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Effects of pentachlorophenol on the detoxification system in white-rumped munia (*Lonchura striata*)

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ARTICLE INFO

Article history:

Received 6 July 2015

Revised 11 October 2015

Accepted 14 October 2015

Available online 19 January 2016

Keywords:

CYP1A4

CYP1A5

Alkoxyresorufin O-dealkylase

Antioxidant enzyme

Lonchura striata

ABSTRACT

Pentachlorophenol (PCP), a priority pollutant due to its persistence and high toxicity, has been used worldwide as a pesticide and biocide. To understand the adverse effects of PCP, adult male white-rumped munias (*Lonchura striata*) were orally administrated commercial PCP mixed with corn oil at dosages of 0, 0.05, 0.5, and 5 mg/(kg·day) for 42 day. Gas chromatography–mass spectrometry (GC–MS) analysis found that PCP was preferentially accumulated in the kidney rather than in the liver and muscle in all exposure groups. To examine the function of CYP1A in pollutant metabolism, we isolated two full-length cDNA fragments (designated as CYP1A4 and CYP1A5) from *L. striata* liver using reverse transcription-polymerase chain reaction and rapid amplification of cDNA ends. PCP induced the expression of CYP1A5, although no obvious change was observed in CYP1A4 expression. Furthermore, PCP significantly elevated the activities of ethoxyresorufin O-deethylase and methoxyresorufin O-demethylase and decreased the activity of benzyloxy-trifluoromethyl-coumarin, with no significant responses observed in benzyloxyresorufin O-debenzylase. PCP induced significant changes in antioxidant enzyme (superoxide dismutase and catalase) activities and malondialdehyde content, but decreased glutathione peroxidase (GSH-Px) and glutathione S-transferase activities and GSH content in the liver of *L. striata*. The present study demonstrated that PCP had hepatic toxic effects by affecting CYP1As and anti-oxidative status.

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Introduction

Pentachlorophenol (PCP) is an organochlorine compound that has been extensively used as a pesticide and biocide in wood preservation worldwide (Seiler, 1991; Zheng et al., 2011). It has become of special interest to studies on pesticides because of its extensive use and ubiquitous appearance in the environment (Letcher et al., 2009; Zheng et al., 2012). Due to its stable aromatic ring system and high chlorine content, PCP is not prone to degradation (Okeke et al., 1997) and has a half-life of up to 200 days in water systems (Law et al., 2003). Due to this

persistence and bioaccumulation, PCP has been detected in surface water and sediment, rainwater, drinking water, aquatic organisms, soil and food, as well as in human milk, adipose tissue and urine (Kondo et al., 2005; Letcher et al., 2009).

PCP is highly toxic to most organisms. The proposed primary mechanisms of toxic action are the uncoupling of substrate oxidation from ATP in the mitochondria and the provoking of oxidative stress in cells and tissues (Xu et al., 2014). PCP exposure can result in many adverse effects and diseases in humans and animals, including liver defects,

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immune system damage, reproductive defects, genetic toxicity, and endocrine disrupting activity (Hurd et al., 2012; Yin et al., 2006; Zha et al., 2006). The target organs of PCP toxicity are the liver, kidney and bone marrow. PCP undergoes oxidative dechlorination to form tetrachlorohydroquinone by CYP450 enzymes, resulting in oxidative stress on cells and tissues, with the liver being the primary target in rodents, for example, and thus, hepatotoxicity generated through oxidative damage may play an important role during the pathophysiological process of liver damage induced by PCP (Fang et al., 2015; Wang et al., 2001).

Cytochrome P450 (CYP450), a superfamily of structurally and functionally related heme enzymes, is involved in the oxidative and reductive metabolism of a range of substrates, including drugs and environmental compounds, as well as endogenous chemicals such as steroids, neurohormones, fatty acids, and prostaglandins (Isin and Guengerich, 2007; Nelson et al., 1993). Cytochrome P450 1A (CYP1A) is a subfamily of CYP450, and plays an important role in the biotransformation of many foreign compounds, including polychlorinated biphenyls (PCBs), 2,3,7,8-tetrachlorodibenzo-p-dioxin and polyaromatic hydrocarbons (PAHs) (Besselink et al., 1998). The CYP1A subfamily has been found in every vertebrate tested to date. In mammals, the CYP1A subfamily generally consists of two isoenzymes, CYP1A1 and CYP1A2 (Teramitsu et al., 2000). In fish, originally only a single CYP1A gene was demonstrated (Chung et al., 2004; Nebert and Gonzalez, 1987), although another CYP1A subfamily member seems to exist in rainbow trout (Gooneratne et al., 1997). Similar to mammals, chickens have two CYP1As (CYP1A4 and CYP1A5), which are orthologous to mammalian CYP1A1 and CYP1A2, respectively (Gilday et al., 1996). Phylogenetic analysis of complete amino acid sequences suggests that the two chicken enzymes cluster together, forming a separate branch distinct from mammalian CYP1A1s/1A2s (Gilday et al., 1996). However, comparison of gene sequences has suggested that avian and mammalian CYP1A paralogs are the result of single gene duplication, gene conversion and positive selection (Goldstone and Stegeman, 2006). Due to its inducibility by xenobiotics and increase in expression levels in response to environmental pollutants, CYP1A is often used as a biomarker of human and wildlife exposure to environmental organic contaminants (Levine and Oris, 1999). Measurement of ethoxyresorufin O-deethylase (EROD) activity and the characterization of CYP1A4 and CYP1A5 mRNA expression are practical methods to quantify CYP1A induction in birds (Herve et al., 2010). Studies have shown that PCP or its dimer is a potential inducer of various CYP450 enzymes in human and wildlife cells (Dubois et al., 1996).

Various studies have reported that PCP induces the production of reactive oxygen species (ROS) and lipid peroxidation, and results in oxidative stress in cells and tissues (Chen et al., 2004; Dong et al., 2009; Naito et al., 1994). The enzymes superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), and glutathione peroxidase (GSH-Px) as well as antioxidant molecules (e.g., glutathione, GSH) are important tools available in cells and tissues to fight against ROS generated during oxidative metabolism (Pastoriza et al., 2015). Lipid peroxidation, estimated by measuring the content of malondialdehyde (MDA), can be defined as the

oxidative deterioration of cell membrane lipids (Amar et al., 2015). The antioxidant defense system has been extensively studied due to the potential use of oxyradical-mediated responses as biochemical biomarkers (Lazar et al., 2015).

As the toxic effects of PCP on birds have not been previously reported, we selected white-rumped munia (*Lonchura striata*), a widely distributed species in Southeast Asia, as an experimental model to understand the hepatic intoxication and antioxidation mechanisms affected by PCP treatment. This passerine bird is a popular subject for neurobiological and ethological studies due to its ease of use in laboratory research, as well as its small body size and high reproductive efficiency (Ueno and Suzuki, 2014). The aim of the present study was to evaluate the *in vivo* uptake of PCP in *L. striata* and test PCP bioaccumulation in different organs. Additionally, we isolated the CYP1A genes and investigated the expression of CYP1A in the livers of PCP-treated birds. We further aimed to clarify the effects of PCP on CYP450 and the antioxidant status of *L. striata* exposed to PCP.

1. Materials and methods

1.1. Reagents

Pentachlorophenol (PCP) ($\geq 99\%$, CAS No. 87-86-5) was purchased from Dr. Ehrenstorfer (Augsburg, Germany). The ^{13}C -pentachlorophenol ($\geq 99\%$) standard was purchased from AccuStandard (USA). All other chemicals used were of analytical grade, with HPLC grade and trace metal analysis grade chemicals used for the analysis of PCP.

1.2. Animals

Eighty four-month-old *L. striata* birds, with an average weight of 13.6 g, were obtained from a local commercial breeder (Beijing, China) and were bred and raised in the breeding colony at Beijing zoo (Beijing, China). Dry seed and fresh water were provided *ad libitum*. The birds were randomly divided into four groups, with 20 birds in each group, and were orally gavaged with consecutive dosages of either 0, 0.05, 0.5 or 5 mg/(kg·day) PCP in corn oil for 42 day, respectively. After exposure, all birds were euthanized and liver, kidney, muscle and serum samples were immediately collected, quickly frozen in liquid nitrogen and stored at -80°C for analysis of PCP concentrations and measurement of biochemical parameters. The animal-based experiments were conducted according to protocols approved by the Animal Ethics Committee of the Institute of Zoology, Chinese Academy of Sciences.

1.3. Chemical analysis of PCP in *L. striata*

Quantification of PCP was carried out by extraction in organic solvent, followed by quantification by gas chromatography with electron capture detection (ECD) (Ge et al., 2007). Briefly, samples (0.5 g wet weight of liver, kidney and muscle) were homogenized with sulfuric acid (8 mL, 50%) in homogenizers, and allowed to stand overnight before extraction. The homogenate was extracted with n-hexane and potassium carbonate solution. Acetic anhydride (0.3 mL) was added to

the potassium carbonate phase, and shaken for 5 min. The mixture was then extracted twice with n-hexane (2 mL each) and dried over anhydrous sodium sulfate and reduced to 1 mL under a gentle stream of nitrogen before injection into the GC-ECD system. The detection of PCP in serum was modified as per Cline et al. (Cline et al., 1989). Serum samples (0.5 mL) and MilliQ water (4.5 mL) were introduced into centrifuge tubes, after which 0.5 M sulfuric acid (0.5 mL) was added. The mixture was then extracted with hexane following the procedure mentioned above.

Concentrations of PCP in extracts were determined by an Agilent 5985–7890 (Agilent Technologies, USA) gas chromatograph equipped with a 63Ni micro-electron capture detector in the selective ion-monitoring (SIM) mode. PCP, along with the internal standard, were separated using a 30 m HP-5 ms column (0.25 mm i.d., 0.25 μ m thickness; Agilent Technologies, USA) under the following conditions: carrier gas: Helium, injection was splitless, injector and detector temperatures were 250°C and 300°C, respectively. The temperature program was 60°C for 2 min, then 20°C/min applied up to 220°C for 3 min, then a temperature gradient of 20°C/min applied up to 250°C for 3 min. Concentrations were calculated using 13 C-PCP as the internal standard. The following ions (m/z) in SIM modus were traced: 266 and 268 for pentachlorophenol acetate and 272 and 274 for 13 C-pentachlorophenol acetate as the internal standard. The method detection limit (MDL) was defined as a value three times the blank signal. Procedural blanks were performed in parallel with the samples from each batch for extraction covering the whole procedure. Average relative standard deviations (RSDs) were less than 10%.

1.4. RNA isolation and cDNA synthesis

Total liver RNA was extracted from 20 mg of frozen liver tissue from each individual *L. striata* using TRIZOL reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA was synthesized via reverse transcription using an oligo-(dT)₁₅ primer and M-MuLV reverse transcriptase (Promega, Madison, USA) according to the manufacturer's instructions.

1.5. Molecular cloning of full-length CYP1A4 and CYP1A5 cDNAs from *L. striata*

1.5.1. Cloning of CYP1A4 and CYP1A5 partial cDNAs

Fragments of *L. striata* CYP1A4 and CYP1A5 cDNA were amplified using primers designed based on the highly conserved nucleotide sequences identified by aligning available homologous sequences from *Gallus gallus* (GenBank no. X99453.1 for CYP1A4 and X99454.1 for CYP1A5), *Phalacrocorax carbo* (GenBank no. AB239444.1 for CYP1A4 and AB239445.1 for CYP1A5), and *Coturnix japonica* (GenBank no. GQ906939.1 for CYP1A4 and GQ906938.1 for CYP1A5). The partial cDNAs were amplified by polymerase chain reaction (PCR) using 2 \times Taq Master Mix (Cwbiotech, China) under the following conditions: 4 min at 94°C, 35 cycles of 15 s at 94°C, 30 s at 65°C for CYP1A4 and 55°C for CYP1A5, and 45 s at 72°C. The isolated gels were purified with a TIANgel Midi Purification Kit (Tiangen Biotech, Beijing, China), ligated with PMD 19-T

Vector (Takara, Japan), transformed into competent JM109 *Escherichia coli* (Dingguo, China), and sequenced (Tsingke, China). All primers are listed in Table 1.

1.5.2. Rapid amplification of cDNA ends (RACE) of CYP1A4 and CYP1A5

To obtain the complete sequences of the CYP1A genes, 5'- and 3'-rapid amplification of cDNA end (RACE) reactions were performed with a SMARTer™ RACE cDNA Amplification Kit (Clontech Laboratories, Inc., USA) following the manufacturer's instructions. The gene-specific primers (GSPs) were designed and synthesized according to the partial sequences of CYP1A4 and CYP1A5 obtained above and listed in Table 1. The 5' end RACE PCR reaction was performed with the cDNA template in a volume of 50 μ L, consisting of 25 μ L 2 \times PrimeSTAR GC buffer (Mg²⁺ plus), 4 μ L dNTP mixture (2.5 mmol/L), 5 μ L 10 \times UPM, 1 μ L GSP1, 2.5 μ L 5'-RACE-Ready cDNA, 0.5 μ L PrimeSTAR® HS DNA polymerase (2.5 U/ μ L) (Takara, Japan) and 12 μ L of nuclease-free water. The first 5'-RACE PCR was carried out under the following conditions: 10 min at 98°C, 30 cycles of 10 s at 98°C, 10 s at 68°C, and 1 min 30 s at 72°C. The expected PCR fragments were purified and used as the template for further 5'-RACE PCR reactions with Takara LA Taq (Takara, Japan). The 3' end RACE PCR reaction was performed with an Advantage® 2 PCR Kit (Takara, Japan). The 5' and 3' PCR products were purified, cloned and sequenced as described above.

1.6. Expression of CYP1A4, CYP1A5 in *L. striata*

Quantitative real-time PCR was performed to evaluate the expression of CYP1A4 and CYP1A5 in the livers of male *L. striata* exposed to PCP. β -Actin was selected as the internal standard due to its stability (Sun et al., 2010). Quantitative real-time PCR was performed using the Stratagene Mx3000P q-PCR system (Stratagene, USA) and the SYBR Green PCR Master Mix Reagent Kit (Tiangen, Beijing, China) following the manufacturer's instructions. Relative fold changes in the expression of target genes CYP1A4 and CYP1A5 were determined using the comparative 2^{- $\Delta\Delta$ CT} (Ct: cycle threshold) method (Livak and Schmittgen, 2001).

Table 1 – Primers designed for PCR.

Primer	Sequence 5'-3'
PCR CYP1A4 (F)	CTGCCCTCTGGAGGAGCA
PCR CYP1A4 (R)	GCTTCTGTGTAGGGCAGCATG
PCR CYP1A5 (F)	GCTTCTGTGTAGGGCAGCATG
PCR CYP1A5 (R)	AAGTGCTCACATCTCTTGTGCT
5' RACE CYP1A4 GSP1	TCCCGGGCACCTCCCAAATTCTGTT
5' RACE CYP1A5 GSP1	GCCACCCGGCTGACTTCATCCCCCTGCT
3' RACE CYP1A4 GSP2	GCCACCCGGCTGACTTCATCCCCCTGCT
3' RACE CYP1A5 GSP2	CCCTTCACCATCCCACACAGCACCA
qPCR β -actin (F)	TGGCAATGAGAGGTTTCAGT
qPCR β -actin (R)	TAGGGATGTCCACATCACACT
qPCR CYP1A4 (F)	TTCGTTGCCAGGTTCA
qPCR CYP1A4 (R)	GGACGGGAGGAGGTTTG
qPCR CYP1A5 (F)	GAGACCTACGACGAGAACTACAT
qPCR CYP1A5 (R)	CGAAGCCTGCTCCAAAG

1.7. Sequence alignments and phylogenetic analysis

Sequences were aligned based on the Blast Program of the NCBI database (<http://blast.ncbi.nlm.nih.gov>). The deduced amino acid sequences of the cloned cDNAs were aligned with homologous sequences in other species, and sequence identities were analyzed using ClustalW multiple sequence software in EBI. The CYP1A4 and CYP1A5 phylogenetic trees were analyzed using MEGA5.05 software according to the amino acid sequences.

1.8. Determination of enzyme activity and MDA and GSH content

The activities of the EROD, methoxyresorufin O-demethylase (MROD), benzyloxyresorufin O-debenzylase (BROD), benzyloxy-trifluoromethyl-coumarin (BFC), GSH-Px and GST were measured using the kits according to the manufacturer's instructions (Genmed Scientifics Inc., USA). The contents of MDA and GSH, as well as the activities of CAT and SOD, were measured by commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions.

1.9. Statistical analyses

All experiments were performed three times to confirm the results, and the experimental results were expressed as means with standard errors (mean \pm SE). Experimental data were analyzed using SPSS 16.0 for windows (SPSS Inc., Chicago, IL, USA). Differences between the control and treatment groups were determined using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Differences were considered significant if $p < 0.05$.

2. Results

2.1. PCP content in liver, kidney, muscle and serum of *L. striata*

To evaluate PCP content in *L. striata*, its concentration in the liver, kidney, muscle and serum samples from the control and

exposure groups was quantified using gas chromatography–mass spectrometry (GC–MS). Results indicated that PCP was rapidly taken up by *L. striata* and assimilated into various tissues. In the liver, kidney and muscle, PCP concentrations increased significantly in a dose-dependent manner (Fig. 1a). In the kidney, the highest content of PCP occurred in the 5 mg/(kg·day) dosage group ($9.75 \pm 1.18 \mu\text{g/g ww}$), which was nearly 7.2-fold higher than that found in muscle and 1.4-fold higher than that found in the liver. In serum, the concentration of PCP also increased in a dose-dependent manner, and the highest concentration was detected in the 5 mg/(kg·day) dosage group ($5.02 \pm 0.47 \mu\text{g/mL}$) (Fig. 1b). The presence of PCP in the serum of the control group may have several origins. PCP may be bioaccumulated from the environment due to its widespread use as a wood preservative (Chhabra et al., 1999) and its recent detection in indoor air and dust samples (Suzuki et al., 2008; Zheng et al., 2000).

2.2. Cloning of full-length *L. striata* CYP1A4 and CYP1A5 cDNAs

The two distinct full-length gene sequences were obtained by 5'- and 3'-RACE. One of the sequences consisted of a 2510 bp cDNA with a 77 bp 5'-untranslated region (UTR) and an 813 bp 3'-UTR. The open reading frame (ORF) was 1590 bp, which encoded 529 amino acid proteins with a predicted molecular mass of 59,505 kDa and a theoretical pI of 8.48. The second gene sequence contained a 1979 bp cDNA with a 169 bp 5'-UTR and a 220 bp 3'-UTR. The ORF was 1590 bp, which encoded 529 amino acid proteins with a predicted molecular mass of 59,915 kDa and a theoretical pI of 8.79 (Fig. S1). The two genes had dissimilar 3'- and 5'-UTRs and 87% identity at the level of cDNA coding sequences and 81% identity between deduced amino acid sequences. In addition, several CYP1A functional motifs were well-conserved in the deduced amino acid sequences of *L. striata* CYP1A enzymes. Both enzymes contained the characteristic heme binding motif, which consisted of the cysteine-rich region (Phe-X-X-Gly-X-Arg-X-Cys-X-Gly), conserved threonine residue, center of α -helix I (FGAGFDT) and proline-rich region (Pro-Pro-X-Pro), which plays a key role in

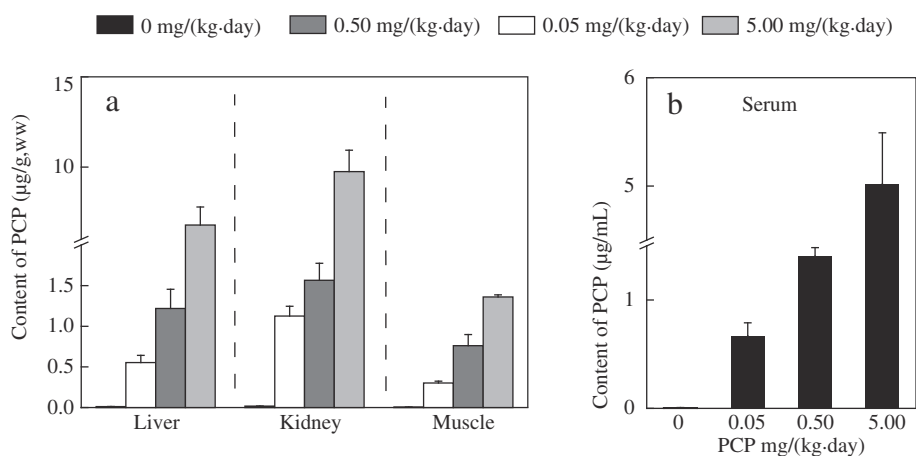


Fig. 1 – Levels of PCP measured in the liver, kidney, muscle, and serum of *L. striata* exposed to 0, 0.05, 0.5, and 5 mg/kg/d PCP doses. (a) Content of PCP in liver, kidney, and muscle; (b) content of PCP in serum. PCP: pentachlorophenol.

protein folding (Fig. 2). The N-terminal amino acid sequences for both enzymes contained the typical hydrophobic signal sequence that anchors CYP in the endoplasmic reticulum

membrane, followed by a halt-transfer signal containing several positively charged amino acids (Werck-Reichhart and Feyereisen, 2000). On the basis of greatest overall amino acid

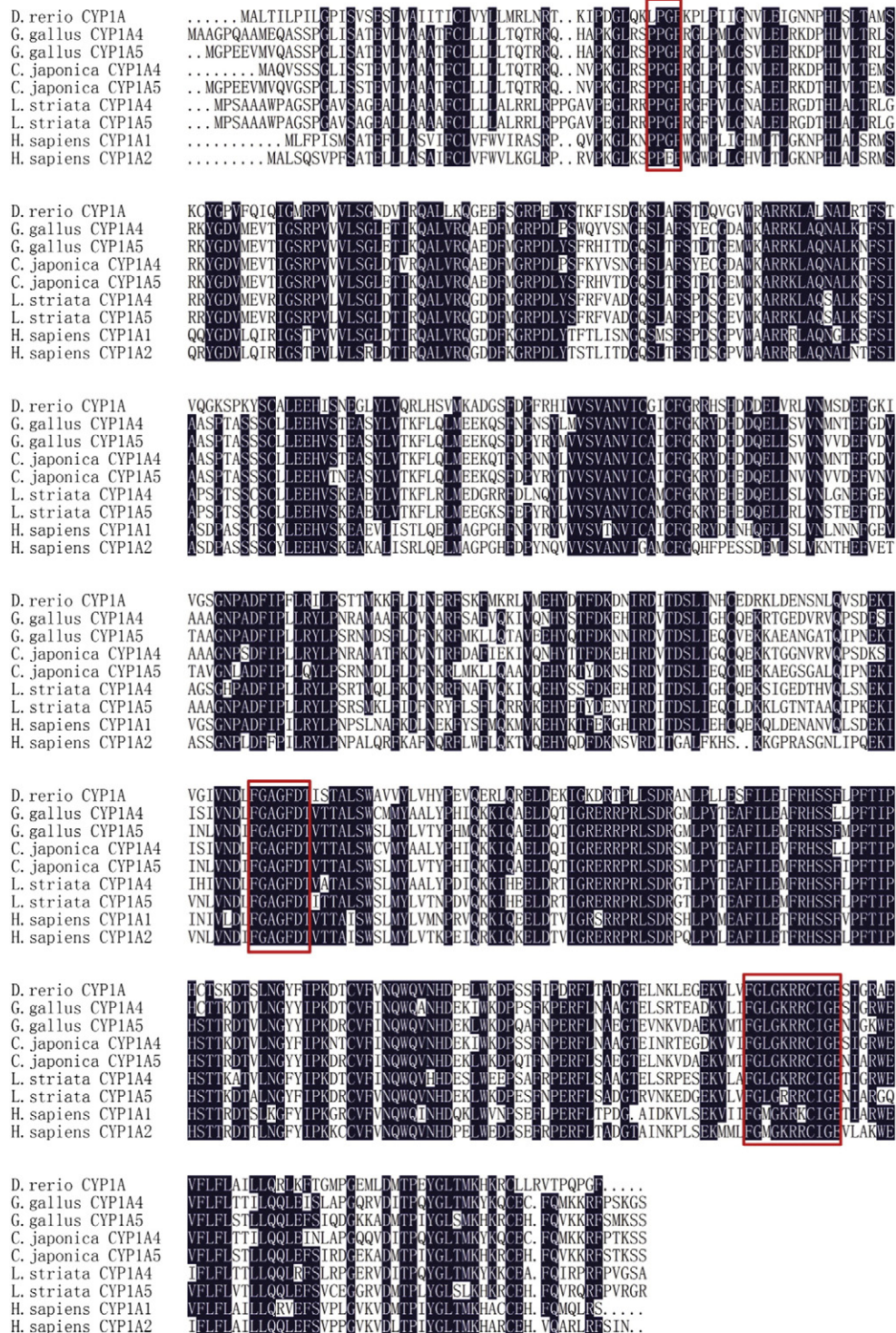


Fig. 2 – Alignment of CYP1A amino acid sequences. The deduced amino acid sequences for *L. striata* CYP1A4 and CYP1A5 were aligned with other CYP1As using ClustalW. The (A) proline-rich region (PPGP), (B) center of alpha-helix I (FGAGFD), and (C) heme-binding motif (FxxGxxxCxG) are boxed. Accession numbers for sequences used are as follows: zebrafish (*D. rerio*) CYP1A (BAB90841.1); chicken (*G. gallus*) CYP1A4 (NP_990478.1); chicken (*G. gallus*) CYP1A5 (CAA67816.1); human (*H. sapiens*) CYP1A1 (NP_000490.1); human (*H. sapiens*) CYP1A2 (NP_000752.2).

identity shared with the Tibetan ground-tit (*Pseudopodoces humilis*) CYP1A4 (90%) and zebra finch (*Taeniopygia guttata*) CYP1A5 (96%), respectively, the two sequences were assigned the names CYP1A4 (GenBank accession no. KM515884) and CYP1A5 (GenBank accession no. KM515885) by D. R. Nelson (University of Tennessee, TN) from the CYP450 Gene Superfamily Nomenclature Committee.

Comparison of the amino acid sequences with other vertebrate CYP1As revealed that *L. striata* CYP1As also exhibited considerable sequence identity to fish CYP1A enzymes, ranging from 59 to 60%, and to *Xenopus laevis* CYP1A1, ranging from 61 to 62%. Based on the putative amino acid sequence, *L. striata* CYP1A4 and CYP1A5 shared 64% and 69% identity to *Alligator mississippiensis* CYP1A5-like, respectively. Only one full-length *A. mississippiensis* sequence was employed in the phylogenetic tree, while there were two cytochrome P450 1A5-like sequences, named isoform X1 and X2, also assigned to CYP1A subfamilies in the CYP database. Moreover, *L. striata* CYP1As also exhibited considerable sequence identity with mammals, including 63–64% identity to mammalian CYP1A1 and 56–62% identity to mammalian CYP1A2 (Table 2).

2.3. Phylogenetic analysis of *L. striata* CYP1A

Based on the amino acid sequences of CYP1As of *L. striata* and other vertebrates, a phylogenetic tree was generated using MEGA5.05 (Fig. 3). Our results showed that *L. striata* CYP1A was more closely related to other avian CYP1A genes than those of

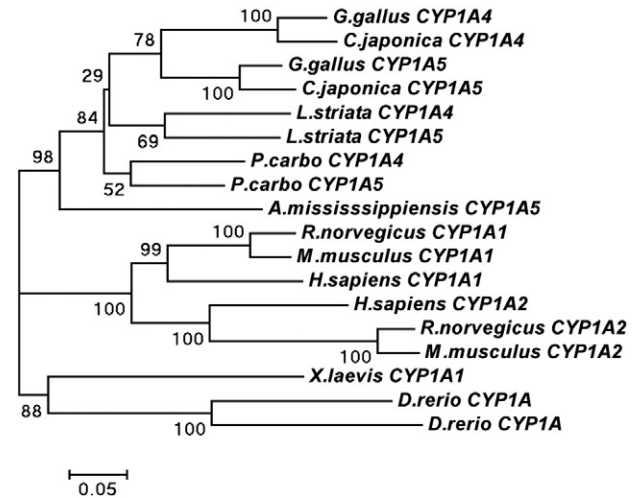


Fig. 3 – Phylogenetic tree of CYP1A created using MEGA5.05 software according to the amino acid sequences of CYP1A. The scale bar represents 0.05 amino acid replacements per amino acid site. GenBank accession numbers of the sequences used in the figure are as follows: *D. rerio* CYP1A (BAB90841.1); *F. heteroclitus* (AAD01809.1); *X. laevis* CYP1A1 (NP_001090541.1); *A. mississippiensis* CYP1A5 (XP_006273206.1); *P. carbo* CYP1A4 (BAE93469.1); *P. carbo* CYP1A5 (BAE93470.1); *G. gallus* CYP1A4 (NP_990478.1); *G. gallus* CYP1A5 (CAA67816.1); *C. japonica* CYP1A4 (ACX54108.1); *C. japonica* CYP1A5 (ACX54107.1); *R. norvegicus* CYP1A1 (NP_036672.2); *R. norvegicus* CYP1A2 (NP_036673.3); *H. sapiens* CYP1A1 (NP_000490.1); *H. sapiens* CYP1A2 (NP_000752.2); *M. musculus* CYP1A1 (NP_034122.1); *M. musculus* CYP1A2 (NP_034123.1).

Table 2 – Percentages of amino acid identities between CYP1A4 and CYP1A5 and other CYP enzymes values given represent the percentage of identities of the derived amino acid sequences for CYP1A4 and CYP1A5 and other CYP enzymes based on multiple comparisons of sequence pairs using the ClustalW method of alignment.

Species	Identity (%)	
	CYP1A4	CYP1A5
Fish CYP1A		
<i>Danio rerio</i>	57	59
<i>Fundulus heteroclitus</i>	58	60
Frog CYP1A1		
<i>Xenopus laevis</i>	61	62
Reptile		
<i>Alligator mississippiensis</i> CYP1A5-like	64	69
Avian CYP1A4		
<i>Phalacrocorax carbo</i>	80	70
<i>Gallus gallus</i>	75	67
<i>Pseudopodoces humilis</i>	90	
Avian CYP1A5		
<i>Phalacrocorax carbo</i>	72	78
<i>Gallus gallus</i>	69	74
<i>Taeniopygia guttata</i>		96
Mammalian CYP1A1		
<i>Homo sapiens</i>	64	64
<i>Rattus norvegicus</i>	63	63
<i>Mus musculus</i>	64	64
Mammalian CYP1A2		
<i>Homo sapiens</i>	61	62
<i>Rattus norvegicus</i>	57	59
<i>Mus musculus</i>	56	58

fish and mammals. In the bird branch, *L. striata* and *P. carbo* CYP1As were segregated according to species, however, *G. gallus* and *C. japonica* CYP1A sequence orthologs formed monophyletic clusters. The tree also showed that *L. striata* CYP1As had a distinct divergence from mammalian CYP1As. In addition, the lower amino acid identities for CYP1A1 and 1A2 (66%, mean for the six species compared in Table 2) than for *L. striata* CYP1A4 and CYP1A5 (84%) suggest that the two mammalian enzymes were more divergent than the two bird enzymes. The relationship displayed in the phylogenetic tree was in good accordance with the concept of traditional taxonomy.

2.4. Effect of PCP on CYP1As mRNA levels in *L. striata*

To further investigate the effect of PCP on the transcriptional expression level of CYP1As, the levels of CYP1A4 and CYP1A5 mRNAs in *L. striata* were measured by quantitative real-time PCR. Our results revealed that CYP1A5 mRNA expression was significantly upregulated in *L. striata* and exhibited an approximate 3.3- and 2.2-fold increase in the 0.5 and 5 mg/(kg·day) PCP treatment groups, respectively, compared with that of the control. In contrast, no significant differences in CYP1A4 mRNA expression were exhibited in any PCP treatment group compared with the control (Fig. 4).

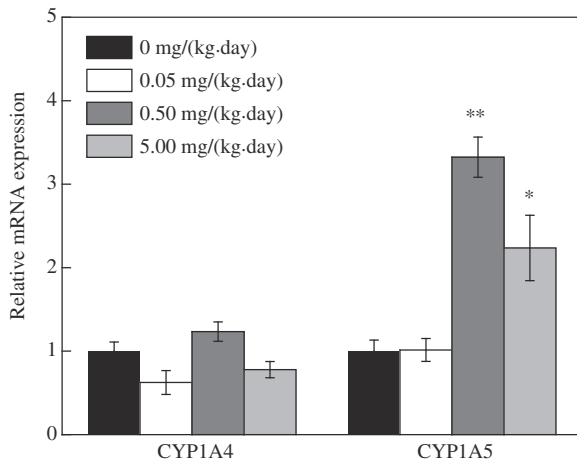


Fig. 4 – Quantitative real-time PCR analysis of mRNA levels of CYP1A4 and CYP1A5 in birds exposed to PCP. Gene expression levels represent relative mRNA expression compared with those in the control. Values are means \pm SE of six individual birds per group. Statistical significance is indicated by * $p < 0.05$ and ** $p < 0.01$ compared with the control. PCP: pentachlorophenol; PCR: polymerase chain reaction.

2.5. Assessment of EROD, MROD, BROD, BFC, SOD, CAT, GSH-Px, and GST activities as well as the contents of MDA and GSH

We focused on the toxic effect of PCP on the liver as it is a major site for detoxification of xenobiotics. Our results showed that hepatic microsomal EROD activity in *L. striata* was significantly greater only in the 0.5 mg/(kg-day) PCP exposure group, with a maximum response approximately 1.36-fold that of the control group ($p < 0.05$). MROD activity showed a significant 1.84-fold increase in the 5 mg/(kg-day) PCP exposure group ($p < 0.05$). However, mean hepatic BROD activity after PCP exposure was not significantly different compared with that of the control. In

addition, hepatic BFC activity in birds from all treatment groups significantly decreased compared with the control by 58.8%, 45.5%, and 51.2%, respectively ($p < 0.01$) (Fig. 5a). The MDA content increased significantly in all PCP exposure groups and reached a maximum value in the high-dosage group (5 mg/(kg-day)). In contrast, GSH concentration decreased in the 0.5 and 5 mg/(kg-day) groups compared with that of the control (Fig. 5b).

In the liver, SOD activity increased significantly in the 0.05 mg/(kg-day) group, and CAT activity increased in the low and intermediate PCP exposure groups compared with that of the control. GST and GSH-Px activities showed distinct decreases at all exposure doses. In the high PCP exposure group (5 mg/(kg-day)), GST and GSH-PX activities decreased by 41.3% and 55.9%, respectively, compared with that of the control (Fig. 5c).

3. Discussion

No mortality occurred during the experiment. The body and liver weights of the treated birds were not lower than those of the control, although body weights of *Anas platyrhynchos* and liver weights of broiler chickens were found to be significantly lowered after PCP exposure (Nebeker et al., 1994; Stedman et al., 1980).

3.1. PCP content in tissues of *L. striata*

PCP has accumulated in the environment, thereby causing a potential risk to animals and humans (Kondo et al., 2005; Maenpaa et al., 2004; Yu et al., 2015). Halogenated phenolic compounds, including PCP, can accumulate in the blood and fetal tissues of birds, fish, cetaceans, and other mammals (Kunisue and Tanabe, 2009; Montano et al., 2013), and has also been found in bird eggs (Berger et al., 2004; Brambilla et al., 2009; Maenpaa et al., 2004). Once absorbed by birds, PCP tends to accumulate in the blood due to its high affinity to plasma

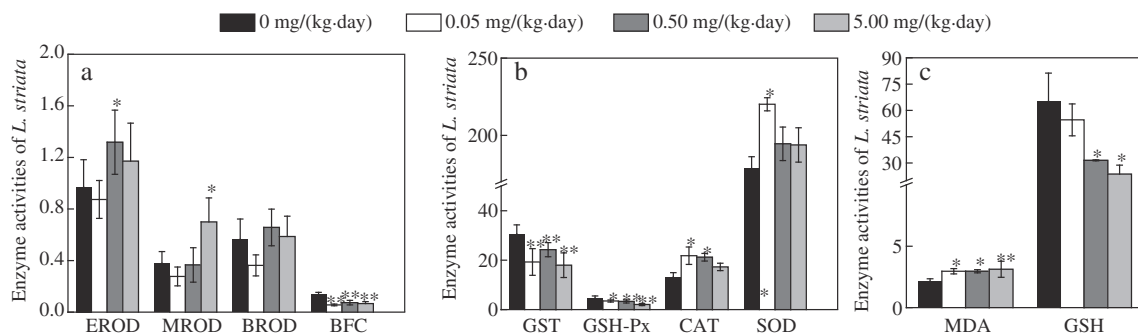


Fig. 5 – Enzyme activities of EROD, MROD, BROD, BFC (a), GST, GSH-PX, SOD and CAT (b), as well as the contents of MDA and GSH (c). Samples were collected from 0, 0.05, 0.5 and 5 mg/kg/d PCP exposure groups (mean \pm SE, $n = 6$). Statistically significant differences between the control and treatments are indicated by * $p < 0.05$, ** $p < 0.01$. EROD, MROD and BROD are expressed as pmol/mg/min, while BFC activities are expressed as nmol/mg/min. Concentrations of MDA and GSH are expressed as nmol/mg protein and μ mol/mg protein, respectively. Activities of GST, GSH-Px, CAT and SOD are expressed as U/mg protein. BROD: benzyloxyresorufin O-debenzylase; EROD: ethoxyresorufin O-deethylase; MROD: methoxyresorufin O-demethylase; BFC: benzyloxy-trifluoromethyl-coumarin; GST: glutathione S-transferase; GSH-PX: glutathione peroxidase; SOD: superoxide dismutase; CAT: catalase.

proteins (Eguchi et al., 2008). Our present study showed that PCP was primarily accumulated in the kidney of *L. striata*. The high residue levels in the kidney and liver may reflect the principal routes of elimination and metabolism. This result was consistent with PCP accumulation studies in broiler chickens, in which PCP levels were greatest in the kidney followed by the liver (Stedman et al., 1980).

3.2. Characterization of CYP1A4 and CYP1A5 in *L. striata*

We focused on gene expression as well as enzyme response in the detoxification process following exposure to PCP. CYP1A plays an important role in organism detoxification. In addition to *G. gallus*, *P. carbo* and *C. japonica*, numerous avian CYP1As have been previously identified, such as CYP1A4/5 in herring gull (*Larus argentatus*) (accession number AY233271/AY330876), CYP1A5 in turkey (*Meleagris gallopavo*) (accession number AY964644) and CYP1A5 in wild jungle crow (*Corvus macrorhynchos*) (accession number AB220967); however, no such information is currently available for *L. striata*. We cloned the CYP1A4 and CYP1A5 genes from *L. striata* liver for the first time to investigate the detoxification mechanisms of PCP exposure. Our results revealed a highly conserved sequence across most species, according to the identity of the deduced amino acid sequences. Alignment of the *L. striata* CYP1A4 and CYP1A5 amino acid sequences with their orthologs revealed that the *L. striata* CYP1A sequences were more closely related to other avian species than to other species such as fish and mammals.

For gene expression levels, PCP induced CYP1A5 in the 0.5 and 5 mg/(kg·day) PCP treatment groups in comparison with the control, though this was not observed for CYP1A4. Thus, PCP might induce CYP1A5-dependent enzyme activity in a noncompetitive manner, but not CYP1A4-dependent activity. However, further studies are required to elucidate the structural basis underlying catalytic differences between CYP1A paralogs, which will allow a mechanistic explanation of enzymatic differences.

3.3. Effect of PCP on activities of alkoxyresorufin O-dealkylase in *L. striata*

Most xenobiotics or environmental pollutants are metabolized or detoxified via the CYP450 system, including the phase I system (Jorgensen et al., 2008). PCP is known as an uncoupler of mitochondrial oxidative phosphorylation and can disturb liver microsomal detoxification functions, including that of CYP450 (Arrhenius et al., 1977). The induction of CYP450 by PCP in humans and animals has been studied both *in vivo* and *in vitro* (Barque et al., 2002; Dubois et al., 1997; Vizethum and Goerz, 1979). Alkoxyresorufin O-dealkylase (AROD) activities, including EROD, MROD and BROD activities, are typical catalytic markers for a variety of CYP450 enzymes, and thus have great potential to elucidate CYP450 induction by chemical exposure. Among them, EROD activity has been used extensively as a sensitive indicator of the effects of exogenous compounds on CYP1A induction in avian species (Kubota et al., 2006; Verbrugge et al., 2001; Watanabe et al., 2005). Our study revealed that PCP increased EROD activity in the 0.5 mg/(kg·day) PCP exposure group ($p < 0.05$) (Fig. 5a),

which is in accordance with the increase in EROD activity following exposure to PCP observed in quail hepatocytes (Verbrugge et al., 2001). Previous studies have revealed that BROD can be induced by phenobarbital, hexachlorobenzene, and PCBs (Li and Hansen, 1996; Parente et al., 2009; Smith et al., 1993). In the present study, however, BROD activity was not significantly different after PCP exposure. The cytochrome P450 monooxygenase 3A4 (CYP3A4) is responsible for the oxidative metabolism of a wide variety of xenobiotics, including an estimated 60% of all clinically used drugs (Lehmann et al., 1998), and both quail and chicken microsomes exhibit CYP3A4-mediated BFC enzymatic activity (Diaz et al., 2010; Mooiman et al., 2014). We found that BFC activity in *L. striata* was inhibited in all exposure groups (Fig. 5a). Thus, it is likely that the observed decrease in specific substrate metabolism was due to competition between PCP and the substrate for the active site of the enzyme. Based on these data, we concluded that exposure to PCP in *L. striata* can induce or inhibit CYP450 activity and thus modify enzymatic pathways mediated by CYP450, allowing CYP450 enzyme activities in the liver to be potent biomarkers of pollution caused by PCP.

3.4. Effect of PCP on antioxidant enzymes in *L. striata* liver

The principal mechanisms of PCP-induced toxicity appear to be related to the generation of ROS (Shan et al., 2013), which affect the antioxidant system (SOD, CAT, GST, GSH-Px and GSH content) and can cause oxidative damage and lipid peroxidation if oxidative stress overwhelms the defense system. In agreement with this, we observed a decrease in GSH and an increase in MDA content in the PCP-treated groups (Fig. 5b). These findings accord with previous studies in which PCP exposed to hepatocytes induced ROS generation, increased lipid peroxidation production (MDA), and decreased levels of GSH (Dong et al., 2009). The depletion of GSH can result in an increase in ROS concentration, which may enhance lipid peroxidation intensity (Chiou et al., 2003). SOD enzymes represent the first line of cellular antioxidant protection against oxidative damage caused by ROS via converting O_2^- to O_2 and H_2O_2 (Li et al., 2014). H_2O_2 is converted to water and O_2 by CAT, which is localized predominantly in the peroxisomes (Mittler, 2002). In the current study, PCP caused an increase in SOD and CAT in the low exposure group, suggesting the generation of free radicals and an adaptive response to oxidative stress and lipid peroxidation (Gehring et al., 2004). GSH-Px catalyzes the reduction of H_2O_2 and lipid peroxides at the expense of GSH and plays a major role in protecting cells against cytotoxic and carcinogenic chemicals by scavenging ROS (Ramesh et al., 2015). The decreased GSH-Px activity in the livers of all treatment groups after 42 d might be attributed to low levels of GSH and important for the conjugation of lipid peroxides. GST plays a role in the detoxification of electrophilic xenobiotic compounds by GSH conjugation (Chatel et al., 2015). Previous studies have also revealed that PCP inhibited equine liver GST activity and inactivated mouse GST- μ *in vitro*, and GST can specifically bind PCP, resulting in the partitioning of PCP from lipid to aqueous compartments (Bain and LeBlanc, 1996; Moorthy and Randerath, 1996). Our results

demonstrated that GST activity was significantly decreased in the PCP exposure groups compared with that of the control ($P < 0.01$) (Fig. 5c), consistent with the above studies.

4. Conclusions

We cloned full-length CYP1A1 and CYP1A4 genes in the liver of *L. striata*, and CYP1A5 expression levels increased following PCP treatment. PCP was primarily accumulated in the kidney and liver of *L. striata*. The changes of alkoxyresorufin O-dealkylase and antioxidant enzymes activities as well as the contents of MDA and GSH suggested that PCP affected CYP450 enzyme activities and promoted an oxidative stress response in the liver of *L. striata*. However, further studies are needed to fully understand the exact mechanisms leading to these outcomes.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jes.2015.10.030>.

Acknowledgments

This work was supported by the Strategic Priority Research Program of the Chinese Academy of Sciences (No. XDB14040202) and the National Natural Science Foundation of China (Nos. 31320103915 and 21377128).

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