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**Characterization of Phase-II conjugation reaction of polycyclic aromatic hydrocarbons in fish species: unique pyrene metabolism and species specificity observed in fish species**

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**Abstract**

Metabolic activity, particularly conjugation, was examined in fish by analyzing pyrene (a four-ring, polycyclic aromatic hydrocarbon) metabolites using high-performance liquid chromatography (HPLC) with fluorescence detector (FD), a mass spectrometry (MS) system, and kinetic analysis of conjugation enzymes. Fourteen fresh water fish species, including *Danio rerio* and *Orizias latipes*, were exposed to aqueous pyrene, and the resulting metabolites were collected. Identification of pyrene metabolites by HPLC/FD and ion-trap MS indicated that the major metabolites were pyrene glucuronide and pyrene sulfate in all 14 species. Differences were observed in pyrene glucuronide:pyrene sulfate ratio and in the total amount of pyrene conjugates excreted between fish species. Furthermore, a correlation was found between the amount of pyrene glucuronide present and the total amount of the pyrene metabolite eliminated. Kinetic analysis of conjugation by hepatic microsomes *in vitro* indicated that the differences in excreted metabolites reflected the differences in enzymatic activities.

Keywords: Fish, pyrene, conjugation, polycyclic aromatic hydrocarbons (PAHs), metabolism, inter-species difference

## 1. Introduction

Organisms vary greatly in their ability to metabolize xenobiotics (Guerrero et al., 2002). This variation is due to differences in the patterns of xenobiotic bioaccumulation amongst organisms, which influences their xenobiotic tolerance. Therefore, it is important to understand the mechanisms of xenobiotic metabolism and to elucidate the physiological functions involved in this process in each organism.

A number of studies have examined the metabolism of xenobiotics in aquatic organisms, including fish. It is known that aquatic organisms living in chemically polluted environments absorb lipophilic organic pollutants (Gobas et al., 2000) and metabolize xenobiotics. Generally, metabolism is divided into three phases. Xenobiotics usually undergo biotransformation in animals through phase-I (functionalization: i.e., oxidation, reduction, etc.), phase-II (conjugation) reactions producing more polar derivatives, which are more readily excreted than the parent compound (Oost et al., 2003), and phase-III involving excretion of metabolites from cells by membrane proteins, such as ATP-binding cassette (ABC) transporters (Cole and Deeley, 1998). Several studies on metabolism of aryl-compounds show that fish can oxidize these compounds and further transform them into glucuronide conjugates, sulfate conjugates, and mercapturic acid. Glucoside conjugates can also be formed, although these are minor conjugates in aquatic species (James, 1987; Willett et al., 2000; Luthe et al., 2002). Oxidation by cytochrome P450 (CYP) and conjugation by enzymes such as UDP-glucuronosyltransferase (UGT), sulfotransferases (SULT), and glutathione S-transferase (GST) participate in the metabolism of aryl-compounds.

Polycyclic aromatic compounds, which include polycyclic aromatic hydrocarbons (PAHs), are a widely studied class of contaminants. They are pervasive contaminants in rivers, lakes, and nearshore marine habitats, and the largest fraction enters marine waters as land-based runoff or

atmospheric deposition. Many polycyclic aromatic compounds are toxic to humans and other organisms. Extensive literature persists describing the effects of PAHs on animals, including fish (Monteiro et al., 2000; Incardona et al., 2004; 2006). Endocrine disruption is one of these effects. Monteiro et al. (2000) reported that PAHs inhibit ovarian steroidogenesis in flounders by strongly blocking the activity of CYP 17, 20 lyase (Monterio et al., 2000). However, the most well-known attribute of PAHs is their toxicity. Due to the production of high molecular weight reactive intermediates by CYP1A, PAHs are carcinogenic and their toxicity is probably mediated through this metabolic pathway. Therefore, it is important to know the metabolic pathways that are affected by PAHs, including the conjugation and excretion of activated phase-I metabolites.

There is currently limited information about xenobiotic metabolism in different fish species. Despite this, fish are often used as environmental indicators and have been used in various ecotoxicological studies. In the Organization for Economic Cooperation and Development (OECD) test guidelines, species such as *D. rerio* (zebrafish) and *O. latipes* (Japanese medaka) are used as standard test organisms in “the fish acute toxicity test,” “the young fish development toxicity test,” and “the fish prolongation toxicity test.” For example, in “the fish prolongation toxicity test,” no observed effect concentration (NOEC), death, and other infections can be caused within 14–28 days of chemical exposure. Due to the widespread usage of fish in these studies, it is important to elucidate the mechanisms and physiological functions involved in xenobiotic metabolism in these species. It is also necessary to categorize inter-species variation in these physiological functions.

This report describes differences in the metabolism of PAHs in 14 freshwater fish species. Pyrene was chosen as a model compound for PAHs because it is one of the most abundant PAHs in the environment (Vives et al., 2005). Moreover, several studies on biotransformation

have already used pyrene as a model substance (Luthe et al., 2002; Akkanen and Kukkonen, 2003; Stroomberg et al., 2004; Ikenaka et al., 2006, 2007; Ueda et al., 2011). In this study, metabolites of pyrene excreted from fish *in vivo* and the kinetic parameters of pyrene metabolism in phase-I (CYP) and phase-II (UGT and SULT) reactions *in vitro* were profiled. Our results revealed new information about pyrene metabolism in different fish species, including standard OECD test species.

## 2. Materials and Methods

### *2.1. Chemicals*

Pyrene and sodium cholate were obtained from Kanto Chemical Co. Inc. (Tokyo, Japan). 1-Hydroxypyrene, sulfatase (from keyhole limpets, Type V; 34 units/mg), β-glucuronidase (from bovine liver, Type B-1; 1240 units/mg), β-glucosidase (from almonds; 3.4 units/mg), and bovine serum albumin and 3′-phosphoadenosine 5′-phosphosulfate (PAPS) were obtained from Sigma-Aldrich (St. Louis, MO, USA). UDP-glucuronic acid (UDP-GA) was purchased from Wako (Osaka, Japan). All chemicals used for high-performance liquid chromatography (HPLC) and mass spectrometry (MS) were HPLC or MS grade and were obtained from Kanto Chemical Co. Inc.

### *2.2. Animals*

Fourteen fish species were used. Their common name, scientific name, and body weight (g, mean ± SD) of the 14 fish species are listed in Table 1 (taken from Nelson, 2006). The phylogenetic tree is illustrated in Figure 1. Fish species chosen included the OECD standard test species and others fish species selected on the basis of the phylogenetic tree. Fish were purchased from local pet shops (Sapporo, Japan). Animals were reared in aged tap water (i.e., tap water kept in the tank under air bubbling for at least 3 days; pH 8) at room temperature (26°C) under a 14/10 h light/dark photoperiod. They were fed once daily. Treatment of all animals was in accordance with the policies of the Institutional Animal Care and Use Committee of Hokkaido University. All fish were held for a minimum of one week before use.

*2.3. Pyrene exposure*

To avoid the bacterial deconjugation effect, fish (n = 3) were food-deprived for 1 day and washed three times with tap water purified by filtration through an ion exchange cartridge and a 0.2-µm membrane filter. Fish were then put in purified tap water for 3 h for acclimatization. Once treatment was completed, animals were exposed to pyrene for 24 h at room temperature (26°C). Exposure medium was prepared by adding 20 µl of pyrene (500 ppm; dissolved in ethanol) to 150 ml of purified tap water to yield a final concentration of 67 ppb. Final concentration used was half of the degree of physicochemical parameter of pyrene water solubility (145 ppb). Two control sets were used: (1) fish in purified tap water without pyrene and (2) pyrene in purified tap water without fish.

To determine whether there was any bacterial deconjugation effect, a glucuronide conjugate purified from *O. latipes* (methanol solution) was added in place of pyrene to both control sets and analyzed at 0 and 24 h..

*2.4. Analysis of pyrene metabolites in exposure medium/water*

To analyze the metabolites, 1 ml of purified tap water was centrifuged (5000 ×g, 3 min) without any extraction in a 1.5-ml Eppendorf tube and was analyzed. Pyrene metabolites were identified using HPLC by fluorescence detector (FD) equipped with an Inertsil ODS-3 column (2.1 mm × 150 mm; GL Sciences, Inc.). HPLC was performed according to the method of Ueda et al. (2011) with slight modification. In brief, mobile phase A consisted of 10 mM ammonium acetate buffer (pH 5.0) and mobile phase B was methanol:acetonitrile:water (38:57:5, v/v/v). Solvent gradient was 29 min in length, with 20% B from 0 to 3 min, followed by a linear gradient to 88% B from 3 to 22 min then increasing to 98% B with a 4 min hold. Injection volume of 10 µl, flow rate of 0.3 ml/min, and column temperature of 45°C were used

throughout. Excitation (Ex) and Emission (Em) wavelengths for FD were 343 and 385 nm, respectively.

*2.5. Enzymatic deconjugation for identification of pyrene metabolites formed by fish*

Deconjugation was performed using the method described by Ikenaka et al. (2007). Briefly, sulfatase, β-glucuronidase, and β-glucosidase were dissolved in 0.1 M sodium acetate buffer (pH 5.0) to final concentrations of 10, 4000, and 17 units/ml respectively. Pyrene metabolites in 30 µl of methanol solution were diluted by adding 270 µl of 0.1M sodium acetate buffer (pH 5.0) and 200 µl of each deconjugation enzyme. In the control treatment, 200 µl of bovine serum albumin (1 mg/ml) was added in place of the deconjugation enzyme. After incubation for 8 h at 37°C in a water bath, 500 µl of methanol was added to stop the reaction. After centrifugation (12,000 ×g for 10 min), the supernatant was analyzed using HPLC/FD (10-µl injection).

For quality control (QC) and quality assurance (QA) of the deconjugation experiment, we prepared the purified standard solution of pyrene glucuronide and pyrene sulfate (methanol solution) from *O. latipes*. In each experiment, deconjugation enzymatic activities were confirmed by using the purified standard solution as a positive control. We confirmed that all positive control samples were deconjugated to 1-hydroxypyrene. Purified standard solution of each conjugated pyrene in methanol was stable for at least 2 years when stored at −20°C.

*2.6. MS condition for identification of pyrene metabolites formed by fish*

HPLC with electrospray ionization ion-trap mass spectrometry (ESI/ion-trap/MS, LTQ Orbitrap; Thermo Fisher Scientific, MA, USA) was used to identify pyrene metabolites produced by fish. ESI conditions were full scan [mass-to-charge ratio (*m/z)* 80 to 800] and

negative mode, with an ion source voltage of −5.0 kV and an ion source temperature of 300°C. Methanol solution, containing pyrene metabolites, was concentrated under a gentle stream of nitrogen. HPLC conditions were as mentioned in section 2.4.

### *2.7. Quantitative determination of pyrene metabolites*

Pyrene glucuronide and pyrene sulfate were deconjugated to 1-hydroxypyrene, and the transformed ratios were 0.21 ± 0.007 and 0.29 ± 0.03, respectively.

Each pyrene metabolite was corrected to the 1-hydroxypyrene intensity using the transformed ratio. Then, it was calculated to its amount (ppb) by the standard curve of 1-hydroxypyrene, and these amounts were concentrated (pmol). The concentration (pmol) was corrected using biomass and liver weight of each fish (Table 1). Liver weight of four fish species were 2.36 ± 0.58 (mean ± SD) for *D. rerio*, 1.73 ± 0.40 for *O. latipes*, 2.53 ± 0.58 for *F. gardneri*, and 5.17 ± 1.22 for *C. labiosa*.

### *2.8. Preparation of microsomes from liver*

Livers were collected from four fish species (*D. rerio, O. latipes, F. gardneri, C. labiosa*) for analysis of enzyme activities. Microsomes were prepared using the method described by Kawahara *et al*. (2009) with a slight modification. In brief, samples consisting of 3–6 pooled livers were used. Livers were homogenized in 400 µl of buffer (0.1 M potassium phosphate, 0.25 M sucrose, 1.0 mM EDTA, and 0.5 mM PMSF; pH 7.4). Microsomal and cytosolic fractions were prepared at 4°C by differential centrifugation. Supernatant of the first centrifugation at 9,000 ×g for 10 min was further centrifuged at 100,000 ×g for 70 min to obtain the cytosolic and microsomal fractions. Microsomal pellets were re-suspended in 150 µl of

buffer and were used to determine UGT activities. Protein concentration in each fraction was measured using the BCA protein assay reagent kit (PIERCE, Rockford, Illinois, USA).

*2.9. Ethoxyresorufin-O-deethylase activity*

Ethoxyresorufin-O-deethylase (EROD) activity reaction was performed in a 100-µl volume, which contained 10 mM G6P, 10 mM MgCl2, 400 µg microsomal protein, and varying ethoxyresorufin concentrations (10, 5, 2.5, 1.25, 0.63, and 0.31 µM) in 100 mM potassium phosphate buffer (pH 7.4). Reaction mixtures were pre-incubated for 5 min at 26°C, and the reaction was initiated by adding a mixture of 10 mM NADPH and 200 U G6PDH, and the mixtures were then incubated for 10 min at 26°C. The reaction was stopped with 200 µl of ice-cold methanol, and samples were centrifuged at 3,000 rpm for 5 min. Supernatant was then injected into the HPLC/FD system. HPLC was performed according to the method of Pikkarainen (2006) with slight modification. Mobile phase A consisted of 10 mM ammonium acetate buffer (pH 5.0) and phase B consisted of 100% acetonitrile. Solvent gradient was 13 min in length with 20% phase B from 0 to 2 min, followed by linear gradient to 88% phase B from 2 to 10 min, then decreased to 20% phase B. Injection volume of 20 µl, flow rate of 0.2 ml/min, and column temperature of 30°C were used throughout. Ex and Em wavelengths for FD were 545 and 588 nm, respectively. Kinetics parameters, including maximal velocity ($V_{max}$), Michaelis–Menten constant ($K_m$), and $V_{max}/K_m$ ratio were determined by the Michaelis–Menten equation using Sigmaplot 11.2 software (Systat Software, Inc., San Jose, CA, USA).

*2.10. UGT dependent 1-hydroxypyrene glucuronidation (HPyG) activity*

UGT activity of 1-hydroxypyrene was assessed using the method described by Ueda et al. (2011) with slight modification. Initially, 15 µl of hepatic microsome solution was mixed with

42 µl of 0.1 M phosphate buffer (pH 7.4). Microsome preparation was then mixed with 3 µl of 1% sodium cholate solution and incubated on ice for 30 min. After this treatment, 50 µl of microsome solution was mixed with 41.5 µl of 0.1 M phosphate buffer (pH 7.4), 5 µl of 50 mM $MgCl_2$, and 1 µl of 1-hydroxypyrene. Samples were then pre-incubated at 26°C for 5 min. Reaction was initiated by adding 2.5 µl of UDP-GA (final concentrations were 100, 50, 25, 12.5, and 6.25 µM) in a final volume of 100 µl. After incubation at 26°C for 10 min, reaction was stopped by adding 400 µl of ice-cold methanol. Reaction samples were then placed on ice for 5 min before centrifugation at 2000 rpm for 10 min. The resultant supernatant was injected into the HPLC system. Mobile phase A consisted of 10 mM ammonium acetate buffer (pH 5.0) and phase B of methanol:acetonitrile:water (38:57:5, v/v/v). Liner gradient was 17 min in length with 90% phase B. Injection volume of 20 µl, flow rate of 0.5 ml/min, and column temperature of 45°C were used throughout. Ex and Em wavelengths for FD were 343 and 385 nm, respectively. Kinetic parameters were determined using the Michaelis–Menten equation, as mentioned in section 2.9.

### *2.11. SULT-dependent 1-hydroxypyrene sulfation (HPyS) activity*

SULT activity for 1-hydroxypyrene was assessed by the method of Ueda *et al*. (2011) with slight modification. Of 500 µg/ml hepatic cytosolic protein solution, 50 µl was mixed with 26.5 µl of 100 mM Tris-HCl buffer (pH 7.4), 10 µl of 50 mM $MgCl_2$, 10 µl of 50 mM $Na_2SO_3$, and 1 µl of 1-hydroxypyrene. Mixtures were pre-incubated at 26°C for 5 min. Reaction was initiated with 2.5 µl of 1 mM PAPS in a final volume of 100 µl. After incubation at 26°C for 10 min, reaction was stopped by adding 400 µl of ice-cold methanol. Reaction samples were then placed on ice for 5 min prior to centrifugation at 2000 rpm for 10 min. The resultant supernatant was injected into the HPLC system. Mobile phase A consisted of 10 mM

ammonium acetate buffer (pH 5.0) and phase B of methanol:acetonitrile:water (38:57:5, v/v/v). The solvent gradient was 17 min in length with a linear gradient of phase B from 50% to 90% from 0 to 7 min, then kept at 90% for 3 min, and decreased to 50%. Injection volume of 20 μl, flow rate of 0.5 ml/min, and column temperature of 45°C were used throughout. Ex and Em wavelengths for FD were 343 and 385 nm, respectively. Kinetics parameters were determined by the Michaelis–Menten equation as mentioned in section 2.9.

**3. Statistical analysis**

Values were represented as mean ± standard deviation (SD) for kinetic analysis and as mean ± standard error (SE) for other experiments. Statistical analysis was performed using Student's *t*-test for the ratios of pyrene glucuronide:pyrene sulfate in each fish species, Dunnett's test for the total amount of pyrene conjugates in each fish species, and Tukey's HSD test for the total amount of pyrene metabolites per liver weight in four fish species. $p < 0.05$ was considered statistically significant for all analyses. All kinetic parameters (Michaelis–Menten equation) were analyzed using Sigmaplot 11.2 software.

## 4. Results

### *4.1. Identification of pyrene metabolites formed by each species*

The results of pyrene exposure experiment revealed two major metabolites in all fish species. The results of enzymatic deconjugation of these metabolites and the MS spectrum identified these conjugates as pyrene-glucuronide (*m/z* 393) and pyrene-sulfate (*m/z* 297; Figure 2).

Representative HPLC/FD chromatograms of pyrene metabolites are shown in Figure 2. These peaks were detected in all pyrene exposure treatment groups; however, they were not detected in the control group. Therefore, they were considered to be pyrene-derived substances and the main pyrene metabolites of fish. Pyrene metabolites, particularly pyrene glucuronide, have been reported to be easily deconjugated by bacteria. For this reason, the experiments were controlled for any bacterial effect. Furthermore, it was confirmed that metabolite compositions detected from pyrene exposed fish and from purified tap water were not different. Moreover, there was no difference in metabolite composition between purified tap water that was used in the exposure experiment and aged tap water that was used for rearing the fish.

The volume of pyrene that remained in purified tap water was also analyzed using *O. latipes* and *C. labiosa*. After 24 h of exposure to 10 µg pyrene in 100 ml water, 0.15 ± 0.01 µg (*O. latipes*) and 0.15 ± 0.006 µg (*C. labiosa*) of the total amount remained. These results indicate that approximately 98.5% of the pyrene was absorbed by the fish body (i.e., the recovery rate of pyrene was more that 99%).

### *4.2. Amount of pyrene metabolites formed by each species*

The amount of pyrene metabolites excreted differed by species (Figure 3). *F. gardneri* excreted a high amount of pyrene glucuronide (92.7 pmol/24 h), whereas *D. rerio* and *O.*

*niloticus* excreted a high amount of pyrene sulfate (27.1 pmol/24 h and 22.3 pmol/24 h, respectively; Tukey's HSD test). *C. labiosa* produced significantly lesser amounts of pyrene metabolite (41.97 pmol/24h), whereas *F. gardneri* produced a larger amount (704.96 pmol/24h). Interestingly, the total amount of eliminated metabolites was significantly larger in species whose main metabolite was pyrene glucuronide than that in species whose main metabolite was pyrene sulfate (Tukey's HSD test; $p < 0.05$). Moreover, the amount of pyrene glucuronide correlated to the total amount of pyrene metabolites (r = 0.94; Figure 5).

*4.3. Profile of pyrene metabolites formed by each species*

Inter-species differences were observed in the ratio of pyrene glucuronide:pyrene sulfate ratio (Figure 6). The major metabolite of *O. latipes*, *F. gardneri*, *T. travancoricus*, *S. nigriventris*, and *A. normai* was pyrene glucuronide ($p < 0.05$). For *C. auratus*, *C. aeneus*, and *C. labiosa*, the major metabolite was pyrene sulfate ($p < 0.05$). In *D. rerio*, *T. albonubes*, *P. reticulate*, *O. niloticus*, *X. maculatus*, and *P. innes*, there was no difference between the amounts of pyrene glucuronide and pyrene sulfate.

*4.4. Comparison of pyrene metabolizing ability among fish species*

Inter-species differences were observed in the total amount and the ratio of pyrene metabolites. Therefore, enzyme activities (CYP1A, UGT, and SULT) were measured, and the relationships to these differences were explored. EROD activity was used to determine phase-I (CYP1A) activity. *D. rerio*, *O. latipes*, *F. gardneri*, and *C. labiosa* were chosen because *D. rerio* and *O. latipes* are the main fish species used in OECD toxicological tests, whereas *F. gardneri* and *C. labiosa* showed high and low total amounts of pyrene metabolites, respectively, *in vivo*.

Enzyme activities were measured *in vitro* using liver microsomes and cytosol to observe hydroxylation, glucuronidation, and sulfation activities.

Activities of EROD, HPyG, and HPyS were different among the four fish species (Figure 7, Figure 8, Figure 9, and Table 2). Typical hyperbolic kinetics were observed for EROD and HPyG activities (Figure 7 and 8). *C. labiosa* showed the lowest enzymatic efficiency for both EROD and HPyG among the four species ($V_{max}/K_m$ values were 3.1 and 0.6, respectively). In case of *D. rerio*, high EROD activities ($V_{max}/K_m$ was 25.5) were found. In contrast to *C. labiosa*, *O. latipes* showed high HPyG activity ($V_{max}/K_m$ was 12.6). *F. gardneri* showed moderate activity of both EROD and HPyG ($V_{max}/K_m$ were 17.6 and 6.8, respectively). In contrast, HPyS activity of the four species demonstrated non-hyperbolic kinetics (Figure 9), fitting with a substrate inhibition model. When examining HPyS kinetic parameters, *O. latipes* showed high HPyS activity ($V_{max}/K_m$ was 14.3) and *C. labiosa* showed the lowest enzymatic efficiency ($V_{max}/K_m$ was 4.3). *C. labiosa* showed the highest inhibition constant ($K_i$ was 117.7), whereas there was no difference in $K_i$ between the other three fish species.

## 5. Discussion

### *5.1. Pyrene metabolites excreted by fish*

Results of pyrene exposure indicated that fish excreted mainly pyrene glucuronide and pyrene sulfate. Differences in both the pyrene glucuronide:pyrene sulfate ratio and the total amount of each excreted metabolite between fish species were observed.

In previous studies, it has been reported that fish can conjugate xenobiotics to sulfuric acid, glucuronic acid, and glutathione (GSH; Beyer et al., 2010; James, 1987). The conjugated metabolite of PAHs in fish is primarily glucuronic acid (Bayer et al., 2010; Hornung et al., 2007), and it is known that glucuronidation of xenobiotics is performed by the action of UGT (Leaver et al., 2007). For example, in *Pleuronectes platessa* hepatocytes, the extent of glucuronide formation was higher than sulfate formation of 1-naphthol and phenolphthalein (Morrison et al., 1985). GSH is also an important conjugate in fish because it contributes significantly to metabolite levels. In Bullhead hepatocytes, benzo[a]pyrene is metabolized to large amounts of GSH conjugates, which are more likely to be retained in the liver than glucuronide or sulfates (Beyer et al., 2010). Glucuronic and glutathione conjugates are major metabolites in fish. In contrast to fish, amphibians and crustaceans have a high capacity to metabolize xenobiotics to sulfate conjugates (Ikenaka et al., 2006; Ueda et al., 2011). For example, crustaceans such as *D. magna* and *N. denticulata* have high ability to predominantly metabolize pyrene to pyrene sulfate (Ikenaka et al., 2006. 2007). However, in this study, differences in the ratio of glucuronide:sulfate conjugation ratios among fish species were seen; these species were divided in three groups on the basis on the differences in the ratio (Figure 6).

Highest amount of pyrene sulfate was excreted by *D. rerio* and *O. niloticus*, which showed no difference in the pyrene glucuronide:pyrene sulfate ratio indicating that the two species excrete pyrene glucuronide to the same extent. In fish species whose main metabolite

was pyrene sulfate, pyrene glucuronide was excreted at a low level, which was one factor behind pyrene sulfate being the main metabolite. These results indicated that species diversity exists in metabolism among different fish species.

*5.2. Liver size, but not diet, affects differences in the total amount and the ratio of pyrene metabolites*

Liver weight may be one of the key factors contributing toward differences in the total amount of pyrene metabolite because larger livers seemed to metabolize more xenobiotics than smaller ones. The liver weight (mg):body weight (g) ratio for four fish species were 7.22 for *O. latipes*, 8.77 for *D. rerio*, 5.39 for *F. gardneri*, and 4.83 for *C. labiosa*. Total amounts of metabolites, normalized per liver weight, in each fish are shown in Table 1. Interestingly, no significant differences in total amount of metabolites were observed in *O. latipes*, *F. gardneri*, and *D. rerio* after normalization. These results indicate that liver size is one of the key factors contributing toward total metabolite excretion. However, *C. labiosa*, still showed a significantly lower elimination of pyrene metabolites compare to other species.

Next, we suspected that diet may be the second factor contributing toward differences in metabolites. In a previous study, it was reported that herbivorous animals, including fish express more CYP than carnivorous animals (Liu et al., 2006; Stegeman et al., 1997). In case of fish, Stegeman et al. (1997) found that CYP2B-like proteins and CYP3A-like proteins are found at high levels in herbivorous fish. However, most of the fish species in this study, including *F. gardneri* and *C. labiosa*, were omnivorous (Bhattacharjee et al., 2009). Therefore, the differences in the total amount and the ratio of pyrene metabolites were not related to diet. These differences were also not related to body weight or the evolution of fish as indicated by the phylogenetic tree as analyzed by Spearman's $\rho$ ($\rho < 0.05$).

*5.3. Comparison of pyrene metabolizing ability among fish species*

EROD and HPyG kinetics parameters ($V_{max}$, $K_m$, $V_{max}/K_m$) were similar to those of previously reported fish species (Gonzalez et al., 2009). Gonzalez and coworkers found bluegill HPyG activity to have $V_{max}$, $K_m$, and $V_{max}/K_m$ values of 263 (pmol resorufin/min/mg protein), 17.5 (μM), and 16.3 (pmol resorufin/min/mg protein/μM), respectively. In this study, *C. labiosa* showed the lowest enzymatic efficiency for both EROD and HPyG activities among the four species. These results were in accordance with the excreted amount of pyrene metabolites *in vivo*.

HPyS activity demonstrated non-hyperbolic kinetics (Figure 9), fitting with a substrate inhibition model. Some types of SULTs showed substrate inhibition for certain substrates such as human SULT1A3 and dopamine (Wang et al., 2007; Barnett et al., 2004). However, when inhibited, the velocity decreased to a plateau rather than to zero. This phenomenon was also shown in a study, which observed partial substrate inhibition for human estrogen sulfotransferase (EST) and estradiol ($E_2$) (Zhang et al., 1998). This indicated that one or more of the kinetic parameters for the reaction were being titrated from one value to another. In the study by Zhang et al. (1998), two $E_2$ were bound to each EST, suggesting that partial inhibition occurred through binding at an allosteric site. From this report, we assumed that HPyS utilized the same mechanism. When examining HPyS activity kinetic parameters, *C. labiosa* showed the lowest enzymatic efficiency. This result was in accordance with the excreted amount of pyrene metabolites *in vivo*. *C. labiosa* showed the highest inhibition constant, whereas there was no difference in *Ki* between the other three fish species. However, because the concentration of pyrene was very low *in vivo* (0.198 μM), it was not necessary to take *Ki* into account when considering the relationship between *in vitro* and *in vivo* experiments. These data indicated that

both phase-I and phase-II metabolic activities potentially contributed toward the observed inter-species differences in the excretion levels of pyrene metabolites in fish.

*5.4. Why are there many variations of metabolism among fish species?*

Mammals have been reported to metabolize PAHs predominantly to sulfate conjugates in the early phase; however, in the late phase, glucuronide conjugates become the main metabolite (Zamek-Gliszczynski et al., 2006). This phenomenon occurs because of sulfate ion depletion. However, aquatic organisms live in an environment where sulfate ions are in abundance. There is a possibility that sulfate conjugation is the fundamental biological defense mechanism against xenobiotics for aquatic organisms because there is no need to be concerned about sulfate ion depletion. It has been reported that the aquatic crustacean *D. magna* and some species of atyidae (the fresh water shrimp decapoda), metabolize pyrene specifically to sulfate conjugates (Ikenaka et al., 2006; 2007). In case of amphibians, which live in wetlands, pyrene is also metabolized mainly to sulfate conjugates (Ueda et al., 2011). In case of fish, sulfate conjugation is the fundamental biological defense mechanism against xenobiotics. Furthermore, the amount of excreted sulfate conjugates was not as variable as that of excreted glucuronide conjugates. Clear positive correlation between the amount of eliminated glucuronide conjugates and the total amount of eliminated pyrene metabolites in fish supported this hypothesis.

Glucuronide conjugates require UDP-glucuronide, which is biosynthesized from UDP-glucose, as a co-factor implying that glucose, an important source of energy for animals, is used for xenobiotic metabolism. Therefore, it is possible that the strategy of xenobiotic metabolism is directly linked to the specific carbohydrate metabolism strategy employed by each fish species. Inter-species differences observed in metabolic patterns appeared to depend on whether glucuronide conjugates were employed for metabolites or not by a default metabolic

strategy. *F. gardneri*, which had high levels of glucuronide conjugates and total metabolites, employed the strategy of metabolizing with glucuronidation in addition to sulfation. In contrast, *C. auratus* metabolized mainly with sulfation, which seemed to be the fundamental metabolite, and little with glucuronidation. From these differences in energy strategy, it seemed that there were variations in the amount and ratio of metabolites in fish species.

## 6. Conclusion

In conclusion, clear inter-species differences were found in both pyrene metabolite characteristics and metabolic capacities for phase-I and phase-II reactions in fish. Metabolites of pyrene were mainly pyrene-glucuronide and pyrene-sulfate. There were also significant differences in the ratio of pyrene conjugates and the total amount of pyrene conjugates. Pyrene-glucuronide seems to be the key factor to determine the total amount of pyrene metabolites. Differences in CYP1A, UGT and SULT activities were also observed between different fish species. Both phase-I and phase-II metabolic activities potentially contributed to the species differences in the excretion levels of pyrene in fish. These large differences in PAH metabolism between fish species were found even between OECD standard test organisms *D. rerio* and *O. latipes*. It is therefore necessary to consider inter-species differences in xenobiotic metabolism during environmental toxicological assessments.

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Tables

**Table 1**

Common name, scientific name, body weight and total concentration of pyrene conjugates (per fish and per liver weight) of 14 fish species. Results were analyzed by Dunnett's test ($p<0.05$) between *Danio rerio* and other species (＊). Mean ± SE, n = 3. Different letters (a,b,c) denote significant differences between four fishes (*O. latipes, F. gardneri, D. rerio, and C. labiosa*; Tukey's HSD test, $p<0.05$). For example, in total concentration of pyrene vonjugate, only *C. labiosa* showed significantly smaller amount than other fish.

| **Common name** | ***Scientific name*** | **Body weight (g) ±SD** | **Concentration of pyrene-glucuronide (pmol/fish/24h) ±SE** | **Concentration of pyrene-sulfate (pmol/fish/24h) ±SE** | **Total concentration of pyrene conjugates (pmol/fish/24h) ±SE** | **Total concentration of pyrene conjugates (pmol/liver weight/24h) ±SE** |
|---|---|---|---|---|---|---|
| Zebrafish | *Danio rerio* | 0.270 ±0.10 | 23.77±3.30 | 27.06±2.38 | 318.47±3.91 (a) | 143.16±10.65 (a) |
| Japanese medaka | *Oryzias latipes* | 0.24 ±0.05 | 20.53±3.88 | 22.96±1.19 | 196.95±4.68 (a) | 113.65±11.65 (a) |
| Guppy | *Poecilia reticulate* | 0.52 ±0.06 | 8.64±0.63 | 8.3±0.65 | 112.96±1.23 ＊ | |
| Goldfish | *Carassius auratus* | 2.72 ±0.50 | 6.31±0.36 | 17.10±2.90 | 156.05±2.73 ＊ | |
| Aphyosemion | *Fundulopanchax* | 0.47 | 92.70±14.52 | 13.05±0.64 | 704.96±15.14 ＊(b) | 136.43±13.11 (a) |

| | | | | | | |
|---|---|---|---|---|---|---|
| gardneri | *gardneri* | | | | | |
| Red gourami | *Colisa labiosa* | 1.07 ±0.41 | 1.61±1.61 | 4.69±2.36 | 41.97±3.33 ＊(c) | 16.57±2.13 (b) |
| Nile tilapia | *Oreochromis niloticus* | 0.42 ±0.05 | 14.21±2.22 | 22.28±5.19 | 243.22±7.08 | |
| African lampeye | *Aplocheilichthys normai* | 0.07 ±0.01 | 39.79±11.49 | 10.83±0.93 | 337.51±10.56 | |
| White cloud mountain Minnow | *Tanichthys albonubes* | 0.17 ±0.07 | 9.22±0.67 | 9.88±2.24 | 127.30±2.47 ＊ | |
| Green spotted puffer | *Tetraodon travancoricus* | 0.15 | 20.15±7.73 | 6.87±0.21 | 180.16±7.92 | |
| Upside-down catfish | *Synodontis nigriventris* | 1.4 | 44.83±19.36 | 9.01±1.99 | 358.96±19.14 | |
| Neon tetra | *Paracheirodon innes* | 0.17 ±0.03 | 5.89±0.92 | 7.32±0.28 | 88.07±1.19 ＊ | |
| Platy | *Xiphophorus maculatus* | 0.93 ±0.15 | 13.66±0.95 | 9.344±0.13 | 153.34±1.02 ＊ | |
| Corydoras | *Corydoras aeneus* | 1.30 ±0.2 | 5.15±0.13 | 10.35±0.82 | 103.30±0.83 ＊ | |

**Table 2**

Kinetic parameters of enzymes in fish.

| EROD activity | $V_{max}$ (nmol/min/mg protein) | $K_m$ (μM) | $V_{max}/K_m$ (nmol/min/mg protein/μM) |
|---|---|---|---|
| *O. latipes* | 179.4 | 10.4 | 17.3 |
| *F. gardneri* | 40.4 | 2.3 | 17.6 |
| *D. rerio* | 28.1 | 1.1 | 25.5 |
| *C. labiosa* | 7.2 | 2.3 | 3.1 |

| UGT activity | $V_{max}$ (pmol/min/mg protein) | $K_m$ (μM) | $V_{max}/K_m$ (pmol/min/mg protein/μM) |
|---|---|---|---|
| *O. latipes* | 609.5 | 48.5 | 12.6 |
| *F. gardneri* | 165.5 | 24.5 | 6.8 |
| *D. rerio* | 286.9 | 47.1 | 6.1 |
| *C. labiosa* | 54.4 | 96.9 | 0.6 |

| SULT activity | $V_{max}$ (pmol/min/mg protein) | $K_m$ (μM) | $V_{max}/K_m$ (pmol/min/mg protein/μM) | *Ki* (μM) |
|---|---|---|---|---|
| *O. latipes* | 6.7 | 0.47 | 14.3 | 9.8 |
| *F. gardneri* | 35.7 | 4.7 | 7.6 | 5.9 |
| *D. rerio* | 21.7 | 2.4 | 9.0 | 9.5 |
| *C. labiosa* | 4.3 | 1.0 | 4.3 | 117.7 |

$V_{max}$: maximum response velocity; $K_m$: substrate concentration at $V_{max}$ /2, $V_{max/}$ $K_m$: enzyme efficiency; $Ki$: inhibition constant

**Figure legends**

**Fig. 1.** Phylogenetic tree of the 14 fish species used in this experiment.

**Fig. 2.** High performance liquid chromatography (HPLC)/fluorescence detector (FD) chromatogram of pyrene metabolites from six fish species (*O. latipes, F. gardneri, P. reticulate, C. auratus, D. rerio*, and *C. labiosa*). Pyrene glucuronide (*m/z* 393) and pyrene sulfate (*m/z* 297) were major metabolites in all the fish species. Excitation (Ex) and emission (Em) wavelengths for FD were 343 nm and 385 nm, respectively.

**Fig. 3.** Concentrations of each pyrene conjugate in each fish species. Results were analyzed by Tukey's HSD test. Mean ± SE, n = 3.

**Fig. 4.** Total concentration of pyrene conjugates in each fish species. Results were analyzed by Dunnett's test ($p < 0.05$) between *D. rerio* and other species (*). Mean ± SE, n = 3.

**Fig. 5.** Relationship between the amount of pyrene glucuronide and the total amount of pyrene metabolites determined by correlation analysis.

**Fig. 6.** Pyrene glucuronide:pyrene sulfate ratio in each fish species. Species were categorized into three groups on the basis of the pyrene glucuronide:pyrene sulfate ratio. Results were analyzed by Student's *t*-test ($p < 0.05$), Mean ± SE, n = 3.

**Fig. 7.** Ethoxyresorufin-O-deethylase (EROD) activity. Data were fitted to nonlinear regression curves using the Michaelis–Menten equation. Plots show the means (± SD) of the triplicate procedures.

**Fig. 8.** UDP-glucuronosyltransferase (UGT) activity. Data were fitted to nonlinear regression curves using the Michaelis–Menten equation. Plots show the means (± SD) of the triplicate procedures.

**Fig. 9.** Sulfotransferases (SULT) activity. Data were fitted to nonlinear regression curves using the Michaelis–Menten equation. Plots show the means (± SD) of the triplicate procedures.