Long-term Exposure to Phenanthrene-Induced Gene Expressions and Enzyme Activities of *Cyprinus Carpio* Below the Safe Concentration

Xin Kang
Donghua University
Dongpeng Li
Donghua University
Xin Cao (caoxin@dhu.edu.cn)
Donghua University https://orcid.org/0000-0002-9516-8222
Xiaoxiang Zhao
Donghua University
Yanfeng Lv
Donghua University
Xi Chen
Chinese Academy of Agricultural Sciences Agricultural Genomes Institute at Shenzhen
Xinshan Song
Donghua University
Xiangyu Liu
Griffith University
Chengrong Chen
Griffith University

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Abstract

Phenanthrene (PHE) is a typical food chain biomagnified compound which endangers human health and is generally accumulated from marine lives. Previous PHE-stressed Carp acute toxicity test showed that the safe concentration of PHE to carp was 1.12 mg/L. In this study, the carp was long-term exposed to PHE below safe concentration up to 25 days. The gene expression levels and cytochrome P450 (CYP1A/EROD (7-Ethoxylesorufin O-deethylase)) and glutathione S-transferase (GST) activities were determined in the carp liver and brain tissues. The results showed that both the *CYP1A* mRNA expression and EROD activity in the liver were continuously stimulated after induction with the increase in exposure time and exposure concentration. However, with the increase of PHE concentration, *GST* mRNA expression in the liver was firstly induced and then inhibited and the induction was significant in the treatment with 0.1 mg/L PHE in the 15th day (almost 2-fold). In the brain, after the 15th day, *GST* mRNA expression was suppressed, but GST activity was induced. Correlation analysis results showed that the CYP1A mRNA expression was significantly correlated with the activity of EROD in both tissues (liver, $r = 0.602$, $P < 0.01$; brain, $r = 0.508$, $P < 0.01$), but the correlation between *GST* mRNA expression and GST activity was poor (liver, $r = 0.385$, $P < 0.01$; brain, $r = 0.293$, $P < 0.01$). This experiment revealed the self-regulation mechanism of carp exposed to lower than safe concentrations of PHE for a long time, indicating the toxicological risk of PHE in the ecosystem.

Highlights

- Carp was long-term exposed to low levels of PHE (1.12 mg/L).
- PHE was accumulated in the liver and brain of carp under safe concentrations.
- Molecular oxidative damage and toxic metabolic pathways were revealed.
- Low levels of PHE significant induced EROD in brain and GST in liver**.**
- The cytochrome P450 system had a closer correlation than the GST.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a widespread pollutant which derived from the incomplete combustion of various fossil fuels and hydrocarbons, such as coal and petroleum. Due to the stable chemical properties and harmful effects, PAHs have become persistent organic pollutants (POPs) and threaten environment and even human beings. Especially, PAHs are known to induce stress and affect marine organisms health (Carmen et al., 2000). According to the combined structure of benzene rings, PAHs can be divided into two categories: fused ring type and non-fused ring type. Phenanthrene (PHE, $C_{14}H_{10}$) is a representative of the fused ring PAHs(Wda et al., 2020). It is a low-molecular-weight PAH-composed of three fused benzene rings with bay-area and K-area, and its carcinogenicity is closely related to K-area (Harvey et al., 1991). Due to the small molecular weight of PHE, it is more easily bio-amplified than other PAHs and produces toxic effects (Maskaoui et al., 2002) like acute lethal effect on aquatic organisms at individual level (Zheng et al., 2020) which further limits individual development

(Lotufo and Fleeger, 1997), reproduction and other behaviors (Nam et al., 2017; Sobanska et al., 2018). At the levels of cells, tissues, and organs, PHE can cause histopathological changes in organisms (Horng et al., 2010; Sun et al., 2019). Carp *(Cyprinus carpio)*, as a traditional freshwater economic fish in China, has been widely used in the ecotoxicity tests of environmental pollutants (Sidika et al., 2018; Woo et al., 2018). However, the study of carp-specific genes expressions and enzymes activities after low dose PHE exposure were seldom reported. Therefore, it is necessary to explore the molecular response of carp-specific genes and characteristic metabolic enzymes in PHE pollution stress.

Current studies on low-molecular-weight PAHs in China have mainly focused on their sources and distributions in rivers and lakes (Huang et al., 2020; Yang et al., 2020). Concentrations of PAHs in the waters of Hangzhou, Qiantang River, and Daya Bay reached 989 ~ 9663 ng/L, 70.3 ~ 1844.4 ng/L and 10984 ~ 19445 ng/L, respectively. The effects of high concentrations of PAHs on aquatic organisms were extensively explored (Mager et al., 2018; Wilson et al., 2018), but the effects of pollutants below safe concentrations on aquatic organisms were still elusive. In the study, the acute toxicity of PHE to carp was tested and the 96-h lethal concentration and safe concentration of PHE to carp were determined. In the experiment, carp were exposed to the PHE at concentrations below safe levels to explore the changes in the activities of characteristic enzymes and characteristic genes expression involved in carp metabolism.

Cytochrome P450 (CYP) enzymes were reported as the biomarkers to estimate the influences of persistent organic pollutants (POPs) on various aquatic organisms (Skjetne Mortensen et al., 2006; Kais et al., 2017; Roy et al., 2019). PAHs, which are known agonists for controlling aromatic hydrocarbon receptors (AhR) (Oost et al., 2003), could induce the expressions of Phase I and Phase II metabolism enzymes including cytochrome P4501A (CYP1A) and glutathione S-transferase (GST).

The study aimed to explore the activity changes of Phase I and Phase II metabolic characteristic enzymes (cytochrome P450 enzyme (EROD) and glutathione S-transferase (GST)) in carp under the exposure to PHE below safe concentration in different organs and determine the changes in the expression levels of characteristic genes of *CYP1A* and *GST*, which controlled the activities of EROD and GST. In addition, the correlations between the expressions of characteristic enzymes (EROD, GST) from metabolic process and the expressions of characteristic genes (*CYP1A*, *GST*) under the exposure to PHE at or below safe concentration were further discussed. We illustrated the responses of carp metabolizing characteristic genes and characteristic enzymes to low concentrations of PHE. Meanwhile, we also explored the differences in the metabolism of heterologous substances in different organs of carp and different enzyme systems induced by PHE.

Materials And Methods

2.1. Main reagents

Phenanthrene (PHE, 95%) was bought from Beijing Braun Technology Ltd. Diethy pyrocarbonate (DEPC), Spin Column Animal Total RNA Purification Kit, and M-MuLV First Strand cDNA Synthesis Kit were

obtained from Sangon Biotech (Shanghai, China). 2×Taq Plus PCR MasterMix was bought from Tiangen Biotech (Beijing, China). Agarose was bought from Aladdin. 50×TAE loading buffer and 6×DNA loading buffer were configured according to general methods (Robert et al., 1999). Fish 7-ethoxyresorufin-o-deethylase (EROD) kit and fish glutathione S transferase (GST) kit came from Jianglai Biotechnology (Shanghai, China). Fish ELISA kits of the enzymes of EROD and GST were bought from Jianglai Biotechnology (Shanghai, China). Albumin from bovine serum (BSA) was bought from Jiancheng Bioengineering(Nanjing, China).

2.2. Fish and treatment

Carp (*Cyprinus carpio*, body weight (9.0 ~ 11.0 g) and body length (8.0 ± 10.0 cm)) were obtained from a fish farm in Songjiang, Shanghai, China. The experimental carps were domesticated in seven groups of 10-L glass tanks under natural conditions for 2 weeks, each group for 10 fish. All tanks were supplied with continuous aeration to maintain nearly saturated dissolved oxygen. Dechlorinated tap water was used at a temperature of 20 ± 2°C, pH 6.8 ~ 7.3, with light intensity of 100 Lux for 10 h/d for 25 d and fed twice a day and clean up metabolites on time to ensure that the mortality rate during domestication is less than 1%, and 4 d before the start of the experiment, ensure that there was no fish death. Three treatment groups were exposed to waterborne PHE in acetone under the concentrations of 0.1, 0.5, and 1.0 mg/L. (the final content of acetone was less than 0.5%) In addition, the 0 mg/L PHE as the negative control (dechlorinated water) was established. The parallel groups of each concentration were set in other three 10-L tanks at 20.0 ± 2°C. After the exposure for 1 days, 5 days, 15 days and 25 days (We define 1–5 days as "early period"), the brain and liver of fish were dissected, placed in 1.5-mL microcentrifuge tubes, frozen in liquid nitrogen immediately, and then stored at − 80°C until further analysis. A part of one sample was ground fully in a vessel with liquid nitrogen to analyze the expressions of relevant genes. Another part of the sample was ground fully with a glass homogenate device with PBS to detect the activities of related enzymes (All samples are homogenized before separation).

2.3. Acute toxicity test

The PHE dose was set to 5, 10, 20, 30, 40, 50 mg/L (the final content of acetone was less than 0.5%) using the aquatic toxicity test method, and each group was 10 pieces. The pre-test first showed that the carp did not die in 96 hours. The highest dose and the lowest dose for all deaths in 24 hours. During the test, no feed was fed, semi-static exposure was performed, and the water was changed 50% every day. The activity status of the carp was observed on time and the number of dead fish was counted in time.

The acute toxicity experiment was conducted according to the SECP-Part 12: Fish Acute Toxicity Test (GB/T 31270.12 − 2014). PHE in eight tanks was set according to equidistant logarithmic concentrations at 5.01, 6.31, 7.94, 10.00, 12.59, 15.85, 19.95, and 25.12 mg/L and there were ten fish in each tank. Three replicates were arranged for each concentration. In the acute test, after 96 h, the mortality was recorded. The carp were not fed during the test period and water was replaced once a day.

Dead fish were identified according to the following method. Carp were transferred to clear water for 30 s and the fishtail was touched with a glass rod. If there was no visible response, carp were considered to be

dead.

The safe concentration (SC) is expressed as follows:

$$(SC) = \frac{(LC50(96h))}{10} \quad . \qquad (1)$$

2.4. RNA extraction and analysis

All samples from the experimental fish were firstly ground into fine powder under liquid nitrogen. Total RNA was extracted with Trizol Reagent (Sangon, China) according to the manufacturer's instructions. Then the OD (Optical density) values of samples at 260 nm and 280 nm were measured. The RNA sample purity was calculated with Eq. (2). Finally, based on the calculation results, the integrity of RNA was detected by 1.5% agarose gel electrophoresis (Fig S4).

$$\text{The purity of RNA is expressed as: } \omega = \frac{A_{260}}{A_{280}} \quad . \qquad (2)$$

If ω is between 1.8 and 2.0, the RNA sample is qualified; if ω is less than 1.8, the sample is contaminated; if ω is larger than 2.0, the RNA sample is degraded.

2.5. Gene primer design and quantitative real-time PCR (qRT-PCR)

After RNA extraction, the RNA was reversely transcribed with the cDNA Synthesis Kit (Sangon, China) according to the manufacturer's protocol. Then, the mRNA levels were expressed as the ratios relative to the transcription level of the 18S rRNA. Primer Premier 5.0 was used to design the primers according to two ends of the target gene based on the sequence of carp *CYP1A* (accession: AB048939.1) and *GST* (accession: LC071505.1) (Table 1).

Table 1
qRT-PCR primers of *Cyprinus carpio* genes

Gene	GeneBank number	Primer sequence	Product size (bp)
β-actin	AF0570 40	F: CCATCTACGAGGGTTACGCC	551bp
		R: AATGCCAGGGTACATGGTGG	
CYP1A	AB048939.1	F: CTGAGCCTGACCGCTATGAG	503bp
		R: CCGCTTCCTACGATCTTCCC	
GSTs	LC071505.1	F: CCGCTTCCTACGATCTTCCC	541bp

PCR primers were synthesized by Sangon Biotech (Shanghai, China). The PCR products of 503 bp (*CYP1A*) and 541 bp (*GST*) were both sequenced to verify the primer specificity. RT-qPCR conditions were as follows: 94°C for 5 min, followed by 35 cycles of 94°C for 5 s, 55°C for 30 s, and 72°C for 40 s. The relative quantification of *CYP1A* mRNA expression was performed with the cycle threshold (Ct) measured with the $2^{-\Delta\Delta}$ Ct method (Du et al., 2002). After the PCR reaction was completed, 5 μL of PCR products were taken for 1.2% agarose gel electrophoresis (180V, 20–30 min) to observe the gel image. The pictures were taken under UV light for data analysis.

2.6. EROD and GST activities

The total protein content was determined with the method of Bradford protein assay (Abel and Lajtha, 1987). EROD activity was determined with the EROD Synthesis Kit (Jianglai, China) according to the manufacturer's protocol. The samples of the brain and liver were homogenized in an ice-cold homogenization buffer (0.125 M $Na_2HPO_4 \cdot 12H_2O$, 0.125 M KH_2PO_4, 0.05 mM Na_2EDTA, pH = 7.7) and the supernatant was prepared by centrifugation (10,000 rpm, 10 min). The sample mixture (50 μL) was firstly placed in an enzyme-labeled plate and then 100 μL of HRP-labeled enzyme-labeled antibody was added for the reaction at 37°C for 10 min. After the supernatant was poured, the PBS solution was added and products were washed for five times. Reactions were stopped by adding 50 μL of 2 M H_2SO_4 into each tube and the supernatant was determined at OD_{450}. The GST activities were determined with GST Synthesis Kit (Jianglaibio, China) according to the manufacturer's protocol. Other steps were similar to those of the EROD kit.

2.7. Statistical analysis

In the statistical analysis, the data were expressed as mean value ± standard deviation (SD). In SPSS 20.0 software, one-way ANOVA was carried out. Duncan's method was used to make the multiple comparisons of the means and $p < 0.05$ indicates a significant difference (*). All figures were drawn by Excel. The correlation analysis was performed to evaluate the relationship between EROD activity and *CYP1A* mRNA expression, as well as the correlation between GST activity and *GST* mRNA expression.

The Gel imaging analysis system(GIS)can quantify the optical density of DNA bands into a spectrum, and automatically integrate it to calculate the response value. The size of the response value can indirectly reflect the content of DNA. Therefore, the amount of mRNA expression from DNA can be obtained by the inverse calculation.

Results And Discussion

3.1. Median Lethal Concentration (LC50) and safe concentration of carp

According to the experimental procedure of 2.3, the acute toxicity dose gradient pre-experiment was first carried out, and the lowest and highest doses of carp death at 24h and 96h were recorded: the highest

dose of carp that did not cause death in 96 h was 5 mg/L, the lowest dose of all death in 24 h was 40 mg/L, and when the dose was 20 mg/L, the 96-hour mortality rate of carp was about 90%. At 30 mg/L, all carps died within 96 h (Data not shown). Therefore, the acute toxicity dose gradient exposure is set in the range of 5–25 mg/L.

The lethal concentration of PHE for carp are shown in Table.2. With the increase in the concentration of PHE, the mortality of carp also increased, displaying a significant dose-toxicity effect. Furthermore, the regression equations between the probability unit of carp mortality and the logarithmic concentration of PHE after 24-h (Eq. (3)) and 96-h (Eq. (4)) cultivation were respectively obtained as follows:

$Y = 3.970\ 2X + 0.2275$, $r^2 = 0.9388$ (3)

$Y = 0.479\ 8X + 0.30$, $r^2 = 0.9229$ (4)

(Y: Probability unit of mortality; X: logarithmic concentration)

Table 2
LC50 of *Cyprinus carpio* after 24-h and 96-h exposure to PHE.

Concentration/(mg·L^{-1})	**5.01**	**6.31**	**7.94**	**10.00**	**12.59**	**15.85**	**19.95**	**25.12**
Log concentration	0.7	0.8	0.9	1.0	1.1	1.2	1.3	1.4
24 h mortality rate/%	0	0	20	20	30	60	70	70
Unit of probability	3.04	3.04	4.16	4.16	4.48	5.25	5.52	5.52
96 h mortality rate/%	10	20	20	30	40	70	90	100
Unit of probability	3.72	4.16	4.16	4.48	4.75	5.52	6.28	6.96

The carp's LC50 value of PHE after 24 h and 96 h were respectively determined as 15.926 mg/L (95% confidence interval: 14.675–17.282 mg/L) and 11.198 mg/L (95% confidence interval: 9.950-12.604 mg/L). According to the classification standard for acute toxicity of fish (Sobanska et al., 2018), PHE is highly toxic to carp. According to Eq. (1), the safe concentration of PHE to carp was calculated to be 1.12 mg/L.

3.2. Gene extraction efficiency

DNA amplification samples were subjected to preset denaturation temperature, cycle parameters, and final repair extension on a gradient PCR instrument. The agarose gel electrophoresis result of 5 μL of samples is shown in Fig. S3. The three genes were loaded and electrophoresed simultaneously. From left to right, they were respectively *CYP1A*, *GST*, and *β-ACTIN, a commonly used* internal reference gene. According to the mobility of each DNA and the indication of the marker, the length of DNA fragments in the PCR sample was between 500 bp and 550 bp. Compared with the β-*ACTIN* gene, the *GST* gene fragment was shorter and had a higher electrophoretic mobility, followed by *CYP1A*. The target genes

were specifically amplified and showed good integrity. Therefore, the samples could be used for the semi-quantitative analysis, recovery, and sequencing for further gene comparison.

The sequencing and splicing results of *CYP1A* and *GST* genes and alignment results obtained from the gene bank (NCBI, National Center for Biotechnology Information) are shown in Table 3. The lengths of the two amplified genes (*CYP1A* and *GST*) were respectively 503 bp and 541 bp. Similarly, the amplified gene had a high similarity of greater than 99% to the target gene in the gene bank. Therefore, the entire RNA extraction, reverse transcription and PCR operations could be qualified.

Table 3
Alignment results of sequenced results of amplified target genes[a].

Genes	Gene length/bp	Accession No.[b]	Gene similarity/%
CYP1A	503	AB048939.1	99.7
GST	541	LC071505.1	99.5

a. The splicing results and peak images of the sequencing results are shown in the supplementary information.

b. The serial number is the gene number in GeneBank (NCBI).

3.3. CYP1A mRNA expression and EROD activity after the exposure to PHE

The *CYP1A* mRNA expression in carp tissues are showed in Fig. 1. We examined the effect of 0 mg/L PHE in the brain and liver as negative control. Pearson correlation indicated the significant correlation between *CYP1A* mRNA concentration and time in both brain and liver. *CYP1A* mRNA expression levels in the liver showed a significant increase compared with those in the control group during 25-day exposure to PHE (Fig. 1a) and the induction trend gradually became more significant during the exposure. *CYP1A* mRNA levels were induced significantly by 0.50 mg/L PHE during the 25-day exposure. In the brain, CYP1A mRNA levels were induced compared with those in the control, but the induction effect was not significantly enhanced with the increase in exposure time or doping concentration (Fig. 1b).

To detect whether the change in *CYP1A* mRNA brought about the change of related metabolic characteristic enzymes, the enzyme activity of EROD was tested (Fig. 1.) We examined the effect of 0 mg/L PHE in the brain and liver as negative control. In the liver, the EROD activity did not change significantly in the initial stage, but all experimental groups resulted in a significant EROD induction in the liver in the 15th day and 25th day (Fig. 1c). In the brain, the activity of EROD in the early period of PHE exposure (Day 1, 5, and 15) was significantly induced compared with the control assays, ($P < 0.05$). However, on Day 25, the induction was not significant (Fig. 1d).

The exposure to PHE showed the induction effect on *CYP1A* mRNA expression in the liver and brain tissues of carp in the entire experimental period. After intraperitoneal injection of TCDD in goldfish, it was also found that the expression of *CYP1A* mRNA was induced in various tissues, and the induction effect was most significant in liver tissues (Lu et al., 2013).It was reported that after the exposure of medaka to pentachlorobiphenyl, CYP1B1 and CYP1C1 mRNA expressions were induced due to the acceleration of Phase I metabolic response (Zanette et al., 2009). If a pollutant entered the carp body, the organism could defend itself by stimulating the production of Phase I- and Phase II-related enzymes degrading external compounds. However, if excessive external substances existed, the body defense mechanism became destroyed.

If fish were exposed to PAHs, an aryl hydrocarbon receptor (AhR) was produced (Wang et al., 2017). AhR can be linked with PAHs to display the protein activity. AhR is a ligand-activating factor in the cytoplasm and can affect the changes in the metabolism of foreign substances and even damage enzymes (Duan and Zhao, 2013). (Oliveira et al., 2007) found that intermediate metabolites produced by the degradation of organic pollutants through bio-converting enzymes were often highly active and toxic electrophilic compounds, which could interact with DNA and cause various DNA damages. These effects could be considered as early warning markers of PAHs pollution.

In the brain and liver of carp, the activities of EROD gradually increased during the exposure to PHE. The similar phenomenon was observed in the liver of tilapia (*Oreochromis niloticus*). The short-term exposure to a low concentration of PHE induced EROD activities in the liver of tilapia *(Oreochromis niloticus)*, whereas the long-term exposure to a high concentration of PHE inhibited EROD activities (Wenju et al., 2009). (Mu et al., 2012) also found that several PAHs with lower molecular weights caused the differential expressions of P450 enzymes. It was also reported that a low concentration of PHE activated EROD in young *Sparus aurata*, but inhibited it under high concentration (Correia et al., 2007). In the brain and liver of carp, the activity of EROD showed an obvious correlation with the expression level of CYP1A. Many compounds could induce EROD activity of fishes and the induction of EROD activity might be impeded since chemicals were competitively bound to the structure of AhR or CYP1A (Whyte et al., 2000). The liver is a key site of detoxification and an important target organ of PAHs (Triebskorn et al., 1997). EROD is the important enzyme assisting in the metabolism of toxic compounds. If pollutants enter the body, the organism starts stress reactions. The differential responses of transcription and expression of characteristic genes can be used as early warning parameters to measure the degree of environmental pollution. The change in CYP1A mRNA expression in the liver and brain of carp was the consequence of the stimulation of the signaling pathway. If the concentration of PHE was too high, it might cause damage and inhibit the gene expression and enzyme activity (Nahrgang et al., 2009).

3.4. GST mRNA expression and GST activity after the exposure to PHE

The GST mRNA expressions in carp tissues are shown in Fig. 2. In the liver, the expression of GST mRNA was always induced when carp was exposed to 0.1 mg/L PHE and the induction effect was weakened in

the 25th day. However, the exposure to 0.5 and 1.0 mg /L PHE showed a significant inhibition effect on GST mRNA expression from the first day to the 25th day (Fig. 2a). In the brain, the experimental groups of different concentrations showed the significant induction in the first day and the induction effect was reduced in the 5th day. During the PHE exposure period, the expression of GST mRNA showed a significant inhibitory effect in the 15th day and this inhibition effect was slightly restored in the 25th day (Fig. 2b). Olsvik et al reported the different fluctuation in Atlantic cod exposed to PAHs and found that only the levels of 2 of the 6 GSTs were up-regulated expressed (Olsvik et al., 2010). Costa et al found no significant difference in GST mRNA expression in Nile tilapia exposed to benzo(a) pyrene (Costa et al., 2012).

In order to detect whether the change in GST mRNA level brought about the change in related characteristic metabolic enzymes, the enzyme activity of GST was tested (Fig. 2). In the liver, the GST activities of all experimental groups were significantly stimulated after induction ($P < 0.05$), but the induction effect was gradually weakened (Fig. 2c). There was no obvious change in the GST activity at the beginning of the experiment (from the first day to the 5th day) in the brain. However, during the PHE exposure period, the GST activities in the 15th and 25th days had a significant induction effect ($P < 0.05$) (Fig. 2d). The reports also found that 5 kinds of PAHs induced the GST activity in the carp liver (Lu et al., 2009). (Yin et al., 2007) also reported that the GST activity in the tissues of catfish was significantly increased after the exposure to PHE. (Nahrgang et al., 2009) reported that benzo (a) pyrene induced the GST activity in cod liver. However, Olinga et al. found that the short-term exposure to PHE inhibited GST activity in the kidney of *Liza aurata* (Olinga et al., 2008).

In the study, the expression of GST mRNA in carp liver and brain showed the early induction and subsequent inhibition effects, indicating that after the Phase I metabolic reactions, the biological activity of GST in the tissues of carp gradually became unbalanced during the exposure. After the exposure to 0.1 mg/L PHE, the induced GST mRNA expression in the liver, but the same phenomenon did not occur in the brain. The difference might be interpreted as follows. The radicals produced by Phase I metabolism are metabolized and the liver is a key organ involved in the metabolism and detoxification of substances. GSTs in most eukaryotic species contain multiple gene families, many of which are expressed in multiple cell types and especially in the liver, its expression accounted for 2–4% of total cytosolic enzymes (Schlenk et al., 2008). In the study, GST was significantly induced in the liver when carp were exposed to PHE for only one day may because the GST signaling pathway was highly expressed in the early exposure stage. In the later exposure period, carp were adapted to the PHE concentration, so GST activity increase was not significant. In the brain, GST activity was significantly induced only after the 15th day of PHE exposure probably. The concentration of PHE was lower than the safe concentration, so there was no induction in the early exposure period.

3.5. Correlation analysis

The correlation analysis was carried out to test the expression levels of characteristic enzymes and characteristic mRNA (Fig. 3). The correlation coefficient indicates that EROD activity in the liver showed a positive correlation with CYP1A mRNA level ($r = 0.602$, $P < 0.01$) (Fig. 3a). In the brain, the correlation

between EROD activity and CYP1A mRNA level was less significant than that in the liver (r = 0.508, $P < 0.01$) (Fig. 3b). However, in the liver or brain, the correlation between GST activity and GST mRNA expression was relatively poor (liver, r = 0.395, $P < 0.05$; brain, r = 0.293, $P < 0.05$) (Fig. 3c and 3d).

In the study, the activity of EROD in the liver was more highly correlated with the activity of *CYP1A* mRNA expression. The liver is an important metabolism organ of heterogeneous organisms and blood mediates the relationship between the brain and other target organs and accelerates the reaction between the characteristic liver enzymes and its mRNA expression. Similar experimental results had been reported in previous studies(Costa et al., 2012; Nam et al., 2017). After the brain and muscle were exposed to soluble components of crude oil, the changes in AchE activity were not obvious compared to other organs (Bettim et al., 2016). In addition, the toxicity induced by CYP1A indicated the chemical exposure and preferential effects in various biological tissues (Whyte et al., 2000).

The cytochrome P450 system showed a highly correlation than the GST system in both the liver and brain. The cytochrome P450 enzyme system played an important role in the heterogeneous biotransformation in Phase I metabolism of fish and other aquatic animals. The activity of ethoxysalolin O-deethylase (EROD) and the *CYP1A* mRNA level seemed to be the most sensitive catalytic probe. The induction response of the cytochrome P450 system had been determined in many studies (Goksoyr, 1992; Anna et al., 2008). The GST is a representative enzyme in Phase II metabolism and play an important role of catalyzing or reducing oxidative substances in the metabolic body. In most cases, only the modest changes in total GST activity were reported (Henson and K., 2004). Similar studies reported that GSTα mRNA expression in all tissues showed no significant difference compared with CYP1A and ABC efflux transporters after the exposure to benzo(*a*)pyrene (Costa et al., 2012).

In addition, there was a significant decrease in GST mRNA in the brain after 15 days of PHE exposure. The higher GST activity and the lower GST mRNA expression in the 15th day might be interpreted as follows. From the first day to the 5th day, the activity of GST increased, thus increasing the clearance rate of PHE and reducing the overall mRNA response and GST synthesis. As a result, the mRNA expression level was reduced (Fig. 2). However, the same phenomenon was not observed in the liver, indicating the differences in the heterogeneous metabolism and PHE-induced enzymes between the brain and liver (Nahrgang et al., 2009).

3.6 Mechanism

The possible mechanism of PHE exposed to carp is shown in Fig. 4. When PHE entered the carp body, the characteristic enzymes of the I-phase reaction were activated to remove pollutants, which result in an increase in the expression of CYP1A gene. At the same time, the free radicals produced by the I-phase reaction are transferred to the II-phase reaction and stimulate the expression of GST and *GST* genes. The difference is that since the liver is an important detoxification organ and free radicals were eliminated in time, the expression of *GST* gene showed a trend of increasing first and then decreasing (as shown in Fig. 2,3). Due to the limited ability of GST clearance, the expression and synthesis of *GST* genes were reduced (Figs. 2,3).

Conclusions

This study demonstrated that PHE stimulated the up regulation of *CYP1A* mRNA and EROD activity in both the liver and brain. However, *GST* mRNA and GST expression in the liver was induced firstly and then inhibited and in brain GST induced but GST mRNA was inhibited. Correlation analysis results showed that CYP1A mRNA expression was closely correlated with EROD activity in both liver and brain, but the differential GST mRNA and GST activities weakened the correlation. This experiment illustrated the difference between Phase I metabolism and Phase II metabolism in different organs and characteristic enzyme systems.

Abbreviations

AhR, aromatic hydrocarbon receptors

BSA, Albumin from bovine serum

CYP1A, cytochrome P4501A

DEPC, Diethy pyrocarbonate

EROD, *7-Ethoxy*lesorufin O-*deethylase*

GST, glutathione S-transferase

PAHs, polycyclic aromatic hydrocarbons

PBS, Phosphate buffer solution

PHE, Phenanthrene

POPs, persistent organic pollutants

qRT-PCR, quantitative real-time PCR

Declarations

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Author Statement

Xin Kang had developed the methodology and conducted a research and investigation process. Xin Kang and Xin Cao had prepared the first draft of the article. Yanfeng Lv and Dongpeng Li had carried out experiments and had helped the experiment testing and data collection. Xiaoxiang Zhao and Xinshan Song had made a contribution to conceptualization. Xin Cao and Xi Chen had reviewed and edited the draft. Xiangyu Liu and Chenrong Chen had revised the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Study data summaries are available; raw data availability may be limited by retrieval labor and overhead costs. Data are available by contacting Xiaoxiang Zhao (zxx@dhu.edu.cn).

Compliance with ethical standards

Animal Research (Ethics) The study has been granted ethics committee approval prior to commencing. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Consent to Participate (Ethics)

The study has been granted ethics committee approval prior to commencing. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Figures

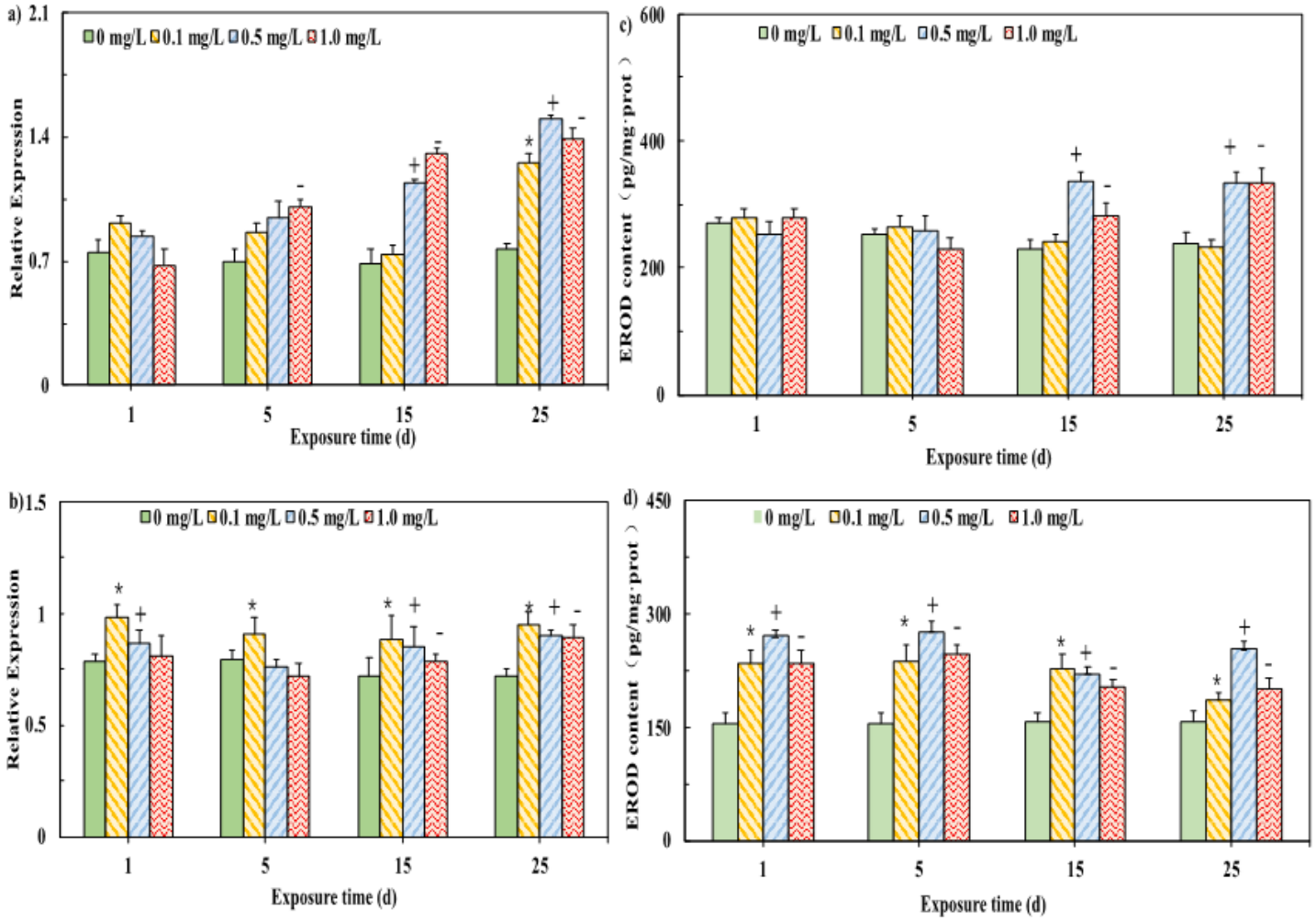

Figure 1

Relative mRNA expression of CYP1A in liver(a) and brain (b) of carp exposed to PHE. Effects of different PHE concentrations on EROD activities in carp (c) in the liver. (d) in the brain (The final expression results are based on the content of characteristic proteins in the mass of each tissue unit of the carp.). Expression was quantified by qRT-PCR. Values are mean ± SD of three replicates. Significant differences, p□0.05 are (*) under 0.1 mg/L versus negative control of 1 d; (+) are under 0.5 mg/L versus negative control of 1 d; (-) are under 1.0 mg/L versus negative control of 1 d, which is based on Duncan's method.

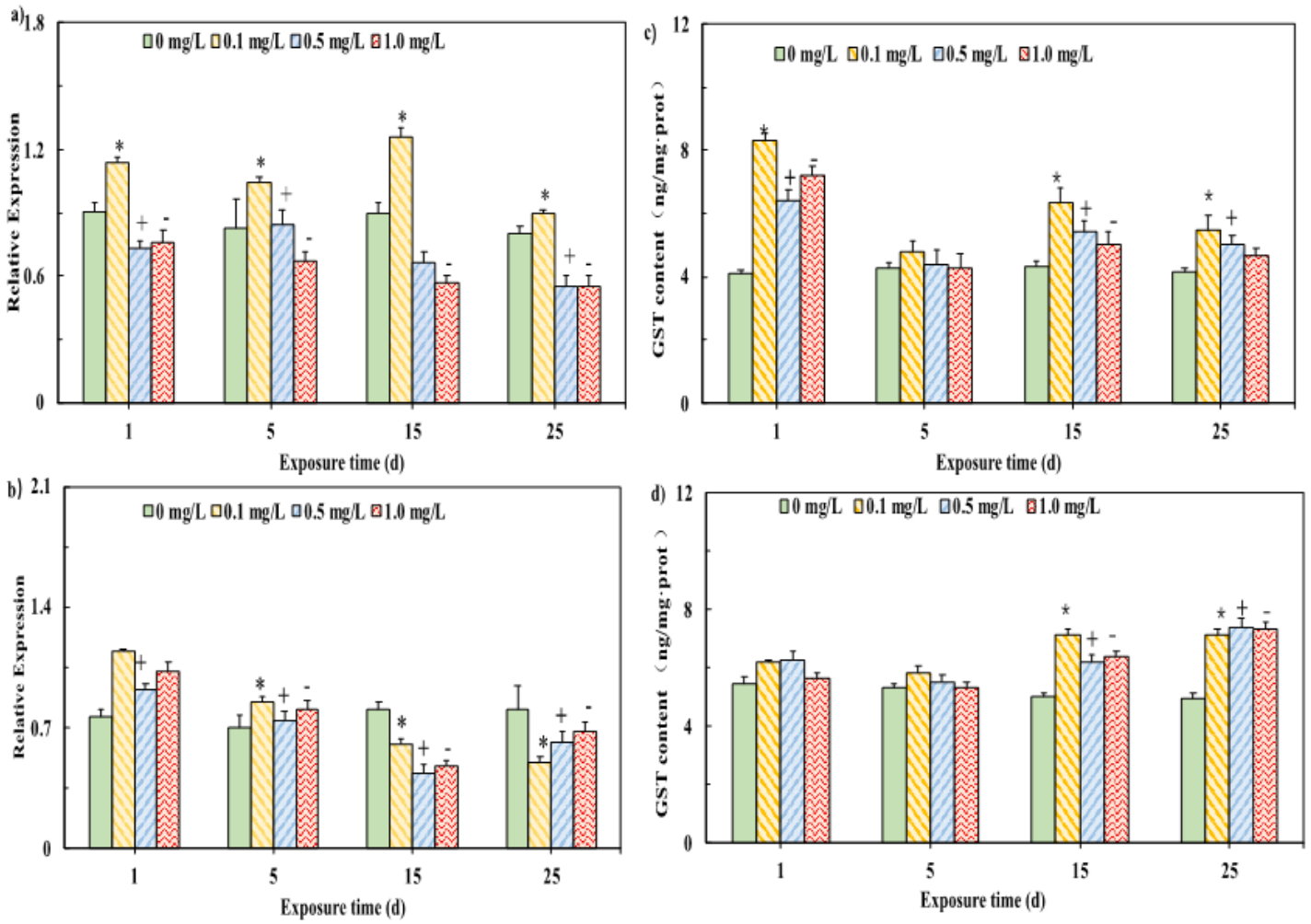

Figure 2

Relative mRNA expression of GST in liver(a) and brain(b) of carp exposed to PHE. Effects of different PHE concentrations on GST activities in the liver (c). and the brains (d) of carp (The final expression results are based on the content of characteristic proteins in the mass of each tissue unit of the carp.) Expression was quantified by qRT-PCR. Values are mean ± SD of three replicates. Significant differences, p□0.05 are (*) under 0.1 mg/L versus negative control of 1 d; (+) are under 0.5 mg/L versus negative control of 1 d; (-) are under 1.0 mg/L versus negative control of 1 d, which is based on Duncan's method.

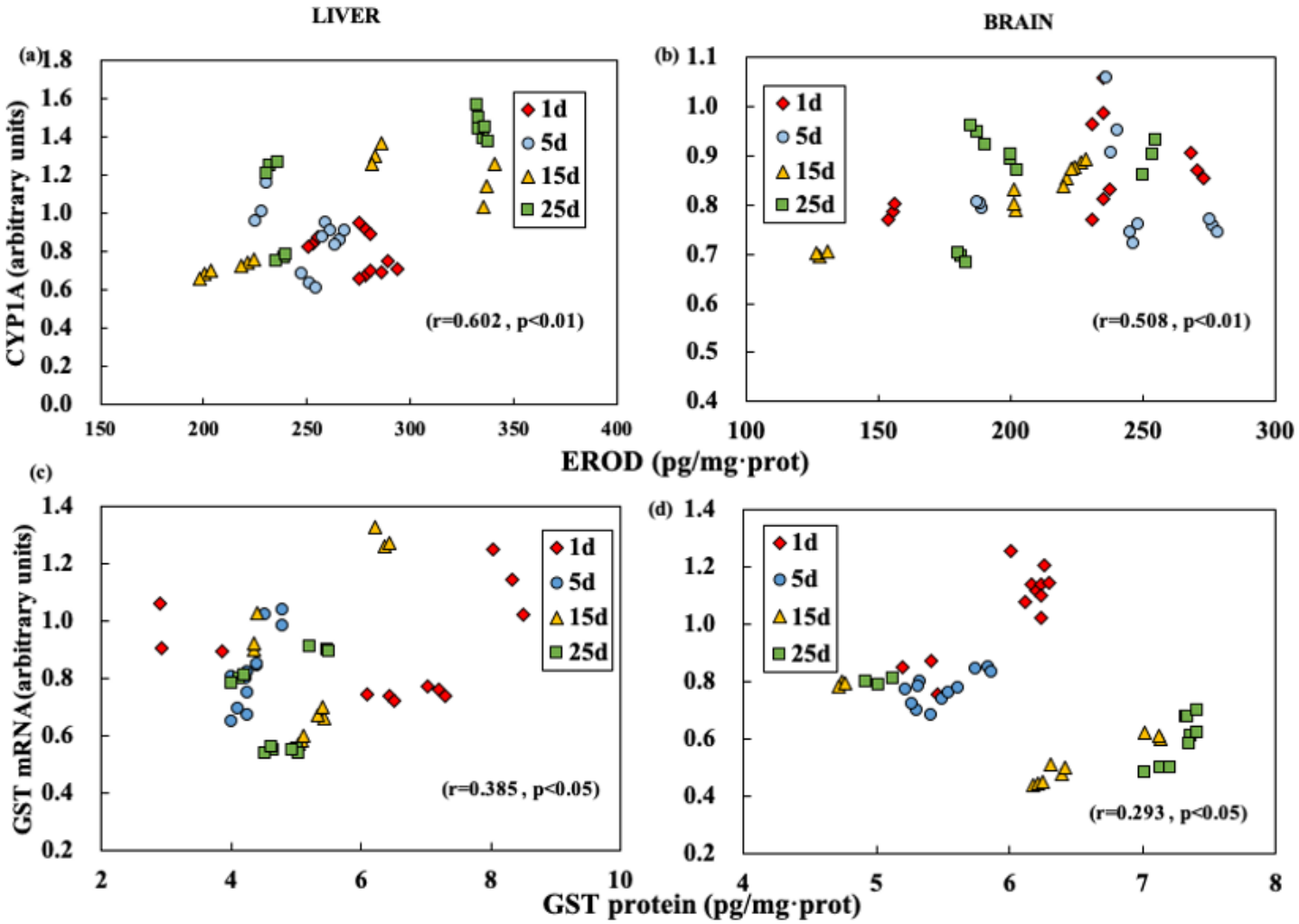

Figure 3

Correlation of EROD and CYP1A mRNA (a, b), GST and GST mRNA (c, d) values in brain (b, d) and liver (a, c) at different exposure time.

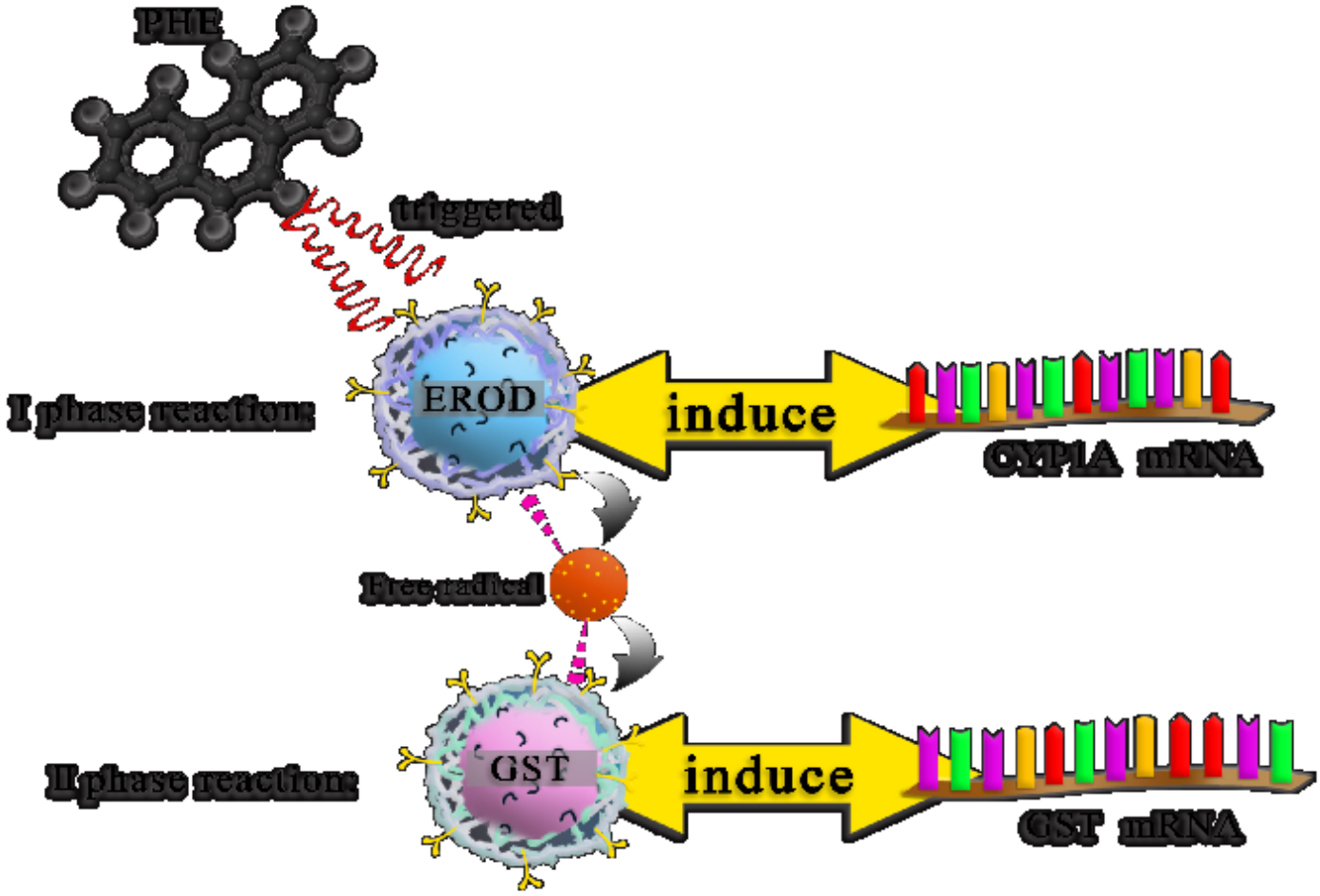

Figure 4

The metabolic mechanism of PHE exposed to carp

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