per experimental group were (chubs, sunfish): Wild (14, 11), Control (9, 5), 25 mg/kg BaP (9, 8), 50 mg/kg BaP (7, 7).
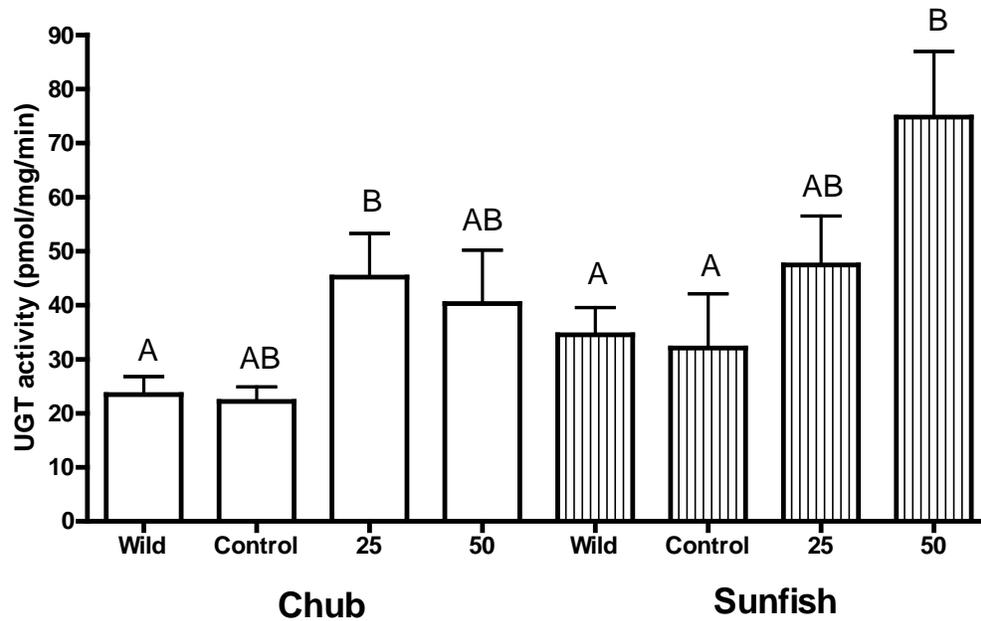
Figure 4. Mean (± se) phenol-type UGT activity, as measured by the conjugation of 9-hydroxy-benzo[a]pyrene, in control and dosed chub and sunfish. Wild animals were sacrificed immediately after collection, control and dosed animals after 3 days of exposure. Control animals were injected with just the vehicle solution while dosed animals received 25 or 50 mg/kg BaP. Groups with different letter designations were significantly different from one another (Factorial ANOVA followed by TukeyHSD post hoc; $p < 0.05$). Within the 4 experimental groups, sunfish UGT activity was only significantly higher than chub UGT activity (Factorial ANOVA followed by TukeyHSD post hoc; $p < 0.05$) in the high dose animals. All other comparisons were not significantly different (Factorial ANOVA, $p > 0.05$), and no significant interaction was observed. Sample sizes per experimental group were (chubs, sunfish): Wild (14, 10), Control (5, 5), 25 mg/kg BaP (8, 9), 50 mg/kg BaP (7, 7).