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Allometric Scaling of Hepatic Biotransformation in Rainbow Trout

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Abstract

Biotransformation may substantially impact the toxicity and accumulation of xenobiotic chemicals in fish. However, this activity can vary substantially within and among species. In this study, liver S9 fractions from rainbow trout (4–400 g) were used to evaluate relationships between fish body mass and the activities of phase I and phase II metabolic enzymes. An analysis of log-transformed data expressed per gram of liver (g liver^{-1}) showed that total cytochrome P450 (CYP) concentration, UDP-glucuronosyltransferase (UGT) activity, and glutathione *S*-transferase (GST) activity exhibited small but significant inverse relationships with fish body weight. In contrast, *in vitro* intrinsic clearance rates ($\text{CL}_{\text{IN VITRO,INT}}$; $\text{mL min}^{-1} \text{mg protein}^{-1}$) for three polycyclic aromatic hydrocarbons (PAHs) increased with increasing body weight. Weight normalized liver mass also decreased inversely with body weight, suggesting a need to express hepatic metabolism data per gram of body weight (g BW^{-1}) in order to reflect the metabolic capabilities of the whole animal. When the data were recalculated in this manner, negative allometric relationships for CYP concentration, UGT activity, and GST activity became more pronounced, while $\text{CL}_{\text{IN VITRO,INT}}$ rates for the three PAHs showed no significant differences across fish sizes. Ethoxyresorufin *O*-deethylase (EROD) activity normalized to tissue weight (g liver^{-1}) or body weight (g BW^{-1}) exhibited a non-monotonic pattern with respect to body weight. The results of this study may have important implications for chemical modeling efforts with fish.

Keywords

Fish; Rainbow trout; Biotransformation; Cytochrome P450; UDP-glucuronosyltransferase; glutathione *S*-transferase; Allometry; Allometric scaling

1. Introduction

Fish, like mammals, metabolically transform many xenobiotic chemicals into more water-soluble metabolites via phase I and phase II reaction pathways (Schlenk et al., 2008; Sijm

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Supplemental data

Measured CYP concentrations, phase I and phase II enzyme activities, and PAH $\text{CL}_{\text{IN VITRO,INT}}$ rates, expressed per mg S9 protein, are presented in Table SI

Declarations of interest: none

and Opperhuizen, 1989). This activity generally results in reduced chemical accumulation and toxicity, although biotransformation may also result in formation of reactive products that are more toxic than the parent compound (i.e., bioactivation). In either case, the potential for a chemical to accumulate in fish and exert a toxic effect may be substantially determined by its rate of biotransformation.

In vitro biotransformation rate and affinity data for fish were previously summarized by Fitzsimmons et al. (2007). These data show that metabolism rates for the same chemical may vary substantially within and among species. Some of these differences can be attributed to species differences in expression of biotransformation enzymes (Wang et al., 2001), while additional variability may be due to methodological differences (e.g., enzyme system; substrate concentration). However, controlled studies indicate that a variety of factors can impact biotransformation rates within the same test animals. For example, Vignier et al., (1996) evaluated the effect of life-stage on hepatic biotransformation by comparing the metabolism of 7-ethoxyresorufin in microsomes collected from Atlantic salmon parr and smolt. Estimates of VMAX were found to be about 10 times greater in smolts. Kennedy and Tierney (2006) investigated the effect of diet and fasting on the metabolism of benzo[a]pyrene in rainbow trout. Measured rates of activity in hepatocytes collected from fasted animals were 2 to 3 times lower than those of fed animals. Additional factors shown to influence biotransformation activity include sexual maturity and gender (Stegeman and Chevion, 1980; Koivusaari et al., 1981, 1984; Förlin and Haux, 1990), and acclimation temperature (Karr et al., 1985; Carpenter et al., 1990).

Across the animal kingdom, many metabolic and physiological processes vary with body size (West and Brown, 2005). The relationship between a biological parameter and body mass is commonly described by the allometric equation $Y = a \times M^b$, where Y is the parameter of interest, expressed on a whole animal basis, M is body mass, *a* is a normalization coefficient, and *b* is the allometric or scaling exponent. In mammals, many physiological processes scale to body weight with fractional exponents of 0.6 to 0.8 (– 0.4 to – 0.2 when the data are expressed on a gram of body weight basis [g BW⁻¹]). These relationships are typically developed using data collected from multiple species. In fish, however, it is difficult to combine data for different species due to the large impact of water temperature on metabolic and physiological processes. For this reason, allometric relationships for fish are generally developed for a single species. This approach eliminates variability due to interspecies difference in measured processes. In addition, many species exhibit indeterminate growth, resulting in large ranges in body mass.

Basal metabolic rate in fish (measured as oxygen consumption; VO₂) typically scales to body mass with a fractional exponent ranging from 0.6 to 0.9 (– 0.4 to – 0.1 on a g BW⁻¹ basis; Clark and Johnston, 1999). Factors shown to affect this relationship include lifestyle (e.g., pelagic vs. benthic; swimming style), temperature, and pH of the holding water (Killen et al., 2010; Ohlberger, et al., 2012; Vaca and White, 2010). To understand the basis for these observed relationships, researchers have investigated the size-dependence of oxidative metabolism at the enzymatic level. In general, the mass-specific activities of oxidative enzymes in white skeletal muscle (e.g., citrate synthetase activity) scale negatively to body weight ($b \approx -0.25$), but scaling exponents differ substantially among species (Somero and

Childress, 1980; Somero and Childress 1990; Vetter and Lynn, 1997; Davies and Moyes, 2007; Moyes and Genge, 2010).

The purpose of this study was to evaluate the size-dependence of hepatic biotransformation in rainbow trout. Of interest was the question of whether fish size represents another factor responsible for observed differences in biotransformation rates within species. To perform this evaluation, we measured total cytochrome P450 (CYP) concentration and the activities of phase I and phase II metabolic enzymes in liver S9 fractions. Trout employed in this study were hatched from the same group of eggs and ranged in size by two orders of magnitude.

2. Materials and Methods

2.1 Chemicals

The enzyme substrates, benzo[a]pyrene (BAP), phenanthrene (PHEN), pyrene (PYR), 7-ethoxyresorufin (ER), *p*-nitrophenol (p-NP), and 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Sigma-Aldrich (St. Louis, MO). The purity of these chemicals was 95% or greater. The incubation cofactors, uridine 5'-diphosphoglucuronic acid (UDPGA, > 98% pure), adenosine 3'-phosphate 5'-phosphosulfate (PAPS, > 60% pure) and reduced glutathione (GSH, > 98% pure) were also purchased from Sigma-Aldrich. β -nicotinamide-adenine dinucleotide phosphate (β -NADPH, > 95% pure) was purchased from Oriental Yeast Company (Tokyo, Japan). All other reagents, unless noted otherwise, were purchased from Sigma-Aldrich and were reagent grade or higher in quality.

2.2 Animals

2.2.1 Source and holding conditions—Rainbow trout (*Oncorhynchus mykiss*, Erwin strain) were obtained as fertilized embryos from the Upper Midwest Environmental Sciences Center (US Geological Survey, Lacrosse, WI) and grown to the sizes needed for this study. All fish originated from the same pool of embryos. The fish were held at 11 ± 1 °C under a 16 h light:8 h dark photoperiod and fed commercial trout food (Classic Trout; Skretting USA, Tooele, UT). The feeding rate and particle size of the food were periodically adjusted to accommodate growth of the fish. Water used for fish holding was obtained directly from Lake Superior (single pass, sand filtered), and was sterilized by UV light before use. Chemical characteristics of the water were: total hardness, 45 to 46 mg L⁻¹ as CaCO₃; alkalinity, 41 to 44 mg L⁻¹ as CaCO₃; pH 7.6 to 7.8; total ammonia, < 1 mg L⁻¹; and dissolved oxygen, 85 to 100% of saturation. All trout holding conditions and experimental procedures were approved by an Institutional Animal Care and Use Committee in accordance with principles established by the Interagency Research Committee.

2.2.2 Selection of fish sizes—Our goal was to evaluate hepatic biotransformation in trout across the widest possible range of fish sizes, taking into account the minimum size required to provide enough tissue for measurement of enzyme activity and the need to avoid fish that were approaching sexual maturity. The minimum amount of pooled tissue needed to perform all planned assays was approximately 2 g. Preliminary experiments indicated that livers from 4 g trout could be cleared of blood and dissected from the body without the gall

bladder attached. The number of 4 g animals needed to provide the required amount of S9 material was ~ 40 animals per pool, which was deemed to be acceptable.

The activities of hepatic phase I and phase II metabolic enzymes in trout change as they approach spawning condition, generally tending to increase, especially in males (Stegeman and Chevion, 1980; Koivusaari et al., 1981, 1984; Förlin and Haux, 1990). The strain of trout employed for this study spawns in the fall. Under the described holding conditions, these fish attain sexual maturity as they approach 3 yr of age, at which point they weigh 600–800 g. To minimize the effects of sexual maturation on enzyme activity, the largest animals were sampled in February, at approximately 2.3 yr of age. The sexual maturity of these animals was evaluated by weighing the gonads from each fish and calculating its gonadosomatic index (GSI; equal to the mass of gonads/body mass $\times$ 100).

Altogether, biotransformation was evaluated in four different sizes of fish. The weights of the animals were equally distributed on a log₁₀ scale across two orders of magnitude, resulting in target body weights of 4, 20, 90 and 400 g. To accommodate individual differences in growth rate, an animal was accepted if its body weight was within 25% of the target value at the time of collection. A small number (< 10%) of 4 g animals were slightly larger.

2.3 Collection of livers and preparation of liver S9 fractions

The body weights of donor fish were routinely monitored by weighing subsets of fish drawn from the population of study animals. Livers were collected whenever the average weight for this sampled subset reached one of the target body weights. Five pools of liver tissue were collected for each size of fish.

S9 fractions were prepared from pooled livers following methods described by Johanning et al. (2012), modified as necessary for smaller animals. The fish were euthanized with an overdose of tricaine methanesulfonate (MS 222, 250 mg L⁻¹; Western Chemical Ind., Ferndale, WA) buffered with 750 mg L⁻¹ NaHCO₃. Previous work has shown that MS 222 has no discernable impact on cytochrome P450 (CYP) protein levels or measured levels of CYP activity (Kleinow et al., 1986; Kolanczyk et al., 2003). All livers were cleared of blood *in-situ* by infusing with Hank's balanced salt solution (pH 7.80; ice-cold; free of CaCl₂, MgSO₄, and phenol red) containing 2.3 mM EDTA and 10 mM HEPES. In the larger fish (20, 90, and 450 g), clearing buffer was delivered through the hepatic portal vein using a hypodermic needle sized to fit the vessel. In the 4 g trout, this approach was not possible due to the small size of the hepatic portal vein. Instead, clearing buffer was administered by inserting a small needle (28 gauge, ½ inch) directly into the liver tissue. Once cleared, the liver was immediately excised from the body, leaving the gall bladder behind. The liver was then rinsed with a small volume of Hank's buffer to minimize possible contamination from bile. Livers were stored on ice in a small volume of homogenization buffer until all livers for a given pool were isolated. The livers were then blotted quickly and weighed. Due to their small size, livers from the 4 and 20 g fish were pooled before weighing. Livers from the 90 and 400 g fish were weighed individually. The livers were homogenized in 2 volumes of homogenization buffer using 4–5 strokes of a Potter-Elvehjem mortar and pestle. The homogenization buffer (pH 7.80 $\pm$ 0.05) consisted of 150 mM KCl, 50 mM Tris, 1 mM

dithiothreitol, 2 mM EDTA and 250 mM sucrose. A small sample (250 μ L) of this homogenate was collected to measure the recovery of microsomal enzymes (see 2.6 Correction for incomplete recovery of enzymes). The remainder was centrifuged at $13,000 \times g$ for 20 min at 4 $^{\circ}$ C. Aliquots (0.1 mL) of the supernatant (S9 fraction) were then flash frozen in liquid nitrogen and stored at -80° C until used. Unpublished data collected in our laboratory indicate that S9 fractions remain viable (i.e., minimal loss of enzyme activity) for at least two years when stored in this manner.

2.4 Measurements

2.4.1 Protein and CYP concentrations—The protein concentration of S9 fractions was measured using Peterson's modification of the Lowry method (Sigma Technical Bulletin TP0300; Sigma-Aldrich, St. Louis, MO). Total CYP concentration was determined using a dithionite difference spectroscopy method (Matsubara, 1976), adapted for studies with trout (Nichols et al., 2013a).

2.4.2 Phase I and phase II enzyme activities—Biotransformation activity was evaluated by performing a set of assays using model substrates for CYP1A, UDP-glucuronosyltransferase (UGT), and glutathione *S*-transferase (GST). All assays were conducted at the physiological temperature ($11 \pm 1^{\circ}$ C) and pH (7.80 ± 0.05) for trout, using saturating substrate concentrations. Substrates were added to the incubation mixtures using a small volume of acetone carrier (0.5% v/v final concentration).

CYP1A activity was characterized by measuring the rate of ethoxyresorufin-*O*-deethylase (EROD; Burke and Mayer, 1974). Incubations were conducted in 1.0 mL reaction mixtures consisting of 1 mg mL⁻¹ S9 protein, 1 mM NADPH, and 5 μ M ER in 100 mM potassium phosphate buffer. Resorufin was measured by fluorescence (BioTek Instruments, Winooski, VT; Ex. 550 nm, Em. 580 nm) and quantified by comparing to a set of resorufin standards.

UGT activity was characterized by measuring the glucuronidation of p-NP as described by Castrén and Oikari (1983), but with the addition of alamethicin to remove latency (Ladd et al., 2016). Incubations (600 μ L) were conducted in 500 mM potassium phosphate buffer containing 100 μ M p-NP, 1 mg mL⁻¹ S9 protein, 12.7 μ M alamethicin, 10 mM EDTA, and 7 mM UDPGA. Buffer, S9 protein, and alamethicin (in methanol, 0.25% v/v final concentration) were mixed and pre-incubated on ice for 15 minutes. Reactions were initiated by adding p-NP and terminated after 30 min of incubation. Substrate (p-NP) was measured by absorbance (BioTek Instruments; 400 nm) and quantified by comparing to a set of p-NP standards.

GST activity was measured in a 1.4 mL reaction mixture containing 100 mM potassium phosphate buffer, 2 mM GSH, and 2 mM CDNB. Reactions were initiated with the addition of S9 protein (0.1 mg mL⁻¹) and absorbance was monitored for 2 min at 340 nm (Agilent Technologies, Santa Clara, CA). Control samples without S9 protein were analyzed simultaneously (dual beam) to determine non-enzymatic changes in absorbance. Enzyme activity was calculated using a molar absorption coefficient of 9.6 mM⁻¹ cm⁻¹ (Habig et al., 1974).

2.4.3 Intrinsic clearance of PAHs—*In vitro* intrinsic clearance ($CL_{IN\ VITRO,INT}$; $mL\ min^{-1}\ mg\ S9\ protein^{-1}$) of PHEN, PYR, and BAP was measured using a substrate depletion approach. These experiments were conducted at $11 \pm 1\ ^\circ C$ in 1.0 mL reaction mixtures consisting of 100 mM potassium phosphate buffer ($pH\ 7.80 \pm 0.05$), $1.0\ mg\ mL^{-1}$ S9 protein, $12.7\ \mu M$ alamethicin, 2 mM β -NADPH, 2 mM UDPGA, 0.1 mM PAPS (corrected for impurities), and 5 mM GSH. Starting substrate concentrations were selected to be well below the enzyme affinity constants (K_M) measured for each of the PAH substrates (Nichols et al., 2017). Buffer, S9 protein, and alamethicin (in methanol, 0.25% v/v final concentration) were pre-incubated on ice for 15 min. Reactions were initiated with the addition of substrate (acetone carrier, 0.5% v/v final concentration) and terminated at pre-determined times by transferring 100 μL aliquots of the reaction mixture into 300 μL of ice-cold acetonitrile (ACN). These samples were vigorously mixed for 20 s and centrifuged at $3000 \times g$ for 5 min ($4\ ^\circ C$) to remove precipitated protein. Supernatants were then transferred to glass liquid chromatography vials and stored at $4\ ^\circ C$ until analyzed within 24 h.

First-order depletion rate constants (k ; min^{-1}) were calculated from the slope of $\log_{10}$ -transformed substrate depletion data:

$$k = -2.3 \times \text{slope} \quad (1)$$

$CL_{IN\ VITRO,INT}$ values were then calculated by dividing k by the concentration of S9 protein in the incubation mixture (C ; $mg\ mL^{-1}$):

$$CL_{IN\ VITRO,INT} = k / C \quad (2)$$

2.4.4 Quality control—Substrate depletion assays were typically conducted 1–2 months after the S9 fractions were prepared. Due to the length of time needed to grow the fish to the largest size, these assays were run over a period of about 2 yr. To evaluate the potential for inter-day differences in performance of these assays, we measured the $CL_{IN\ VITRO,INT}$ of PYR using an independent reference pool of S9 (positive control) during every substrate depletion experiment. The results showed that the inter-day variance in assay performance was quite low ($CV < 5\%$; data not shown).

2.5 Analysis of PAHs

Samples from the substrate depletion assays were analyzed for PHEN, PYR, and BAP by high-performance liquid chromatography (HPLC; Nichols et al., 2017). The samples were injected onto an Agilent 1260 HPLC system equipped with a fluorescence detector. Parent PAHs were separated from metabolic products using a reverse phase Hypersil Green PAH column ($5\ \mu m$; $100 \times 2.1\ mm$; Thermo Fisher Scientific, Waltham, MA). Solvent A consisted of 90% water (Millipore, Billerica, MA) and 10% ACN. Solvent B was made of 5% water and 95% ACN. The solvents were run isocratic at a flow rate of $1.0\ mL\ min^{-1}$ but the proportions of solvents A and B were changed for each PAH to optimize retention time and peak symmetry. Excitation and emission wavelengths (nm) of the fluorescence detector

were: BAP, 255/420; PHEN, 260/350; PYR, 237/390. The limits of detection for PHEN, PYR, and BAP were approximately 0.5, 0.2, and 0.1 nM, respectively. The content of PHEN, PYR, and BAP in the samples was quantified by comparing peak areas to those of known standards.

2.6 Correction for incomplete recovery of enzymes

Measured CYP concentrations, enzyme activities, and $CL_{IN\ VITRO,INT}$ rates were initially expressed in units of $\text{pmol mg S9 protein}^{-1}$, $\text{pmol min}^{-1} \text{mg S9 protein}^{-1}$ and $\text{mL min}^{-1} \text{mg S9 protein}^{-1}$, respectively. To calculate the true activity of the intact tissue, it was necessary to correct for incomplete recovery of phase I and phase II enzymes, which may be lost to the mitochondrial pellet during centrifugation of the whole liver homogenate. Liver S9 fractions contain microsomal enzymes that are localized to the endoplasmic reticulum of cells (e.g., CYPs and UGTs), as well as enzymes that are present in the cell cytoplasm (e.g., GSTs). Due to these differences in subcellular localization, recovery of microsomal enzymes can differ markedly from that of cytosolic enzymes (Statham, et al., 1977).

Recovery of microsomal enzymes was estimated by measuring the activity of a marker enzyme, glucose 6-phosphatase (G6P), in samples of S9 and whole liver homogenate (Schultz and Hayton, 1999; Nichols et al., 2013a). Like the CYPs and UGTs, G6P is localized to the endoplasmic reticulum (Hübscher and West, 1965). The fractional recovery of microsomal enzymes for each pool of livers was calculated as

$$\text{G6P activity in liver S9 fraction} / \text{G6P activity in whole liver homogenate} \quad (3)$$

Corrected values for CYP concentration, EROD activity, UGT activity and $CL_{IN\ VITRO,INT}$ of the PAHs were then calculated as

$$\begin{aligned} & \text{amount or activity} / \text{mg S9 protein} \times 1 / \text{fractional enzyme recovery} \\ & = \text{corrected amount or activity} / \text{mg S9 protein} \end{aligned} \quad (4)$$

A complete description of the G6P assay is presented in Nichols et al. (2013a).

Nimmo et al. (1981) measured GST activity toward CDNB in subcellular liver fractions from rainbow trout following differential centrifugation. Expressed as a percent of total activity recovered, the authors found 1.4% of activity in the nuclear pellet, 2.5% in the heavy and light mitochondrial pellets, 2.3% in the microsomal pellet, and 93.8% in the cytosolic fraction. Because the amount of activity measured in the nuclear and mitochondrial pellets was quite low (< 5%), we used these findings to correct GST activity measured in the present study. Thus, GST activity was corrected by employing a fractional recovery value of 0.961 (sum of microsomal and cytosolic values).

2.7 Extrapolation to the intact liver and the whole animal

Corrected values for each pool, expressed per mg of S9 protein, were extrapolated to the intact liver using group-specific scaling factors which reflect the protein concentration of S9 fractions and the volume of S9 obtained from a gram of tissue

$$\begin{aligned} & \text{corrected amount or activity} / \text{mg S9 protein} \times \text{mg S9 protein} / \text{mL S9} \times \text{mL S9} / \text{g liver} \\ & = \text{corrected amount or activity} / \text{g liver} \end{aligned} \quad (5)$$

To describe the metabolizing capabilities of whole fish, corrected values expressed per gram of liver tissue were multiplied by an additional group-specific scaling factor which describes liver size as a fraction of total body weight (BW)

$$\begin{aligned} & \text{corrected amount or activity} / \text{g liver} \times \text{g liver} / \text{g BW} \\ & = \text{corrected amount or activity} / \text{g BW} \end{aligned} \quad (6)$$

2.8 Data transformations and statistical analyses

Corrected values for CYP concentrations, enzyme activities, and $CL_{\text{IN VITRO,INT}}$ rates were log-transformed and plotted against log-transformed trout body weights. The data were evaluated for monotonicity, as outlined in the Organization for Economic Co-operation and Development guidance document for statistics (OECD, 2014). Data sets exhibiting monotonic behavior were then fitted to the equation $\log Y = b \times \log M + \log a$, which is the linear form of the allometric equation $Y = a \times M^b$, where Y is the parameter of interest, M is body mass, a is a normalization coefficient, and b is the allometric exponent. The value for b (slope of the linear form) characterizes the rate of change in a parameter in relation to changes in body mass. Linear regression analyses were performed on the log-transformed data to determine whether there was a significant change with increasing body weight. The null hypothesis was that the data do not change (i.e., the slope of the fitted relationship is zero).

Differences in measured S9 protein concentration (mg g liver^{-1}) within and among groups were evaluated by two-factor ANOVA. Fish body weights within each group were compared using single factor ANOVA. Single factor ANOVA was also employed to evaluate group-specific differences in S9 volume (mL g liver^{-1}) and fractional recovery of G6P. Differences in G6P recovery among the four groups were evaluated using Tukey's test for multiple comparisons. All statistical analyses were conducted using a significance level of 0.05.

3. Results

3.1 Fish and S9 pools

Summary data for each pool of liver S9 are presented in Table 1. Within each group, there were no significant differences in mean body weight between pools. GSI values measured for the 400 g trout ranged from 0.02% to 0.06% for males, and 0.18% to 0.40% for females. A comparison of these values to published values for trout indicates that these animals were

in very early stages of sexual development (Gomez et al., 1999; Le Gac et al., 2001). Thus, enzyme activities were unlikely to have been affected by sexual maturation. Previous work performed using fish from the same source and strain as that employed in the present study indicates that biotransformation rates in sexually immature trout do not differ by gender (Nichols et al., 2013b; Fay et al., 2014).

The protein concentration of S9 pools ranged from 21.0 mg mL⁻¹ to 26.0 mg mL⁻¹, and did not differ within or among groups. Recovery of G6P activity differed between groups. Significantly higher values were measured for the 4 g trout, suggesting that the smallest livers were homogenized more efficiently. G6P recoveries for the other 3 groups were not significantly different and did not exhibit a clear pattern with respect to body weight. Overall, G6P recovery values were similar to values reported previously for fish (35–60%; Schultz and Hayton, 1999). The lack of an apparent relationship between G6P recovery and the protein concentration of S9 fractions indicates that microsomal protein comprises a small fraction of the total protein content of these samples. There were no significant differences in S9 volume (mL g liver⁻¹) between groups.

There was a significant linear relationship between log liver weight and log BW (Fig. 1A). The slope of this fitted relationship was 0.887 ($r^2 = 0.99$; Table 2), which is similar to the value (0.885) previously reported for carp (Goolish and Adelman, 1981). When expressed in terms of a hepatosomatic index (HSI = liver weight/BW $\times$ 100; Table 1), fractional liver weights were shown to decrease from about 1.5% of BW in the two groups of smaller fish (4 and 20 g) to about 0.95% in the larger animals (90 and 450 g). A plot of log HSI against log BW revealed a significant linear relationship (Fig. 1B) with a fitted allometric exponent (b) of -0.113 (Table 2).

3.2 CYP concentration and CYP1A activity (phase I)

The total CYP concentration of liver tissue, expressed on a g liver⁻¹ basis, varied inversely with trout body weight, but the fitted allometric exponent for this relationship was relatively small ($b = -0.064$; Table 2 and Fig. 2A). A stronger inverse relationship ($b = -0.177$) was obtained when total CYP concentration was expressed on a g BW⁻¹ basis (Table 2 and Fig. 2B). In contrast to CYP concentration, CYP1A activity (EROD) expressed in either manner (g liver⁻¹ or g BW⁻¹) exhibited a non-monotonic pattern with respect to body weight (Figs. 2A and 2B). As such, the results for CYP1A activity were inconclusive.

3.3 UGT and GST enzyme activities (phase II)

Log-transformed UGT and GST activities, expressed on a g liver⁻¹ basis, are shown in Figure 3 A. In both cases, the regression analysis revealed a significant inverse relationship between measured activity and log BW (Table 2). For GST activity, the allometric exponent was relatively small ($b = -0.043$), while that for UGT activity was somewhat higher ($b = -0.097$). Stronger inverse relationships were obtained when GST and UGT activities were expressed on a g BW⁻¹ basis. For GST activity, the allometric exponent was -0.156 , while that for UGT activity was -0.209 (Fig. 3B and Table 2).

3.4 Intrinsic clearance of PAHs

When expressed on a g liver^{-1} basis, log-transformed $\text{CL}_{\text{IN VITRO,INT}}$ rates for PHEN, PYR, and BAP were found to increase with body weight, except for rates measured in 20 g animals, which were 17–34% lower than values measured in 4 g animals (Fig. 4A). This pattern is similar to that observed for EROD activity. However, unlike EROD activity, data for all three PAHs passed the formal test for monotonicity. Allometric exponents obtained by regressing $\text{CL}_{\text{IN VITRO,INT}}$ against body weight ranged from 0.148 to 0.164 (Table 2). Overall, $\text{CL}_{\text{IN VITRO,INT}}$ rates expressed on a g liver^{-1} basis varied by a factor of 2.1 to 2.7 across the different sizes of fish.

This apparent increase in PAH clearance with increasing fish size disappeared, however, when $\text{CL}_{\text{IN VITRO,INT}}$ rates were expressed on a g BW^{-1} basis (Fig 4B). Although the slopes of these relationships were still positive (range from 0.035 to 0.051), these fitted terms were not statistically different from 0. Thus, the ability of trout to clear PAHs, when expressed on a whole animal basis, did not appear to vary with fish size.

4. Discussion

The goal of this study was to investigate the size-dependence of hepatic biotransformation in rainbow trout. All measurements were made using liver S9 fractions. The S9 fraction contains the full complement of microsomal and cytosolic enzymes involved in hepatic biotransformation (Johanning et al., 2012). Extrapolation of S9 data to the intact tissue requires the application of sample-specific scaling factors to account for differences in recovery of microsomal enzymes (utilizing G6P recovery as a surrogate measurement), S9 protein concentration, and S9 volume. Apart from a modest increase in G6P recovery for the smallest fish tested, there were no significant differences among groups with respect to these scaling factors. It is unlikely, therefore, that differences in tissue processing contributed to observed size-related differences in metabolic activity.

Measured rates of activity were initially expressed on a g liver^{-1} basis. These calculated values represent the activity of the tissue itself. However, liver size as a fraction of total body weight was found to vary inversely with animal size. This observation suggested a need to express metabolism data on a g BW^{-1} basis. Values calculated in this manner represent the metabolic potential of the whole organ and, by extension, the animal itself, assuming the liver to be the dominant site metabolism. When expressed on a g liver^{-1} basis, total CYP concentration, UGT activity, and GST activity decreased with increasing fish size. These relationships were strengthened when measured values were expressed on a g BW^{-1} basis. PAH clearance expressed on a g liver^{-1} basis increased with increasing fish size while EROD activity was unchanged. When expressed on a g BW^{-1} basis, there were no apparent relationships of PAH clearance or EROD activity to fish size.

EROD, UGT, and GST activity were measured at saturating substrate concentrations. Therefore, any size-related differences in activity are likely due to differences in the concentration of enzymes responsible for catalyzing these reactions (i.e., V_{MAX}). In contrast, assays designed to measure $\text{CL}_{\text{IN VITRO,INT}}$ were run under first-order conditions (substrate concentration $\ll K_M$). Under these circumstances, $\text{CL}_{\text{IN VITRO,INT}}$ may be

interpreted as the ratio $V_{\text{MAX}}/K_{\text{M}}$ (Nichols et al., 2006). Thus, size-related differences in $\text{CL}_{\text{IN VITRO,INT}}$ could be due to changes in V_{MAX} or K_{M} . However, a change in K_{M} would suggest the involvement of different enzyme isoforms. For this reason, changes in $\text{CL}_{\text{IN VITRO,INT}}$ with fish size are also likely reflect differences in enzyme content unless there are life stage-dependent differences in expression of specific enzyme isoforms.

The primary pathway for PAH metabolism in fish involves phase I hydroxylation of one or more aromatic rings, followed by phase II glucuronidation and sulfation of the hydroxylated products (Schlenk et al., 2008; Varanasi et al., 1989). The rate limiting step in this sequence of reactions is ring hydroxylation. Previous studies of BAP metabolism have shown that ring hydroxylation is catalyzed primarily by CYP1A, although other CYP isozymes (CYP2, CYP3) may contribute. Dealkylation of 7-ethoxyresorufin to the fluorescent metabolite resorufin is also catalyzed by CYP1A (Schlenk et al., 2008). Therefore, the correspondence between EROD activity and $\text{CL}_{\text{IN VITRO,INT}}$ of PHEN, PYR, and BAP, is not surprising. Taken together, the results of the present study suggest that CYP1A activity in trout, expressed on a g BW^{-1} basis, does not vary with body weight.

However, CYP1A is only one of many CYPs that comprise the total CYP content of liver tissue. As indicated previously, size-related differences in CYP concentration were shown to be significant; thus, values measured in 4 g trout were approximately 2 times greater than those measured in 400 g animals (g BW basis^{-1}). The finding suggests that activities associated with other CYP isozymes might have scaled negatively to body weight, had they been measured. Limited data exist to support this suggestion. Addison and Willis (1982) reported a negative allometric relationship ($b = -0.211$) for hepatic ethoxycoumarin *O*-deethylase activity (ECOD; $\text{nmol min}^{-1} \text{g liver}^{-1}$) in brook trout. ECOD is generally thought to reflect CYP2 activity. This earlier finding is complicated, however, by the fact that some of the brook trout were approaching sexual maturity (reported to have “maturing gonads”). Furthermore, the body weights of these test animals varied by only a factor of 4 to 5.

The UGTs and GSTs catalyze phase II reaction pathways that involve conjugation of chemical substrates with glucuronic acid and glutathione, respectively (James, 1986; Nimmo, 1987; Clarke et al., 1991; Schlenk et al., 2008). Both reaction pathways play an important role in promoting elimination of phase I reaction products. Additional compounds possessing functional groups with hydrophilic (UGTs) or electrophilic (GSTs) properties are conjugated directly. In the present study, measured rates of UGT and GST activity (g BW basis^{-1}) were about 2 times greater in the 4 g fish than in the 400 g animals.

We are unaware of any reported allometric relationships for biotransformation in mammals. Because cardiac output in mammals scales to a fractional exponent of body weight, it is common practice to assume similar scaling of renal clearance, as the mechanisms responsible for renal clearance (glomerular filtration, active secretion) are closely linked to renal blood flow. Empirical data support this approach (Lin, 1998). In theory, hepatic clearance (CL_{H}) will be approximately equal to the rate of hepatic blood flow if the rate of intrinsic clearance is high (i.e., when CL_{H} is blood flow-limited; Rowland et al., 1973; Wilkinson and Shand, 1975). For these high clearance compounds, an allometric relationship between CL_{H} and body size might be expected. When intrinsic clearance is low,

differences in CL_H are largely due to differences in enzyme activity, which can vary substantially among species. Such differences would be likely to obscure any underlying allometric relationships.

Allometric scaling relationships figure prominently in computational models used to describe chemical bioaccumulation in fish. For example, the model given by Arnot and Gobas (2003) employs mass-specific allometric exponents of -0.4 and -0.15 to scale the weight-dependence of chemical uptake across the gills and gut, respectively. These allometric exponents are based, in turn, on empirical relationships developed by previous authors (Gobas and Mackay, 1987; Gobas et al., 1993). A model provided by Arnot and Gobas (2004) employs an empirically-derived allometric exponent of 0.65 to scale ventilation rate, which is used to calculate a gill uptake rate constant. A second allometric exponent of 0.85 is used to scale size-dependent changes in feeding rate. Similarly, Nichols et al. (2007) scaled total gill area to an allometric exponent of 0.85 , based on data given previously by Hughes (1984). The same authors used a mass-specific allometric exponent of -0.1 to model the size-dependence of cardiac output. This value was based on an empirical equation developed earlier by Erickson and McKim (1990).

If biotransformation impacts the accumulation of a compound, it becomes important not only to represent this activity in the model, but also to adopt reasonable assumptions regarding the size-dependence of biotransformation rate parameters. In general, there are two approaches used to estimate biotransformation rates employed in computational modeling efforts. The first involves an experimental design wherein small fish are dosed with the chemical of interest. The biotransformation rate is then determined from observed effects on the extent of chemical accumulation (Arnot et al., 2008), or its impact on parent chemical kinetics (typically during the depuration phase of an uptake-depuration exposure protocol; Sijm et al., 1990; Fisk et al., 2000; Konwick et al., 2006). In such cases, the relevant question is how to apply these rates determined using small fish to larger animals, particularly when modeling chemical accumulation that occurs over long periods of time (i.e., when fish are predicted to grow).

The second approach involves *in vitro* determination of metabolic activity followed by extrapolation of this information to the intact animal (Nichols et al., 2006, 2009). These *in vitro* studies are generally performed using biological material from relatively large fish. Intrinsic clearance rates determined using this material may then be used to estimate the rate of biotransformation in much smaller animals, such as those routinely employed for *in vivo* bioaccumulation testing (Nichols et al., 2013a). In this case, concern regarding the size-dependence of biotransformation exists largely because of methodological constraints that make it difficult to measure *in vitro* activity in the small fish of interest.

The results of this study suggest that it would be reasonable to scale liver size in trout to a negative exponent of -0.10 ($HSI; g\ BW^{-1}$) when modeling biotransformation impacts on chemical accumulation. The size dependence of metabolic activity expressed on a $g\ tissue^{-1}$ basis varied with the type of activity measured, but was generally not pronounced. Therefore, in the absence of information regarding the size dependence of a particular reaction, it may be advisable to scale activity directly to body weight since any errors

associated with this assumption are likely to be small. Additional work is needed to determine the generalizability of these findings to other biotransformation pathways and fish species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIG 1A-B.

Log-log plots of liver and fish body weights. Panel A – Liver weight. Panel B – Hepatosomatic index (HSI). In this and Figures 2-4, each point illustrates the value for a single pool of liver S9. Dashed lines show linear regressions fit to each data set. Regression parameters and summary statistics are presented in Table 2 Liver weight and HSI values for the 4 and 20 g trout were determined from pooled liver samples; data presented for 90 and 400 g trout are mean values determined for individual fish.

FIG 2A-B.

Log-log plots of cytochrome P450 concentration (CYP; gray circles), ethoxyresorufin-*O*-deethylase activity (EROD; white circles), and fish body weight. Panel A – Data expressed per gram of liver (g liver⁻¹). Panel B – Data expressed per gram of body weight (g BW⁻¹). Data points illustrate the mean of 3 (CYP) or 4 (EROD) replicate determinations.

FIG 3A-B.

Log-log plots of fish body weight and the activities of UDP-glucuronosyltransferase (UGT; gray circles) and glutathione *S*-transferase (GST; white circles). Panel A – Data expressed per gram of liver (g liver⁻¹). Panel B – Data expressed per gram of body weight (g BW⁻¹). Each point represents the mean of 4 replicate determinations.

FIG 4A-B.

Log-log plots of fish body weight and *in vitro* intrinsic clearance ($CL_{IN\ VITRO,INT}$) of phenanthrene (PHEN; gray circles), pyrene (PYR; white circles), and benzo[a]pyrene (BAP; black circles). Panel A – Data expressed per gram of liver ($g\ liver^{-1}$). Panel B – Data expressed per gram of body weight ($g\ BW^{-1}$). Each point represents the mean of 3 replicate determinations.

Table 1.

Summary of fish data, protein concentration, S9 concentration and microsomal enzyme recovery for each pool of liver S9 fractions.

	Pool 1	Pool 2	Pool 3	Pool 4	Pool 5	Group
4 g trout						
Number of fish	42	40	42	42	42	
Body weight (g)	4.1 ± 0.6	4.2 ± 0.6	4.0 ± 0.5	4.2 ± 0.8	4.2 ± 0.7	4.1 ± 0.1
HSI (%)	1.53	1.47	1.57	1.35	1.46	1.48 ± 0.08
S9 protein (mg mL ⁻¹)	21.9 ± 0.5	23.1 ± 0.4	22.0 ± 0.3	23.7 ± 0.4	25.0 ± 0.4	23.1 ± 1.3
S9 content (mL g liver ⁻¹)	2.03	2.12	2.14	2.14	2.15	2.12 ± 0.05
G6P recovery	0.46	0.53	0.52	0.42	0.51	0.49 ± 0.05
20 g trout						
Number of fish	7	7	7	7	7	
Body weight (g)	18.9 ± 1.9	19.9 ± 2.9	19.1 ± 2.9	20.6 ± 3.0	19.0 ± 1.4	19.5 ± 0.7
HSI (%)	1.44	1.46	1.36	1.41	1.50	1.43 ± 0.05
S9 protein (mg mL ⁻¹)	26.0 ± 0.5	25.8 ± 0.3	22.1 ± 0.8	22.1 ± 0.5	23.8 ± 0.2	23.9 ± 1.9
S9 content (mL g liver ⁻¹)	1.92	2.03	2.14	2.24	1.93	2.05 ± 0.14
G6P recovery	0.37	0.41	0.31	0.33	0.32	0.35 ± 0.04
90 g trout						
Number of fish	2♀, 1♂	2♀, 1♂	2♀, 1♂	1♀, 2♂	2♀, 1♂	
Body weight (g)	84.5 ± 8.3	93.7 ± 8.0	89.1 ± 2.2	97.8 ± 4.4	86.3 ± 8.1	90.3 ± 5.5
HSI (%)	1.01 ± 0.08	1.01 ± 0.20	1.03 ± 0.07	0.85 ± 0.05	0.81 ± 0.06	0.94 ± 0.10
S9 protein (mg mL ⁻¹)	24.5 ± 1.4	24.8 ± 1.1	21.0 ± 0.4	23.3 ± 0.8	24.3 ± 0.4	23.6 ± 1.5
S9 content (mL g liver ⁻¹)	1.93	1.93	2.04	2.02	1.94	1.97 ± 0.06
G6P recovery	0.29	0.34	0.26	0.27	0.38	0.31 ± 0.05
400 g trout						
Number of fish	1♀, 2♂	2♀, 1♂	3♀	3♀	3♂	
Body weight (g)	463 ± 31	440 ± 25	431 ± 6	440 ± 25	425 ± 25	440 ± 14
HSI (%)	0.94 ± 0.04	0.98 ± 0.10	0.83 ± 0.07	1.03 ± 0.06	0.97 ± 0.07	0.95 ± 0.07
GSI, females (%)	0.29	0.31 ± 0.12	0.32 ± 0.07	0.23 ± 0.08	N/A	
GSI, males (%)	0.06 ± 0.00	0.06	N/A	N/A	0.04 ± 0.01	
S9 protein (mg mL ⁻¹)	23.8 ± 0.1	24.6 ± 0.2	24.7 ± 0.2	25.3 ± 0.3	25.7 ± 0.3	24.8 ± 0.7
S9 content (mL g liver ⁻¹)	2.05	1.98	1.95	2.03	2.05	2.01 ± 0.05
G6P recovery	0.38	0.35	0.36	0.46	0.45	0.40 ± 0.05

Abbreviations: HSI – hepatosomatic index; G6P – glucose 6-phosphatase; GSI – gonadosomatic index; N/A – not applicable. Values are reported as the mean ± standard deviation (SD). HSI values reported for the 4 and 20 g trout were calculated as the pooled liver weight divided by the sum of the body weights × 100; HSI values reported for 90 and 450 g trout are the mean ± SD of values determined for individual fish. S9 protein concentration was determined from n = 4 (4 and 20 g trout) or n = 3 (90 and 450 g trout) replicated measurements.

Table 2.

Summary of allometric exponents (*b*), log normalization coefficients (*a*), and statistical results for allometric linear regressions.

	<i>b</i>	<i>p</i> -value for <i>b</i>	log <i>a</i>	<i>r</i> ²
Liver weight (g)	0.887	1.2 E –21	– 1.75	0.99
HSI (%)	–0.113	1.2 E –06	0.25	0.74
CYP cone, (pmol g liver ^{–1})	–0.064	0.002	4.15	0.43
CYP cone, (pmol g BW ^{–1})	–0.177	5.1 E –09	2.40	0.86
UGT activity (nmol min ^{–1} g liver ^{–1})	–0.097	0.002	2.33	0.43
UGT activity (nmol min ^{–1} g BW ^{–1})	–0.209	1.7 E –05	0.58	0.65
GST activity (nmol min ^{–1} g liver ^{–1})	–0.043	0.007	4.54	0.34
GST activity (nmol min ^{–1} g BW ^{–1})	–0.156	3.1 E –07	2.79	0.77
CL _{IN VITRO,INT} (mL min ^{–1} g liver ^{–1})				
PHEN	0.164	0.0004	0.09	0.50
PYR	0.149	0.004	1.25	0.38
BAP	0.148	0.0005	1.50	0.50
CL _{IN VITRO,INT} (mL min ^{–1} g BW ^{–1})				
PHEN	0.051	0.13	–1.65	0.12
PYR	0.036	0.36	–0.50	0.05
BAP	0.035	0.18	–0.25	0.10

Abbreviations: HSI – hepatosomatic index; CYP – cytochrome P450; BW – body weight; UGT – uridine diphosphate glucuronosyltransferase; GST – glutathione *S*-transferase; CL_{IN VITRO,INT} – *in vitro* intrinsic clearance; PHEN – phenanthrene; PYR – pyrene; BAP – benzo[a]pyrene.