Effects of diclofenac on the expression of Nrf2 and its downstream target genes in mosquito fish (*Gambusia affinis*)

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ABSTRACT: Diclofenac (DCF) is one of widely used non-steroidal anti-inflammatory drugs. Recently, this drug has been universally detected in aquatic environment. However, its potential adverse effects and oxidative stress toxic mechanisms on fish remain unclear. In the present study, we first cloned the crucial partial sequences of some key oxidative stress related genes, which include NF-E2-related factor 2 (Nrf2), NAD(P)H: quinoneoxidoreductase (NQO1), glutamate-cysteine ligase catalytic subunit (GCLC), Cu-Zn superoxide dismutase (SOD2), catalase (CAT), alpha-glutathione S-transferase (GSTA), and UDP-glucuronosyltransferases (UGT) in mosquito fish (*Gambusia affinis*). We also deduced amino acids of Nrf2 and then constructed the phylogenetic trees of Nrf2, NQO1 and GCLC, respectively. Results showed that a high identity percentage was founded between *G. affinis* and other bony fish species, such as *Xiphophorus maculatus* and *Poecilia reticulata*. The transcriptional expression of these genes and partly related enzymes activities were then investigated under the included environmental relevant concentration DCF exposure (0 μmolL^{-1} , $1.572 \times 10^{-3} \mu\text{molL}^{-1}$, $1.572 \times 10^{-2} \mu\text{molL}^{-1}$, $0.1572 \mu\text{molL}^{-1}$ and $1.572 \mu\text{molL}^{-1}$) for 24 h and 168 h. The expression of *Nrf2* was inhibited at 24 h but induced at 168 h, exhibiting a significant time and/or dose-effect relationship under DCF exposure. Similar observation was found in its downstream target genes. However, Nrf2-mediated antioxidant enzymes activities displayed differently under the same concentration of DCF exposure for the same time. Under DCF exposure for 168 h, the genes exhibited dramatic induction trend, but there were no significant changes in enzyme activities and MDA content. Overall, mRNA responses were more sensitive than enzyme changes in mosquito fish under DCF exposure.

Keywords: Diclofenac; Mosquito fish; Nrf2; Oxidative stress

Highlights:

1. *Nrf2* and *NQO1*, *GCLC*, *GST*, *UGT* and *SOD2* genes in mosquito fish were first cloned;
2. Diclofenac could affect the expression of *Nrf2* and its mediating target genes;
3. Responses of *Nrf2* and its downstream genes are sensitive to diclofenac than their enzyme activity.

Abbreviations: PPCPs: Pharmaceuticals and personal care products; DCF: Diclofenac; tBHQ, tert-butylhydroquinone; Nrf2: NF-E2-related factor 2; NQO-1: NADPH quinine oxidoreductase 1; GCLC: r-glutamylcysteine synthetase catalytic subunit; GSTA: alpha-glutathione S-transferase; UGT: UDP-glucuronosyltransferase; SOD: Cu-Zn superoxide dismutase (SOD2); CAT: catalase;

ROS: reactive oxygen species.

1. Introduction

Pharmaceuticals and personal care products (PPCPs) are widely used not only in human daily life, but also in aquaculture, animal husbandry and other agricultural activities. However, residual PPCPs discharged in environment may pose potential threats to non-target organisms in aquatic ecosystem (Edhlund et al., 2006; Liu et al., 2013). Diclofenac (DCF) as one of typical PPCPs, has being broadly used in form of its sodium and potassium salts owing to its good anti-inflammatory, antipyretic, analgesic and anti-rheumatism effects. Recently, many investigations were reported that DCF could be detected generally in aquatic environment, ranging from ngL^{-1} to μgL^{-1} level (Lonappan et al., 2016; Feito et al., 2012). For example, the concentration of DCF was 22-302 ngL^{-1} in a river in Finland (Brozinski et al., 2003), and detected with the mean concentration of 2.1 ngL^{-1} in the 49 sewage treatment plant effluents in France, Greece, India and Sweden (Ferrari et al., 2003). Studies found that DCF can accumulate in living organisms and cause chronic toxicity for long-term exposure. A typical example was the decrease of vulture population in Pakistan which decimated to nearly extinction owing to the long-term DCF exposure in the environment (Oaks et al., 2004). Some negative effects were also observed in the kidney, liver and gill of rainbow trout under the long-term (28 d) DCF exposure (Triebskorna., 2004). Although many studies about the toxic effects of DCF on organisms were done (Groner et al., 2017; Stepanova et al., 2013; Triebskorna., 2004), the toxic mechanisms of DCF exposure to organisms, especially involved in oxidative stress responses, still need more and further investigations.

Mosquito fish (*Gambusia affinis*) is a kind of small fish and described as the most widespread freshwater fish attributing to its notable adaptability to harsh conditions and its global introduction into many habitats for the mosquito control (Pyke, 2008). Because of its broad distribution and being captured easily in natural aquatic fields and its convenient culture in laboratory condition, mosquito fish may be considered as a candidate sentinel in ecotoxicological research. Studies found that environmental pollutants such as chlorpyrifos may bring about the sensitive change of antioxidant enzymes, acetylcholinesterase and locomotor behavior in mosquito fish, indicating that the species may be regarded as indicator for biomonitoring and risk assessment of environmental pollution (Kavitha et al., 2008).

It is known that most of environmental contaminants adsorbed by aquatic livings could be metabolized by phase I detoxification enzymes (P450 families, CYPs) (Bard et al., 2002; Uno et al., 2012), phase II conjugation enzymes (GST, UGT etc.) (Li et al., 2008) and phase III transportation proteins (ABC families) (Torre et al., 2012; Luckenbach et al., 2014). During the process, some reactive oxygen species (ROS) may produce and possibly damage the biomolecules like DNA, proteins and lipids (Liu et al., 2008). However, antioxidant system in organisms can prevent ROS accumulation to maintain the balance of ROS production and elimination. NF-E2-related factor 2 (Nrf2) is a basic leucine zipper (bZIP) protein that regulates the expression of multiple antioxidant genes including *NQO1*, *GCLC*, *SOD*, *CAT*, *GST* and *UGT* etc, and protects against oxidative damage triggered by contaminants exposure, injury and inflammation (Gold et al., 2012; Osburn and Kensler, 2008). Thus Nrf2 plays a crucial role in the process of dealing with oxidative stress and quenching of reactive oxygen species (ROS) derived from the metabolism of environmental pollutants.

NQO1 is a prototypical protein that catalyzes the reduction and detoxification of highly

reactive quinones that could cause redox cycling and oxidative stress (Venugopal et al., 1996). Glutathione (GSH) is a very powerful important endogenous antioxidant and GCLC is the key enzyme for the rate-limiting step in the synthesis of GSH (Solis et al., 2002). SOD and CAT are mainly responsible for eliminating ROS both derived from endogenous and exogenous sources (Podder et al., 2010) and thus play an important role for the ROS elimination in organisms (Samanta et al., 2014; Wang et al., 2016). Alpha-glutathione S-transferase (GSTA) is one of keystone antioxidant enzymes in organisms, which participates in many important conjugation reactions with intermediate metabolites of many xenobiotics (Armknacht et al., 1998; Chattopadhyay et al., 2011; Corcoran et al., 2015; Ji et al., 2016). UGT is a cytosolic glycosyltransferase that catalyzes the transfer of the glucuronic acid component of UDP-glucuronic acid to a small hydrophobic molecule (King et al., 2000; Hoeger et al., 2008). Many researches showed that the contribution of UGT is determinant in the mechanism of protection against some toxic dietary components, various environmental pollutants (Bartsch et al., 1992; Jin et al., 1993; Court and Greenblatt, 1997; Christen and Fent, 2014).

Previous studies suggested that Nrf2 and its target genes are involved in antioxidant responses under the exposure of environmental contaminants both *in vivo* and *in vitro* (Li et al., 2005; Osburn and Kensler, 2008). However, the role of Nrf2 and its downstream target genes in the detoxification of DCF has not been fully elucidated in fish. In the present study, we selected mosquito fish (*G. affinis*) as the experimental model owing to its representative in local environment, its sensitivity to the exposure of environmental contamination, and its easy culture in laboratory (Huang et al., 2016).

The objectives of our study are (1) to clone the genes related to oxidative stress response including *Nrf2*, *NQO1*, *GCLC*, *SOD2*, *CAT*, *GSTA* and *UGT* in *G. affinis*; (2) to determine the effects of diclofenac on *Nrf2* and its downstream target genes at mRNA transcriptional levels, as well as the activity of the corresponding enzymes such as GST, SOD and CAT activity, GSH and MDA content in mosquito fish; (3) to provide more insights into the mechanism of detoxification in mosquito fish under DCF exposure.

2. Materials and methods

2.1 Chemicals and reagents

DCF sodium salt was obtained from Anpu Co. in Shanghai (Tokyo Chemical Industry LTD, Japan), paracetamol was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany), and tert-Butylhydroquinone (tBHQ) was obtained from Tixiai Co. (Shanghai, China). All other reagents were analytical grade and obtained from commercial sources.

2.2 Sample collection and exposure scenario

The mosquito fish (*G. affinis*) with 3.0 ± 0.35 cm in length and 0.62 ± 0.21 g in mass ($n = 96$) were provided by the Pearl River Fisheries Research Institute in China. Considered the gender difference, only selected female mosquito fish were employed in our experiment. Healthy fish with similar size were selected and maintained in a 100 L tank with dechlorinated fresh water (aerated for 72 h to dechlorinate water, pH 6.69 ± 0.12 , hardness 54 mg L^{-1} , alkalinity 67 mg L^{-1} and total dissolved oxygen of 6.8 mg L^{-1} - 7.5 mg L^{-1}) at 25 ± 3 °C with a natural light/dark cycle of 16/8 h before experiment. Selected fish used for experiment were starved for 3 d before experiment and then were exposed to different concentration of DCF ($1.572 \times 10^{-3} \text{ } \mu\text{mol L}^{-1}$, 1.572

$\times 10^{-2} \mu\text{molL}^{-1}$, $0.1572 \mu\text{molL}^{-1}$ and $1.572 \mu\text{molL}^{-1}$) for 24 and 168 h, respectively. tBHQ ($3.008 \mu\text{molL}^{-1}$) and paracetamol ($6.614 \times 10^{-2} \text{mmolL}^{-1}$) exposure were employed as positive controls in the same conditions for 24 and 168 h. Three tanks with 3 fish per tank were set as parallels for each concentration and the control group without the DCF. Each tank (capacity of 10 L volume) contained a 6 L exposure solution. The exposure was set in the dark room so as to avoid target chemical photo-degradation. According to the previous study (Buser et al., 1998), the degradation of diclofenac in the dark is negligible, therefore a half medium solution was refreshed every 24 h to keep relative constant of DCF concentration during the experimental periods. The fish were not fed without aeration during the periods of experiment. No fish died during the exposure period.

2.3 Primer design

Gene sequences were not available for *Nrf2*, *NQO1*, *GCLC*, *SOD2*, *CAT*, *GSTA* and *UGT* of *G. affinis*, so we designed the PCR primers to obtain the partial sequences of these genes mentioned above with the Primer Premier 5 software according to the conserved regions of the corresponding DNA sequences that identified from NCBI GenBank (<http://www.ncbi.nlm.nih.gov>) and the Ensembl^{ASIA} database (<http://asia.ensembl.org/index.html>) in the following species. The primers designed for *Nrf2*, *NQO1*, *GCLC*, *SOD2*, *CAT*, *GSTA* and *UGT* were listed in Table 1.

The Nrf2 primers were designed by Nrf2 sequences of the following species: HQ916348.1 (*Oncorhynchus mykiss*), XM_005172511.1 (*Danio rerio*) and ENSXMAT00000000447 (*Xiphophorus maculatus*, from the Ensembl^{ASIA} database). The NQO1 primers were designed by NQO1 sequences of the following species: ENSXMAT00000013518 (*Xiphophorus maculatus*, from the Ensembl^{ASIA} database), NM_001204272.1 (*Danio rerio*), NM_001200463.1 (*Ictalurus punctatus*). The GCLC primers were designed by GCLC sequences of the following species: KC540904.1 (*Acanthopagrus schlegelii*), XM_003441123.2 (*Oreochromis niloticus*). The SOD2 primers were designed by SOD2 sequences of the following species: ENSXMAT00000003343 (*Xiphophorus maculatus*, from the Ensembl^{ASIA} database), XM_005802615.1 (*Xiphophorus maculatus*). The CAT primers were designed by CAT sequences of the following species: XM_005815279.1 (*Xiphophorus maculatus*), BT045615.1 (*Salmo salar*). The GSTA primers were designed by GSTA sequences of the following species: ENSXMAT00000010346 (*Xiphophorus maculatus*, from the Ensembl^{ASIA} database), NM_001279635.1 (*Oreochromis niloticus*), NM_001160559.1 (*Oncorhynchus mykiss*), and NM_001141492.2 (*Salmo salar*). The UGT primers were designed by UGT sequences of the following species: XM_003445329.2 (*Oreochromis niloticus*), NM_001200233.1 (*Ictalurus punctatus*), and ENSXMAT00000004986 (*Xiphophorus maculatus*, from the Ensembl^{ASIA} database), ENSXMAT0000002018 (*Xiphophorus maculatus*, from the Ensembl^{ASIA} database).

According to Lee et al (2001) and Court et al (2000), the target genes *Nrf2*, *NQO1*, *GCLC*, *SOD2*, *CAT* and *GSTA* of *G. affinis* were induced under the exposure of $6.016 \times 10^{-2} \text{mmolL}^{-1}$ tBHQ solution for 24 h and the *UGT* gene was induced under the exposure of $6.614 \times 10^{-2} \text{mmolL}^{-1}$ paracetamol solution for 24 h. The partial cDNAs were amplified by PCR (Applied Biosystems, USA) in a 25 μL solution of 17.25 μL ddH₂O, 2.5 μL 10 $\times$ PCR Buffer, 2 μL dNTP Mixture, 1 μL F primer (10 μM), 1 μL R primer (10 μM), 1 μL cDNA and 0.25 μL rTaq DNA polymerase under the following conditions: initial denaturation at 95 $^{\circ}\text{C}$ for 4 min; 35 cycles of denaturation at 94 $^{\circ}\text{C}$ 30 s, primer annealing at 55 $^{\circ}\text{C}$ for 40 s, an elongation at 72 $^{\circ}\text{C}$ 1 min; and 72 $^{\circ}\text{C}$ for 10 min. The cDNA templates were reverse transcribed by RNA isolated from *G. affinis* induced by the tBHQ

and paracetamol. The PCR products were analyzed on 2% agarose gels. The isolated gels were purified with a Gel Extraction Kit (Omega, USA), ligated with (Takara, Japan), and transformed into DH5 α competent cells (Cwbiotech, China). Sequencing was performed by BGI (Shenzhen, China) and the identification of the target genes was confirmed using the NCBI Blast program.

2.4 Total RNA isolation and cDNA synthesis

Livers were collected from *G. affinis* (n = 9 for each concentration) and immediately stored at -80°C . Total RNA was isolated from 50 to 100 mg frozen liver with Trizol reagent (Invitrogen, USA), following the manufacturer's instructions. Total RNA doses and purity were detected on a Thermo Scientific Nano Drop 2000 (USA) and stored at -80°C until further processing. Reverse transcription was performed by using a PrimeScriptTM RT reagent Kit (Takara, Japan), according to the manufacture's protocol.

2.5 Sequence alignments and phylogenetic analysis

The sequences were aligned based on the Blast Program of the NCBI database (<http://blast.ncbi.nlm.nih.gov>). Deduction of the sequences of these genes and comparison analysis of putative heme-binding domain and open reading frame (ORF) were performed by Vector NTI Advance 11.5.1 software. The phylogenetic trees of Nrf2 in mosquito fish were analyzed by the MEGA5.05 software according to the amino acid sequence.

2.6 Quantitative real-time PCR (qPCR)

Primers designed for targeted genes qRT-PCR and relevant different annealing temperatures were listed in Table 2. β -actin (GenBank accession no.AB182330.1) was selected as an internal standard for its stability. First-strand cDNAs were reverse transcribed from the total RNA isolated from the fish following DCF exposure by the PrimeScriptTM RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, Japan).

Expression of *Nrf2*, *NQO1*, *GCLC*, *SOD2*, *CAT*, *GSTA* and *UGT* of *G. affinis* was quantified by RT-PCR on the Bio-Rad CFX Manager by SYBR Premix Ex TaqTM (Takara, Japan) under the following conditions: 95°C 30 s; 45 cycle of 95°C 5 s, and 60°C 30 s. At the end of each run a melting curve analysis was done (plate read every 0.5°C from 55°C to 95°C) to determine the formation of the specific products. Reactions were conducted in a 20 μL volume containing 10 μL SYBR Premix Mix Taq (2 $\times$), 0.8 μL primer (10 μM) each, 0.4 ROX, and 2 μL cDNA, and brought to required volume with ultrapure ddH₂O. Samples were run in duplicate. To determine the threshold cycle (CT), we initially calculated the slopes and regression curves of the standard curves to determine the efficiency of the PCR reactions at least with 7 dilutions of the template (10^9 – 10^3 copies/ μL). The PCR efficiency (E) was calculated according to the equation: $E = 10^{[-1/\text{slope}]}$ – 1. The relative changes in gene expression was analyzed by using the $2^{-\Delta\Delta\text{CT}}$ method, where $\Delta\Delta\text{CT} = (\text{CT}_{\text{T,Target}} - \text{CT}_{\text{T,Actin}})_{\text{sample}} - (\text{CT}_{\text{T,Target}} - \text{CT}_{\text{T,Actin}})_{\text{control}}$ (Pfaffl., 2001).

2.7 Measurement of enzyme activity and other content indicators

Liver samples were homogenized in 4 volumes buffer (0.25 M sucrose, 0.1 M Tris-HCl, 1 mM EDTA, pH 7.4) and centrifuged at $10,000\times g$ for 20 min at 4°C . Methods of enzyme activity and other biomarkers were referred to the instruction of kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China): Numbers of CAT, SOD, GST enzyme activity kits are A007-1, A001-1 and A004, respectively and numbers of GSH and MDA content kits are A006-1

and A003-1, respectively. Activity was expressed in international units per mg of protein. Protein levels were estimated spectrophotometrically by the method of Bradford (1976) using bovine serum albumin as a standard.

2.8 Statistical analysis

Statistical analysis was performed with SPSS Statistics 21.0 software (SPSS Inc., USA). Differences between genes expression and enzymes activity in different treatments (different concentrations and different exposure times) were evaluated by one-way ANOVA followed by Dunnett's t-test (2-sides). Enzyme activity was evaluated by LSD post hoc tests at a 5% significance level.

3. Results

3.1. Nucleotide sequence and phylogenetic analysis of *Nrf2*

A length of 803 bp *Nrf2* cDNA in mosquito fish with an open reading frame (ORF) of 758 bp encoding for 252 amino acids (a predicted molecular weight of 28, 757 kDa and a theoretical pI of 4.54) were obtained (Fig. 1). To access the phylogenetic relationships between mosquito fish and other different species, a phylogenetic tree was constructed using the MEGA5.05 software according to the amino acid sequences of *Nrf2*, *NQO1* and *GCLC*. Results showed that *Nrf2* of *G. affinis* was more closely related to other fish *Nrf2* genes such as platy fish (*X. maculatus*), rainbow trout (*O. mykiss*) and zebrafish (*D. rerio*); and *NQO1* of *G. affinis* also had the higher similarities with fish species such as platy fish (*X. maculatus*). So did *GCLC* of *G. affinis* compared to the fish species of *Cyprinodontiformes* as showed in the phylogenetic tree (Fig. 2).

The mosquito fish belong to *Cyprinodontiformes poeciliidae* in taxonomy. In accordance with our experimental predicted expectations, the partial sequences of *Nrf2*, *NQO1*, *GCLC*, *SOD2*, *CAT*, *GSTA* and *UGT* in *G. affinis* should be closer to bony fish species in *Cyprinodontiformes poeciliidae* (e.g. *X. maculatus*). As showed in Table 3, the maximum identity of each cloned gene all appeared in *Cyprinodontiformes*, such as *X. maculatus*, *P. formosa* and *O. niloticus*. The maximum identities of these genes are: *Nrf2* compared to *X. maculatus*, up to 96%; *NQO1* compared to *X. maculatus*, up to 98%; *GCLC* compared to *X. maculatus*, up to 97%; *SOD2* compared to *X. maculatus*, up to 96%; *CAT* compared to *P. formosa*, up to 98%; *GSTA* compared to *P. formosa*, up to 96%; *UGT* compared to *O. niloticus*, up to 81%. These results are in agreement with the taxonomic position and predicted expectations of these reported fishes genes.

3.2. Effects of DCF on the expression of *Nrf2*, *NQO1*, *GCLC*, *SOD2*, *CAT*, *GSTA* and *UGT* at transcriptional levels

qPCR was employed with β -actin as the housekeeping gene to assess the effects of DCF on *Nrf2*, *NQO1*, *GCLC*, *SOD2*, *CAT*, *GSTA* and *UGT* expression. The qRT-PCR efficiencies (%) for β -actin, *Nrf2*, *NQO1*, *GCLC*, *SOD2*, *CAT*, *GSTA* and *UGT* were measured at 99.2%, 99.65%, 105.3%, 103.73%, 97.5%, 98.93%, 95.9% and 104.9%, respectively. Based on the work, we analyzed the genes expression changes of tested organism under DCF exposure. Control groups were treated under the same conditions, but without DCF. $CT_{\beta\text{-actin sample}} = 29.6 \pm 0.92$ during the experiment had no significant differences compared to $CT_{\beta\text{-actin control}}$. Thus, β -actin could be used as the housekeeping gene.

Results showed that *Nrf2*, *NQO1*, *CAT* and *GSTA* genes exhibited inhibition trends after 24

h DCF exposure in most treatments (Fig.3 and Fig.4), and *GCLC* and *SOD2* genes showed only inhibition at low concentration group ($1.572 \times 10^{-3} \mu\text{molL}^{-1}$) compared to the control. However, *UGT* had no significant inhibition change at 24 h. But with the prolonged exposure time, all gene expressions up-regulated at 168 h compared to 24 h. *Nrf2* gene expression showed an obvious dose-effect relationship at 168 h, which increased with rising concentrations except at the highest concentration. Moreover, *NQO1*, *GCLC*, *CAT* and *GSTA* at transcriptional level had similar time-effect relationships. As the positive controls, tBHQ ($3.008 \mu\text{molL}^{-1}$) and paracetamol ($6.614 \times 10^{-2} \text{mmolL}^{-1}$) exposure did obviously increase the transcript levels of the tested genes both for 24 h and 168 h (Fig. 3 and Fig.4), which were in agreement with expectations.

3.3. Response of the enzyme activity of CAT, GST, SOD and change of GSH and MDA content to DCF exposure

The enzymes activities (GST, SOD and CAT) as well as the changes of GSH and MDA content were assessed following DCF exposure, the control groups were treated the same as the condition mentioned above. Results showed that GST enzyme activities displayed a slight inhibition at 24 h, $1.572 \mu\text{molL}^{-1}$, but had no significant differences compared to the control. No significant change was observed for GST with the prolonged exposure time. SOD exhibited similar change patterns to GST and there was no time-dependent relationship for different treatments. However, CAT showed a trend of inhibition with the extension of exposure time, displaying significant decreasing at 168 h compared to that at 24 h (Fig.5). GSH significantly decreased at $1.572 \times 10^{-3} \mu\text{molL}^{-1}$, $1.572 \times 10^{-2} \mu\text{molL}^{-1}$ and $0.1572 \mu\text{molL}^{-1}$, but had a significant rising at $1.572 \mu\text{molL}^{-1}$ at 24 h, and showed significantly decreasing at lower concentrations ($1.572 \times 10^{-3} \mu\text{molL}^{-1}$ and $1.572 \times 10^{-2} \mu\text{molL}^{-1}$) but significant increasing at higher concentrations ($0.1572 \mu\text{molL}^{-1}$ and $1.572 \mu\text{molL}^{-1}$) at 168 h. MDA content, as a typical oxidative product, was significantly increased at $1.572 \times 10^{-3} \mu\text{molL}^{-1}$ and $0.1572 \mu\text{molL}^{-1}$ at 24 h compared to the control, but with time extend, MDA content became lower, there was no difference in comparison with the control at 168 h (Fig.5). Under the exposure of tBHQ ($3.008 \mu\text{molL}^{-1}$) for 24 h, SOD, CAT, GSH and MDA except GST increased significantly compared to the control, but under tBHQ exposure for 168 h, just CAT and GSH displayed increasing in comparison with the control (Fig.5).

4. Discussion

4.1 Sequences analysis of *Nrf2*, *NQO1*, *GCLC*, *SOD2*, *CAT*, *GSTA* and *UGT* in *G. affinis* and their taxonomy comparison

In this study, we focused on investigation of the gene expressions and the enzyme responses of the key transcriptional factor *Nrf2* and its downstream target genes in mosquito fish exposed to different concentrations of DCF. The sequences of *Nrf2*, *NQO1*, *GCLC*, *SOD2*, *CAT*, *GSTA* and *UGT* in *G. affinis* have not been previously identified (<http://www.ncbi.nlm.nih.gov/nuccore>). Therefore, we firstly cloned the partial sequences of these genes in *G. affinis*, and thus prepared for next steps of investigating the detoxification mechanisms of mosquito fish under DCF exposure.

Results revealed a high conservation of these genes across most species according to the identity percentage of the deduced amino acid sequences, especially in bony fish. Phylogenetic analysis also showed that *Nrf2*, *NQO1* and *GCLC* in *G. affinis* were more closely related to the fishes of *Cyprinodontiformes poeciliidae* in taxonomy, such as *X. maculatus*, *P. formosa* and *O.*

niloticus than other mammals, like frogs and birds (Fig. 1 and Fig.2). Furthermore, BLAST results indicated that the key sequences of *Nrf2*, *NQO1*, *GCLC*, *SOD2*, *CAT*, *GSTA* and *UGT* in *G. affinis* have high identities with *Poeciliidae* fish species, which was in agreement with the taxonomic position (Table.3). This is first report for the key sequences of *Nrf2*, *NQO1*, *GCLC*, *SOD2*, *CAT*, *GSTA* and *UGT* in *G. affinis*. The present works will be benefit for the utilization of the global distribution small fish species as an ecotoxicological candidate indicator.

4.2 Transcriptional changes of *Nrf2*, *NQO1*, *GCLC*, *GSTA*, *UGT*, *CAT* and *SOD2* in *G. affinis* to DCF exposure

In the present study, *Nrf2*, *NQO1*, *GCLC* and *GSTA* gene expressions showed similar time and /or dose-effect patterns (Fig.3 and Fig.4), which were in agreement with expectations of *NQO1*, *GCLC* and *GSTA* as the target genes of *Nrf2*. Several researches found the expressions of *NQO1*, *GCLC* and *GSTA* were up-regulated in a *Nrf2*-dependent manner (Solis et al., 2002; Nioi et al., 2003). At 24 h, *Nrf2*, *NQO1* and *GCLC* gene expressions were repressed at lower concentrations, similar observation was also showed in Deng's report (Deng et al, 2016). We speculated the uptake of DCF during initial stage of exposure was not enough to trigger the accumulation of reactive oxygen species (ROS) in fish and subsequently induced transcriptional expression of antioxidant-related genes.

However, with the prolonged exposure time, *Nrf2*, *NQO1*, *GCLC* and *GSTA* genes expressions exhibited obvious up-towards trends in all treatments at 168 h (Fig.3 and Fig.4). Perhaps it might be attributed to the chronic toxicity effect of DCF. Because mosquito fish exposed to DCF for a week could largely absorb and accumulate the drug which may cause the accumulation of ROS to initiate antioxidant system, thus exhibiting up-regulation of *Nrf2* and its downstream genes. Toufexi et al. (2016) reported that DCF may produce $\bullet\text{O}_2^-$ and NO free radical species via NADPH oxidase and NO synthetase. Similar performance was reported in previous studies of *NQO1* under the exposure of pollutants (Zhu et al., 2005; Thummabancha et al., 2016).

SOD2 and *CAT* are known to belong to antioxidant enzymes. Superoxide radical anion (O_2^-), as a minor product of mitochondrial respiration (Oost et al., 2003) and/or deriving from the metabolism of contaminants, is transformed by *SOD* to hydrogen peroxide (H_2O_2), which was continuously captured and metabolized by *CAT* via dismutation of H_2O_2 to H_2O (Barata et al., 2005). Both coordination between *SOD* and *CAT* was commonly reported. As showed in Fig.4, mRNA changes of *SOD2* and *CAT* displayed the opposite trend at 24 h. *SOD2* increased with rising concentrations. However, *CAT* gene expressions inhibited at higher concentrations, which was similar to the *SOD* and *CAT* profiles in the gill tissues of crucian carp exposed in hexavalent chromium (Li et al., 2013). We inferred that the opposite performance in *SOD2* and *CAT* mRNA at 24 h might be associated with their similar roles in antioxidant system, in which there is a mutual complementation between them. But with the extend of exposure time, *SOD2*, *CAT* all had dramatic inductions at 168 h, as mentioned above, DCF exposure for a prolonged time may cause the accumulation of ROS in mosquito fish, and thus required more higher response in antioxidant system (Zhu et al., 2005; Thummabancha et al., 2016).

No significant change was observed for *UGTs* at 24 h under DCF exposure. Kropf et al also found the mRNA expression of *UGTs* had changes but no significant differences in early life stages of rainbow trout (Kropf et al., 2016), similar to the *UGT* performance in the present study. But with the prolonged exposure time, *UGT* gene expression exhibited inverted “U”-shape change

pattern with rising concentrations at 168 h, and similar changes was observed in the livers of Nile tilapia (Thummabancha et al., 2016). There were many reports of genes expression performance as U-shape, displaying the induction at lower concentrations and inhibition at higher concentrations (Ma et al., 2012; Brox et al., 2016). This phenomenon might be attributed to the activation of the detoxification system at lower concentrations exposure, but under higher concentrations excess and accumulation of ROS (H_2O_2) that blocked the transcriptional expressions, exhibiting a trend of inhibition (Solis et al., 2002; Nioi et al., 2003; Deng et al., 2016).

4.3 Enzyme response of GST, SOD and CAT, the change of GSH and MDA content in G. affinis to DCF exposure

Oxidative stress occurs when the balance between ROS and antioxidant system is disrupted. Thus, prooxidant/antioxidant balance and ROS scavenging are essential for living organisms to maintain cellular homeostasis (Valavanidis et al., 2006). Antioxidant enzymes such as GST, SOD, CAT and some reductive small molecule like GSH can protect organisms against ROS-mediated oxidative stress and damage (Soldatov et al., 2007). It was reported that DCF affected mitochondrion and consequently oxidative phosphorylation, thus potentially increasing ROS production, in particular the O^{2-} anion (Asensio et al., 2007; Islas-Flores et al., 2013). Felice et al. (2011) demonstrated that DCF-induced toxicity was involved not only in mitochondrial dysfunction and apoptotic pathway but also oxidative stresses.

In this experiment, no significant concentration-dependent effects (except for CAT at 168 h) was found for GST, CAT and SOD under DCF exposure as reported previously in many studies (Wang et al., 2002; Soldatov et al., 2007), which was obviously not in line with their roles in antioxidant system. Many studies suggested that DCF may be hydroxylated through the P450 complex to 4'-hydroxy DCF and 5'-hydroxy DCF which are oxidized to benzoquinonimine intermediates (Grillo et al., 2003). This DCF electrophilic reactive metabolites are responsible for diverse ROS such as the superoxide anion radical (O^{2-}) and hydroxyl radical ($OH\bullet$) formation (Boelsterli., 2003; Stülten et al., 2008; Pourahmad et al., 2011; Islas-Flores et al., 2013). But we do not found significant responses of antioxidant enzymes such as GST, SOD and CAT as predicted to the potential oxidative stress derived from DCF exposure.

It is interesting for the performance of GST and GSH at 24 h. GST was induced (except $1.572 \mu\text{molL}^{-1}$) at primary stage of exposure in spite of no significant difference compared to the control, concomitantly, GSH content decreased significantly at 24 h (except $1.572 \mu\text{molL}^{-1}$). But with the prolonged exposure time, GSH content decreased at lower concentration and increased at high concentration DCF exposure (168 h), meanwhile, GST enzyme activity became a little lower, but without any significant differences in comparison with the control. This might be related to DCF exposure time, we speculated that for a short time exposure it may be associated with the consumption of GSH pool and for the long time exposure it may be involved in the de novo synthesis of GSH. As known to all, GSH is a reductive "pool" to GSTs, thus GSTs activity may be restrained ultimately with GSH content (Boyer., 1989; Mukanganyama et al., 2011). Similar reports were found in many researches in which it was explained that the detoxification system may cope with the insults of xenobiotics via the existing reductive pool at low concentrations, but with rising concentrations, the accumulation of ROS ultimately blocked the responses of antioxidant system at the higher concentration (Ma et al., 2012; Deng et al., 2016).

MDA, as a typical oxidative product, its content significantly increased at 1.572×10^{-3}

μmolL^{-1} and $0.1572 \mu\text{molL}^{-1}$, corresponding to the decrease of GSH to some extent at 24 h. But with prolonged time, although MDA content became lower, but no significant differences compared to control at 168 h, suggesting weak oxidative damage to mosquito fish in the present investigation. Similar reports were recorded that DCF displayed relatively lower toxicity to fish species (Gröner et al., 2015; Lubiana et al., 2016). This could be related to the DCF exposure concentration and period, obvious damage of DCF to animals usually occurred at a very high concentration and more long-term exposure (Yang et al., 2011; Stepanova et al., 2013; Tribskorna., 2004; Ji et al., 2006) and this need further investigation for a more longer exposure.

4.4 Relationships between the mRNA expression, enzyme activities and other oxidative stress indicators in G. affinis to DCF exposure

As we known, the regulations of genes are usual dependent-time and dose patterns. The slight inhibition in short-term exposure and great induction in long-term exposure for genes associated with stress responses are typical in organisms exposed to contaminants. In the present investigation, most genes were inhibited to some extent at 24 h to DCF exposure but significantly induced at 168 h, displaying an obvious time-effect. Possible owing to its higher lipophilicity with respect to other NSAIDs, DCF may be slowly absorbed into mosquito fish at initial stage of exposure, with the extension of exposure period and increasing concentration, bioaccumulation DCF increases and thus consequently toxicity effects occurs gradually. Similar changes were reported in rainbow trout (Cuklev et al., 2011).

The overall transcriptional change of *Nrf2* and its downstream target genes in mosquito fish were more obvious than the change of enzyme activities and other indicators under the DCF exposure. For example, *CAT* and *SOD2* at mRNA level sharply induced at 168 h, but there was no significant change for their corresponding enzyme activity at 168 h. The same is also true for GST, in which *GSTA* mRNA expression displayed significant up-regulation at 168 h, but the corresponding GST activity had no significant differences. GSH just kept the consistence at higher concentration with the *GCLC* mRNA expression but not at lower concentration. It was explained that the enzyme activity and the mRNA expression actually reflected the different scales of detoxifying responses under xenobiotics exposure *in vivo*. Because transcriptional expression was usually instantaneous *in vivo*, and as a kind of transient changes, was acuter and easier to be observed. The responses of detoxification-related enzymes and proteins may be relatively lagging compared with transcriptional changes. Furthermore, there are multiple signaling pathways in the regulation of detoxification-related metabolism *in vivo*, and the differences between the mRNA expressions and enzyme activities may be also related to the post-transcriptional mechanisms such as microRNA regulation (Tang et al., 2013; Yu et al., 2012; Duru et al, 2015). Moreover, other post-translational modifications (e.g. methylation, acetylation, ubiquitylation etc.) may also affect these detoxification and antioxidant enzymes (Rhee et al., 2005; Nioi et al., 2007; Regoli et al., 2011). Ultimately, there might be less dramatic changes in enzyme activity level through a variety of complex regulatory actions (Li et al., 2013).

In summary, this is the first study to simultaneously examine the mRNA expression of *Nrf2* and its downstream related genes *NQO1*, *GCLC*, *GSTA*, *CAT*, *SOD2* and *UGT* and the changes of CAT, SOD, GST activity, reductive small molecule GSH and peroxidative byproduct MDA content in the liver of mosquito fish under DCF exposure. Overall, the results of the present study demonstrated that DCF may exert effects on the transcriptional expression involved in antioxidant

defenses system, depending on the exposure concentration and time. The low certain degree of coherency between mRNA and enzyme changes to DCF exposure reflected the complexity of detoxification metabolism regulations in organisms to deal with PPCPs exposure. Particularly the changes of the key transcriptional factor Nrf2 and its downstream target genes may have multiple regulation signal pathways. More research will be required on the understanding of exact detoxification mechanisms of DCF, especially in post-transcriptional regulation level.

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Capitals and Legends:

Figure 1. Opening reading frame and deduced amino acid sequence of the partial *Nrf2* sequences from *Gambusia affinis*

Figure 2. Phylogenic trees of *Nrf2*, *NQO1* and *GCLC* in *Gambusia affinis* were conducted by using the MEGA5.05 software according to the amino acid sequences of *Nrf2*, *NQO1* and *GCLC*, respectively.

A: *Nrf2*:

The GenBank accession numbers of the sequences used in the figure are as follows: *Oncorhynchus mykiss* (HQ916348.1); *Xiphophorus maculatus* (XM_005806271.2); *Homo sapiens* (NM_001145413.3); *Mus musculus* (NM_010902.4); *Danio rerio* (XM_005172511.2); *Bos taurus* (NM_001011678.2); *Xenopus tropicalis* (NM_001007489.2); *Chrysemys picta* (XM_005300456.2); *Oryzias latipes* (XM_011489925.1); *Poecilia latipinna* (XM_015051945.1); *Poecilia mexicana* (XM_015003229.1); *Oryctolagus cuniculus* (NM_001082067.2); *Nothobranchius furzeri* (XM_015966274.1); *Physeter catodon* (XM_007131156.1).

B: *NQO1*:

The GenBank accession numbers of the sequences used in the figure are as follows: *Xiphophorus maculatus* (ENSXMAT00000013518, from the EnsemblASIA database); *Danio rerio* (BC065622.1); *Gallus gallus* (NM_001277619.1); *Homo sapiens* (NM_000903.2); *Ictalurus punctatus* (GU588627.1); *Macaca mulatta* (NM_001260998.1); *Mus musculus* (NM_008706.5); *Oryzias latipes* (AF128816.1); *Canis lupus* (XM_848524.4); *Chelonia mydas* (XM_007062453.1); *Cynoglossus semilaevis* (XM_008310231.1); *Equus caballus* (XM_005608375.2); *Lepisosteus oculatus* (XM_015368092.1); *Takifugu rubripes* (XM_003969730.2); *Tursiops truncatus* (XM_004312066.1); *Xenopus tropicalis* (NM_001016468.2).

C: *GCLC*:

The GenBank accession numbers of the sequences used in the figure are as follows: *Acanthopagrus schlegelii* (KC540904.1); *Oreochromis niloticus* (XM_003441123.2); *Homo sapiens* (NM_001197115.1); *Mus musculus* (NM_010295.2); *Drosophila melanogaster* (NM_001298072.1); *Danio rerio* (NM_199277.2); *Xenopus tropicalis* (NM_001079335.1); *Gallus gallus* (XM_419910.4); *Bos taurus* (NM_001083674.1); *Nothobranchius furzeri* (XM_015976378.1); *Poecilia latipinna* (XM_015060264.1); *Poecilia mexicana* (XM_014981845.1); *Poecilia reticulata* (XM_008429790.1); *Poecilia formosa* (XM_007546141.2); *Xiphophorus maculatus* (XM_005811878.2); *Oreochromis niloticus* (XM_003441123.3).

Figure 3. Transcriptional expression of *Nrf2*, *NQO1*, *GCLC* and *UGT* in *Gambusia affinis* under diclofenac exposure for different time. Samples were collected under the exposure of diclofenac sodium salt ($1.572 \times 10^{-3} \mu\text{molL}^{-1}$, $1.572 \times 10^{-2} \mu\text{molL}^{-1}$, $0.1572 \mu\text{molL}^{-1}$ and $1.572 \mu\text{molL}^{-1}$) and tBHQ ($3.008 \mu\text{molL}^{-1}$) and paracetamol ($6.614 \times 10^{-2} \text{mmolL}^{-1}$) as positive control for different periods (24 h, 168 h). The mean values of gene expression were compared to the control. All tests were performed in duplicate, and data were shown as the means $\pm$ SD (n=9). Statistical analysis was carried out using one-way ANOVA followed by Dunnett's post hoc test. Asterisks (* $p < 0.05$, ** $p < 0.01$) indicated that values were significantly different from the control.

Figure 4. Transcriptional expression of *SOD2*, *CAT*, *GSTA* in *Gambusia affinis* under diclofenac exposure for different time. Samples were collected under the exposure of diclofenac sodium salt ($1.572 \times 10^{-3} \mu\text{molL}^{-1}$, $1.572 \times 10^{-2} \mu\text{molL}^{-1}$, $0.1572 \mu\text{molL}^{-1}$ and $1.572 \mu\text{molL}^{-1}$) and tBHQ ($3.008 \mu\text{molL}^{-1}$) and paracetamol ($6.614 \times 10^{-2} \text{mmolL}^{-1}$) as positive control for different periods (24 h, 168 h). The mean values of gene expression were compared to the control. All tests were performed in duplicate, and data were shown as the means $\pm$ SD (n=9). Statistical analysis was carried out using one-way ANOVA followed by Dunnett's post hoc test. Asterisks (* $p < 0.05$, ** $p < 0.01$) indicated that values were significantly different from the control.

Figure 5. Change of CAT, GST and SOD activities and content of GSH and MDA in *Gambusia affinis* under the exposure of diclofenac for different times. Samples were collected under the exposure of DCF ($1.572 \times 10^{-3} \mu\text{molL}^{-1}$, $1.572 \times 10^{-2} \mu\text{molL}^{-1}$, $0.1572 \mu\text{molL}^{-1}$ and $1.572 \mu\text{molL}^{-1}$) and tBHQ ($3.008 \mu\text{molL}^{-1}$) and paracetamol ($6.614 \times 10^{-2} \text{mmolL}^{-1}$) for different periods (24 h and 168 h). The mean values of gene expression fold were compared to the control. All tests were performed in duplicate and data were shown as the means $\pm$ SD (n = 9). Statistical analysis was carried out using one-way ANOVA followed by LSD post hoc test. Asterisks (* $p < 0.05$, ** $p < 0.01$) means that the values were significantly different from the control.

Fig. 1.

	10	20	30	40	50	60
1	CCGCGTACGGGCGGAGCTTGAGGAGGAGAAGAGCTGCACTTGATGCAGGAGCAGGAGAAG					
	70	80	90	100	110	120
61	GCCCTGCTGGCGCAGCTGCAGCTGGATGAGGAAACGGGAGAGTACATACCCCGCCCGCCT					
21	L	L	A	Q	L	Q
	130	140	150	160	170	180
121	CCCAGCGCCCCCTCTGCAGCCGACCATCACACCCCTGGAGGTTACTCAGAATGCCGGCTTC					
41	P	S	A	P	L	Q
	190	200	210	220	230	240
181	ACACAGGAGACAGGTGACAGCATGTCAATTTGATGAGTGTTCAGCTCCTGGCAGAGACG					
61	T	Q	E	T	G	D
	250	260	270	280	290	300
241	TTTCCCATAGAGCAAACCTCCGACTCCCCCGGTTTGCTGGATGCACCTGCTGTTTCAGTA					
81	F	P	I	E	Q	T
	310	320	330	340	350	360
301	CCCATGATGATGTCCCCAAAGCAGCCAGCTCTGTGCGCCAGCCACCCTGTCTCAGGTCAG					
101	P	M	M	M	S	P
	370	380	390	400	410	420
361	CCGACTCCGCCACAGAGAATGTCTCCCGATCTGGAGGAGGCTTGGATGGAGCTCTTGTCA					
121	P	T	P	P	Q	R
	430	440	450	460	470	480
421	CTCCCTGAGCTACAGCAATGTCTGAACATGCAAATGGAAGACAGTTGCAGACCACAACA					
141	L	P	E	L	Q	Q
	490	500	510	520	530	540
481	TACCCTCTCCAGCCAATGCTGAAGCACAGACTCCAAGCTACCCTAACTATCCCCCTGTC					
161	Y	P	L	P	A	N
	550	560	570	580	590	600
541	CAAGCTCCTATTTTAAACCCCTAAACAGCATCAACAATGTCAAAACAAACAGTCTGAAT					
181	Q	A	P	I	F	N
	610	620	630	640	650	660
601	GTTGGCCCCGAGAGTTTATGAATACATTGGATGGCTCCATTCCCAGCTTGGCTCCGTCA					
201	V	G	P	A	E	F
	670	680	690	700	710	720
661	AATGCTATTAGACAGATCGAAGTGGACGCCCCAACCTAGATACAAGCTTCAGTGGAGA					
221	N	A	I	Q	T	D
	730	740	750	760	770	780
721	AGGTTTTGGTGGGGTGTTCCTTATCCAACAAGTGGATGAGTGCAGCAGTCAGCACA					
241	R	F	W	W	G	V
	790	800				
781	ACCAAGAAGAAAATGAAAGAATG					
261						

Fig.2.

Fig.3.

Fig.4.

Fig.5.

Table 1 PCR primers designed for targeted genes *Nrf2*, *NQO1*, *GCLC*, *CAT*, *SOD2*, *GSTA* and *UGT* in *Gambusia affinis*.

Primer	Sequence 5'-3'	Designed product length (bp)	Annealing temperature (°C)
PCR Nrf2(F)	ACCACCGCCAGAAAGAG	1338	52.3
PCR Nrf2(R)	ACGGGCAGGTTGATTAT		47.4
PCR GSTA(F)	ACCTGCCAGTATTTGAAAAG	301	47.9
PCR GSTA(R)	TGGAAAGGGTCCTGTGAT		50.5
PCR CAT(F)	TTCGGCTACTTTGAGGTC	979	50.5
PCR CAT(R)	TGTTGGGGTAGTAGTTTGGA		51.7
PCR SOD2(F)	TGGAGAACTACGCAGGTG	957	52.8
PCR SOD2(R)	TGGGTATGAAAAGCAAGAG		42.1
PCR NQO1(F)	ACGGCGATGAGACGATG	353	52.3
PCR NQO1(R)	CTGAAACCCGCAGAAAGT		49.8
PCR GCLC(F)	GCWGCYTCACCCTTCTACA	610	58.1
PCR GCLC(R)	RTAAAACATGCCCTCCWGGAC		51.7
PCR UGT(F)	CTGCTTTAGGGAACAACACTATG	434	54.9
PCR UGT(R)	GTAGGAATACCAGGGCATCTT		54.2

Note: F: forward primer; R: reverse primer.

Table 2 qRT-PCR primers designed for targeted genes *Nrf2*, *NQO1*, *GCLC*, *CAT*, *SOD2*, *GSTA* and *UGT* in *Gambusia affinis*.

Primer	Sequence 5'-3'	GenBank accession no.	Designed product length (bp)	Annealing temperature (°C)
qPCR β -actin(F)	CACCTTCTACAATGAGCTCCGT	AB182330.1	129	41.6
qPCR β -actin(R)	GCAGGAGTGTTGAAGGTCTCAA			52.3
qPCR Nrf2(F)	GACAGATCGAAGTGGACGCC		121	57.9
qPCR Nrf2(R)	TTTCTTCTTGGTTGTGCTGACTG			54.9
qPCR GSTA(F)	CCTGCTGATGGCTGGCTTTA		122	55.8
qPCR GSTA(R)	CAGATGTGCTCCTGGTTGAATG			56.4
qPCR CAT(F)	GCAGCCACCAACCCAGATT		167	55.5
qPCR CAT(R)	GGGAACTCCTTATGCGACCA			55.8
qPCR SOD2(F)	TACTGGGCACTGACTAAGGAGC		156	58.3
qPCR SOD2(R)	GGCATTGATGTATGGGAGCA			53.8
qPCR NQO1(F)	TCTACTGGTTCAGCGTCCCG		130	57.9
qPCR NQO1(R)	AACGACAGCAGAGCCTTCTTG			56.1
qPCR GCLC(F)	GACAGGACCCAAGAGGAACG		107	57.9
qPCR GCLC(R)	TCCCCACAGCAAGACAGGTAG			58.1
qPCR UGT(F)	AGTCAACAGTCATTTTGGTCACATC		106	55.5
qPCR UGT(R)	CCAGTTCTAGGCATTCCACTTATC			56.9

Note: F: forward primer; R: reverse primer.

Table 3 The BLAST results of cloned genes in *Gambusia affinis* in comparison with other fish species in NCBI database.

Gene	The specie of max identity	GenBank accession NO	Identity
Nrf2	<i>Xiphophorus maculatus</i>	XM_005806271.2	96%
NQO1	<i>Xiphophorus maculatus</i>	XM_014472679.1	98%
GCLC	<i>Xiphophorus maculatus</i>	XM_005811878.2	97%
SOD2	<i>Xiphophorus maculatus</i>	XM_005802615.1	96%
CAT	<i>Poecilia formosa</i>	XM_007546784.2	98%
GSTA	<i>Poecilia formosa</i>	XM_007546133.2	96%
UGT	<i>Oreochromis niloticus</i>	XM_013275912.1	81%