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Phylogenetic signals in detoxification pathways in Cyprinid and Centrarchid species in relation to sensitivity to environmental pollutants

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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.

1. 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, 2017). 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

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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.

2. Materials and methods

2.1. 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.

2.1.1. 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′ × 14′ × 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.

2.1.2. 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.

2.1.3. Preparing liver post-mitochondrial (S9) fractions

Livers were weighed, thawed and individually homogenized with a glass Potter-Elvehjem homogenizer in approximately 5 vols of cold homogenization buffer (van den Hurk, 2006). Liver homogenates were then centrifuged at 10,000 × *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.

2.1.4. 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, 5 mM 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 to 800 nM.

2.1.5. 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.

2.1.6. 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).

2.1.7. 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 × *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