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Ploidy-, gender-, and dose-dependent alteration of selected biomarkers in *Clarias gariepinus* treated with benzo[*a*]pyrene

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ABSTRACT

Naturally-occurring and artificially-induced polyploids have been documented in various fish species but to date no comparison has been reported of the impacts of ploidy on fish biomarker responses to organic pollutants. This study describes effects of ploidy, gender, and dose on biliary fluorescent aromatic compound (FAC) concentrations, hepatic ethoxyresorufin-O-deethylase (EROD) and glutathione S-transferase (GST) activities in one of the most commonly cultured warm-water species, the African catfish *Clarias gariepinus*. Recently matured male and female diploid and triploid fish were intraperitoneally (i.p.) injected with 0, 5 or 25 mg/kg benzo[*a*]pyrene (BaP) and liver and gallbladder were sampled 48 hr later. No significant differences were found between ploidies in bile concentrations of 7,8 dihydrodiolbenzo[*a*]pyrene (7,8D BaP), 1-hydroxybenzo[*a*]pyrene (1-OH BaP) or 3-hydroxybenzo[*a*]pyrene (3-OH BaP). However, concentrations of the biliary FACs did differ between males and females at different dose of injection with generally higher concentrations in females at the low dose of BaP and higher concentrations in males at the higher BaP concentration. Hepatic EROD activity did not exhibit gender-dependent difference, whereas it was significantly higher in triploids than diploids. GST activities were not significantly influenced by any of the tested factors. This work advanced our understanding of the role of ploidy, gender, and dose in biotransformation of pollutants in fish.

Introduction

Fish are the most diversified and wide spread vertebrates on the planet (Nelson, 2006) increasing the chance of their exposure to a wide range of pollutants discharged into aquatic environments. Some studies have highlighted the influence of confounding factors (*e.g.*, age, gender, length, water temperature, and nutritional status) on fish physiological responses towards water pollutants (Široká and Drastichova, 2004; Napierska and Podolska, 2005). However, reports on the influence of ploidy on fish responses to pollutants are scarce.

Polyploidy occurs naturally in several orders of fish including the Perciformes and the Cypriniformes (Comber and Smith, 2004). Triploid cells possess 50% more genes as compared to the diploids. Number and expression of genes and their interactions define organisms' responses to

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environmental conditions (Ching et al., 2009). Triploid fish have fewer but bigger cells than their diploid counterparts (Benfey, 1999). This phenomenon reduces the ratio of cell surface area to volume and, therefore, could affect cellular performance (*e.g.*, enzyme abundance, recovery after stress, diffusion) (Hyndman et al., 2003). Some studies have reported inconsistent trends of responses of diploid and triploid fish towards stressors. Juvenile triploid silver catfish (*Rhamdia quelen*) exhibited greater sensitivity than diploids to ammonia during the first 48 hr of exposure but comparable sensitivity after 96 hr (Weiss and Zaniboni-Filho, 2009). Atkins and Benfey (2008) reported higher metabolic rates in triploid than diploid Atlantic salmon (*Salmo salar*) and brook trout (*Salvelinus fontinalis*) at low temperatures but lower metabolic rates at high temperatures. Orrego et al. (2008) reported ethoxyresorufin-O-deethylase (EROD) induction and elevated plasma vitellogenin levels in triploid rainbow trout (*Oncorhynchus mykiss*) exposed to pulp mill effluent but no comparison was made with diploid fish. Despite the enormous importance of triploid fish for the aquaculture industry, no study has yet investigated the effects of organic pollutants on any triploid fish species.

The role of gender on toxicity of polycyclic aromatic hydrocarbons (PAHs) in aquatic organisms is well documented (Fossi et al., 2002). In sexually mature fish, females often exhibit lower levels of detoxifying enzymes than males and may therefore have lower levels of biliary fluorescent aromatic compounds (FACs) compared to males (Vuorinen et al., 2006). In Turbot (*Scophthalmus maximus* L.), sexually mature females showed lower cytochrome P4503A (CYP3A) levels than males (Arukwe and Goksøyr, 1997). Similarly, Winzer et al. (2002a) reported decreased supply of hepatocytic NADPH, reduced and/or delayed NADPH-dependent activity of CYP450 and a lower capacity of reduced glutathione (GSH) in female than male European flounder (*Platichthys flesus*) exposed to BaP.

Polycyclic aromatic hydrocarbons are a ubiquitous group of organic pollutants in aquatic environments (Wang et al., 2014). Fish possess phase I enzymes of mixed-function monooxygenase that convert PAHs into more hydrophilic components such as phenols, dihydrodiols and quinines which can then be eliminated from the body (Liu et al., 2014). Hence, quantifying PAHs in fish tissue may underestimate the exposure level (Beyer et al., 2010). Following conjugation with some molecules (*e.g.*, glutathione) through phase II enzymes (*e.g.*, glutathione S-transferase (GST)), biotransformed PAHs are stored in the gall bladder before excretion from the body (Varanasi et al., 1989). Benzo[*a*]pyrene is a member of the PAHs family and is a well-known oxidative stress inducer (Winzer et al., 2002b; Wang et al., 2014). Biliary FACs are reliable and sensitive biomarkers of recent PAH exposure in fish (van Schanke et al., 2001). The three main BaP FACs reported in fish are 7,8-dihydrodiolbenzo[*a*]pyrene (7,8D BaP) and the two phenolic compounds, 1-hydroxybenzo[*a*]pyrene (1-OH BaP) and 3-hydroxybenzo[*a*]pyrene (3-OH BaP) and were therefore used as the selected biliary FACs in this study (van Schanke et al., 2000; Telli-Karakoç et al., 2002; Karami et al., 2012b).

The main objective of this study was to evaluate the role of gender and ploidy in *Clarias gariepinus* on concentrations of glucuronides and sulfate conjugated biliary FACs, EROD and GST activities at different doses of BaP following intraperitoneal (i.p.) injection. Injection of substances into the peritoneal cavity is commonly used for exposing small laboratory animals to target compounds (Turner et al., 2011).

African catfish (*C. gariepinus*) is among the leading food fishes in tropical and subtropical countries (Adewolu et al., 2008). To our knowledge no study to date has investigated the role of ploidy on fish biomarkers in combination with gender and/or dose of organic pollutants.

1. Materials and methods

1.1. Chemicals

Colchicine, BaP, 1-chloro-2,4-dinitrobenzene (CDNB), dithiothreitol (DTT), GSH, 7-ethoxyresorufin (7-ER) and nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma Chemical (USA); β-glucuronidase/arylsulfatase (30/60 U/mL, from *Helix pomatia*) and Giemsa from Merck (Germany); 7,8-D BaP, 1-OH BaP, and 3-OH BaP were supplied by the Mid-west Research Institute (USA); methanol and acetone (HPLC grade) were purchased from JT Baker (USA); 0.2 μm syringe filter was obtained from Whatman (UK); and distilled water (HPLC grade) was produced in the laboratory.

1.2. Broodstock

Immature *C. gariepinus* were purchased from local farmers in Selangor, Malaysia and raised for 4 months until reaching maturity in 1000-L fibreglass tanks under a 12-hr light and dark cycle (12:12). Throughout the study, fish were fed *ad-libitum* with commercial pellets (Cargill, Malaysia). The mean (±SE) water temperature and dissolved oxygen were 27.2°C (±0.1) and 6.9 mg/L (±0.09), respectively.

1.3. Fish breeding and triploidy induction

Male and female broodstocks were injected with 0.25 mL/kg and 0.5 mL/kg body weight of Ovaprim®, respectively, 10 hr prior to breeding. Eggs and milt were mixed together and then divided into two batches 3 min later. The second batch was cold-shocked 3 min after fertilization at 5°C for 40 min to induce triploidy as described by Richter et al. (1986). Shocked and unshocked eggs were incubated separately in fibreglass tanks filled with 200 L of UV-sterilized water. Primary triploidy induction success rate was evaluated through chromosomal preparation of 2 day post-hatch larvae according to Karami et al. (2010). Chromosomal spreads with 56 and 84 chromosomes were considered as diploid ($2n = 56$) and triploid ($3n = 84$), respectively (Miskolczi et al., 2005).

Fish from both ploidy groups were fed three times a day *ad-libitum* and reared for 5 months in 1000-L fibreglass tanks prior to the start of the experiment. Recently matured fish from diploid and triploid groups were sexed according to the size, shape and colour of genital papilla and size of the belly. Individuals were labelled by plastic T-bar anchor tags. Triploidy was confirmed through evaluating size and shape

of erythrocyte cells and nuclei of individual fish (Karami et al., 2010).

1.4. BaP injection and sampling

Fish were fasted 36 hr prior to the injection and during the experiment. Groups of male diploid (192.91 ± 5.67 g), female diploid (188.24 ± 8.71 g), male triploid (181.33 ± 7.47 g) and female triploid (165.8 ± 5.56 g) *C. gariepinus* were injected i.p. with 5 and 25 mg/kg body weight BaP dissolved in corn oil as the carrier. Control groups were injected with corn oil only. Six fish were considered per treatment (ploidy × gender × dose; $n = 6$). Active carbon bags were used to absorb minor BaP leakages from the injection sites. Water was changed partially (50%) every day. No mortality was observed during the experiment. Forty-eight hours later fish were killed by clove oil overdose (0.2 mL/L). Livers and gall bladders were carefully removed, snap-frozen in liquid nitrogen and stored at −80°C until further analysis.

1.5. Biliary FACs determination

Bile samples were prepared for high-performance liquid chromatography with fluorescent detection (HPLC/FL) as described by Karami et al. (2012b). Briefly, gall bladders were thawed on ice and sonicated. In the first stage, 20 μL of bile was mixed with 460 μL of HPLC grade distilled water and 20 μL β-glucuronidase/arylsulfatase. After 2 hr of incubation at 37°C in a water bath shaker, reactions were terminated by the addition of 2000 μL chilled acetone. The mixture was centrifuged at 11,000 ×*g* at 2°C for 6 min and the supernatant was collected for FACs analysis. For the second stage, pellets were resuspended in distilled water and 10 μL of glucuronidase/arylsulfatase enzyme solution was added. Cocktails were incubated in the water bath, 1250 μL chilled acetone was added, and finally the mixture was centrifuged. Supernatant was analysed for BaP metabolite quantification. Values from the first and second stages were added together representing the maximum concentration of each metabolite. Deconjugated metabolites were separated and quantified using an Agilent 1200 series HPLC (Agilent Technologies Deutschland GmbH, Waldbronn, Germany) equipped with an auto sampler, a vacuum degasser, a fluorescent detector and a A201TP54 reversed-phase C18 analytical column (Vydac; 250 × 4.6 mm ID) (van Schanke et al., 2000).

1.6. Enzymatic activities

Livers were weighed and homogenized in KCl-HEPES buffer (0.15 mol/L KCl, 0.02 mol/L HEPES, pH 7.5) followed by centrifugation at 10,000 ×*g* for 20 min at 2°C. The supernatant (S9 fraction) was used for GST and EROD activity determination (Hodson et al., 1991).

EROD activity was determined by the method of Hodson et al. (1991) assigned to microplate by Frasco and Guilhermino (2002). Briefly, 180 μL of substrate solution [7-ethoxyresorufin 0.002 dissolved in dimethylsulfoxide (DMSO) and diluted into EROD buffer (0.1 mol/L Tris–HCl, 0.1 mol/L NaCl, pH = 7)] was added to 100 μL S9 fraction and 20 μL NADPH solution (0.9 mmol/L) in each well. The reaction was recorded at 570 nm every minute for 10 min using a microplate reader (**VersaMax, Molecular Devices Corporation, Sunnyvale, CA, USA**).

To determine GST activity, 100 μL of liver S9 fraction was combined with 200 μL of reaction solution (containing 60 mmol/L CDNB in methanol and 10 mmol/L GSH in 0.1 mol/L phosphate buffer (pH = 6.5)) in a microplate well according to the method of Habig et al. (1974) assigned to 96-wells microplate by Frasco and Guilhermino (2002). Absorptions were recorded every minute for 10 min at 340 nm using the microplate reader. Protein concentration of S9 fractions was quantified according to Bradford (1976).

1.7. Statistics

To improve normality and homogeneity of variance (Tabachnick and Fidell, 2001) FAC concentrations, and EROD and GST activities were log-square-root transformed. Afterwards, a three-way multi-factor analysis of variance (MANOVA) was employed to assess the effect of the three independent variables (ploidy, gender and dose) on biliary FAC concentrations. Separate three-way analyses of variances (ANOVAs) were used to evaluate the changes on EROD and GST activities across the independent variables. The MANOVAs and ANOVAs were followed by post-hoc Duncan's multiple comparison tests when significant differences were detected. All statistical comparisons were performed through IBM SPSS (V. 21, IBM, Armonk, USA). Descriptive statistics were computed by Statistix software (V. 10, Analytical Software, Tallahassee, FL, USA).

1.8. Ethics statement

All experiments were conducted in accordance with Malaysian legislation and the Code of Practice and accreditation criteria of the University Federation of Animal Welfare, UK (UFAW) (Hubrecht and Kirkwood, 2010).

2. Results

2.1. Biliary FACs

There were no significant effects of ploidy (Fig. 1a–c) or gender (Fig. 2a–c) on biliary FAC concentrations (three-way MANOVA, $P > 0.05$) but there was a significant effect of BaP dose (three-way MANOVA, $F_{2,60} = 16.06$, $P < 0.001$, partial eta squared = 0.39). Among potential interactions of the three main effects only gender × dose was significant (three-way MANOVA, $F_{2,60} = 2.33$, $P = 0.03$, partial eta squared = 0.08; Table 1). Tests of between-subject effects of dose and gender × dose are presented in Table 1. Duncan's multiple range tests were run on the concentrations of 7,8D BaP, 1-OH BaP, and 3-OH BaP over treatment combinations (gender × dose, Fig. 3a–c). Concentrations of all metabolites did not differ between the males and females in the control group. However, following the injection of 5 mg/kg BaP females showed significantly ($P < 0.05$) higher concentrations of 1-OH BaP than males (Fig. 1b). In contrast, male fish showed significantly ($P < 0.05$) higher concentrations than females of

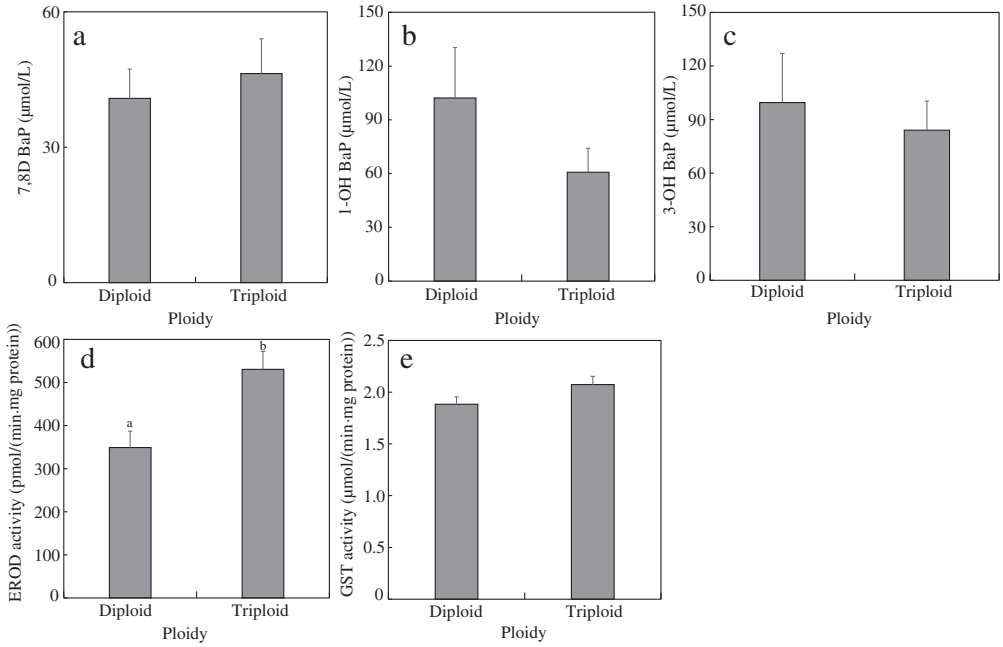

Fig. 1 – Ploidy-dependent biliary (a) 7,8D BaP, (b) 1-OH BaP, and (c) 3-OH BaP concentrations, and (d) ethoxyresorufin-O-deethylase (EROD) and (e) glutathione S-transferase (GST) activities in *C. gariepinus*. N = 36 fish for each bar, and the data shown here are Mean + SE. Bars surmounted by different letters are significantly different from each other ($P < 0.05$, Duncan's multiple range test).

all three biliary FACs following the injection with 25 mg/kg BaP (Fig. 1a–c).

2.2. Enzymes

Hepatic EROD activities were significantly higher in the triploids than diploids (three-way ANOVA, $F_{1,60} = 14.01$, $P < 0.001$; Fig. 1d). Furthermore, no significant differences on EROD activities were detected between males and females (Fig. 2d) and among different doses of BaP injection. However, changes on EROD activities were significantly different between diploids and triploid over doses of injection (three-way ANOVA, $F_{2,60} = 5.98$; Fig. 4). In contrast, hepatic GST activities did not differ significantly ($P > 0.05$) between ploidies (Fig. 1e), genders (Fig. 2e), doses of injection or among their interactions.

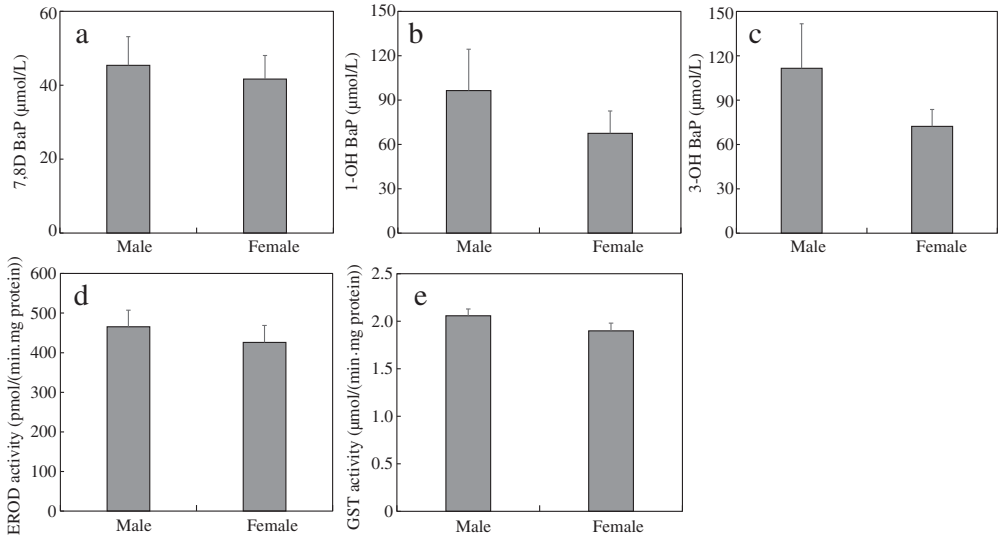

Fig. 2 – Gender-dependent biliary (a) 7,8D BaP, (b) 1-OH BaP, and (c) 3-OH BaP concentrations, and (d) EROD and (e) GST activities in *C. gariepinus*. N = 36 fish for each bar, and the data shown here are Mean + SE. Bars surmounted by different letters are significantly different from each other ($P < 0.05$, Duncan's multiple range test).

Table 1 – Tests of between-subject effects for dose, gender, and on the selected biliary FACs (7,8-D BaP, 1-OH BaP, 3-OH BaP); a Bonferroni adjusted alpha level of 0.016 was applied; *df* = 2; *df* error = 60.

Source of variation	Biliary FAC	*F*	*P*	Partial eta squared
Dose	1-OH BaP	14.30	<0.001 *	0.27
	3-OH BaP	32.15	<0.001 *	0.45
	7,8D BaP	74.46	<0.001 *	0.66
Dose × gender	1-OH BaP	4.83	0.01 *	0.11
	3-OH BaP	4.58	0.01 *	0.10
	7,8D BaP	4.75	0.01 *	0.11

* Significant difference at <0.016 level.

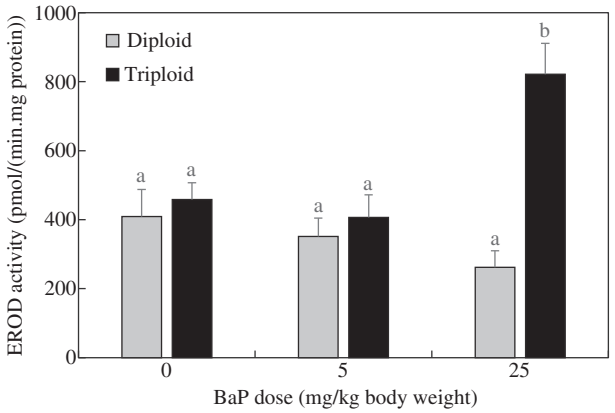

Fig. 4 – EROD activities over different ploidy and dose of BaP injection in *C. gariepinus*. N = 12 fish for each bar, and the data shown here are Mean + SE. Bars with different letters are significantly different from each other (*P* < 0.05, Duncan's multiple range test).

3. Discussion

Polyploidy is an important phenomenon of evolutionary and phylogenetic studies in fish (Comber and Smith, 2004). Triploid fish are used for controlling populations due to their sterility (Maxime, 2008; Gause et al., 2012) and for their potential economical profits because of faster growth compared to their diploid counterparts (Tiwary et al., 2004). Unlike mammals and birds, in which polyploidy induction is typically fatal (Otto, 2007), triploid fish are often able to survive (Mizgireuv et al., 2004). This quality provides a unique opportunity to evaluate the impacts of the extra set(s) of chromosome on physiological responses towards environmental stimuli.

Triploid *C. gariepinus* are sterile (Karami et al., 2011b), hence might have lower levels of sex hormones than diploids. Sex hormones, particularly 17-β Estradiol (E_2), are believed to modulate mixed-function monooxygenase reactions (Arukwe and Goksøyr, 1997) including phase I and II enzymes (Solé et al., 2003). This physiological difference might be the reason for the greater EROD induction in triploid fish than diploid fish exposed to BaP in this study. Alternatively, differences in gene expression and/or gene interaction are potential reasons for the observed differences in EROD activity between diploids and triploids as triploids have three copies of a genes compared to two in diploids. Following bath exposure to three carcinogens, the incidence of tumours in the swim bladders, stomachs, kidneys, and livers of triploid *O. mykiss* was lower than in diploids suggesting lower likelihood that all copies of tumour suppressor genes were mutated in the triploids (Thorgaard et al., 1999). Our observation of no significant EROD induction in diploid fish exposed to BaP is consistent with the study of Mdegela et al. (2006a) who reported no changes in hepatic EROD activities in *C. gariepinus* following water-borne exposure to BaP. Undetectable EROD activity in *C. gariepinus* individuals was also reported by several other studies (Mdegela et al., 2006b; Karami et al., 2011a). As well, lower EROD activity was observed in *C. gariepinus* exposed to BaP than Nile tilapia (*Oreochromis niloticus*) (Hassanain et al., 2007). These findings may suggest that BaP biotransformation in *C. gariepinus* is not an aryl hydrocarbon receptor (AhR)-dependent pathway.

7,8 dihydrodiolbenzo[*a*]pyrene was one of the major biliary metabolites in this study. In BaP-injected *S. maximus*, 7,8D BaP represented 70% of the total metabolites (Telli-Karakoç et al., 2002). This compound is the potent tumorigenic precursor which, after conversion into 7,8-diol 9,10-epoxide BaP (BPDE), is able to bind to cellular macromolecules to form adducts (*e.g.*, BPDE-DNA) (Shiizaki et al., 2013). The bioactivation process is mediated by metabolizing enzymes (*e.g.*, CYP1A1) (Rojas and Esponda, 2001). The strong relationship between dose of injection and 7,8D BaP concentration in the pool of diploid and triploid *C. gariepinus* (partial eta squared = 0.66; Table 1) suggests a novel area of research using polyploids as the vertebrate model and BaP as a tumour inducer.

In the present study, diploid and triploid *C. gariepinus* did not differ in the concentrations of biliary FACs. These

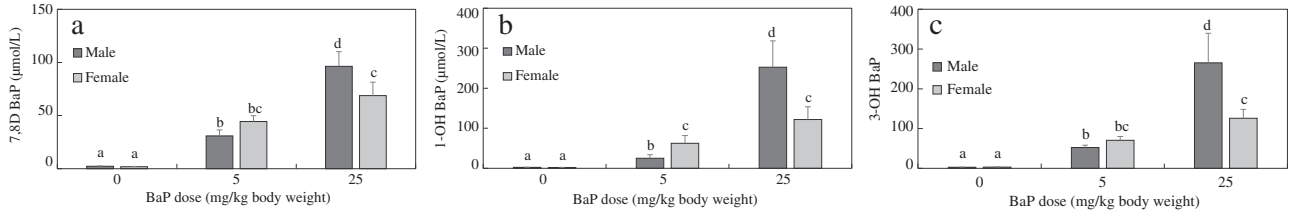

Fig. 3 – Dose- and gender-dependent concentrations of biliary (a) 7,8D BaP, (b) 1-OH BaP, and (c) 3-OH BaP in *C. gariepinus*. N = 12 fish for each bar, and the data shown here are Mean + SE. Bars surmounted by different letters are significantly different from each other (*P* < 0.05, Duncan's multiple range test).

findings may suggest comparable activities of the enzymes involved in biotransformation of BaP between diploid and triploid *C. gariepinus*. However, further experiments incorporating multiple sampling times and a wider range of BaP doses will be required to confirm similar biliary FAC levels between diploid and triploid *C. gariepinus*.

The present study highlighted that, regardless of the ploidy status, concentration of FACs across doses of BaP injection was significantly different between the males and females (dose × gender). Comparable concentrations of 7,8D BaP and 3-OH BaP between males and females of 5 mg/kg BaP-injected fish were documented. In another study on *C. gariepinus*, the concentrations of BaP-type fluorescent compounds, hepatic EROD and GST activities were not different between the males and females following waterborne exposure to BaP (30 μg/L corresponding to 5 mg/kg body weight) (Mdegela et al., 2006a). Similarly, the concentrations of BaP- and naphthalene (NAPH)-type metabolites were comparable between male and female brown bullhead (*Ameiurus nebulosus*) collected from some United States rivers (Yang and Baumann, 2006). No sex-dependent difference was observed in PCB concentrations in the liver and muscle of the thicklip grey mullet (*Chelon labrosus*) from Mondego estuary (Baptista et al., 2013). Males and females of some trout species in some high mountain lakes in Europe and Greenland showed comparable PAH content in the liver (Vives et al., 2004). However, in the current study, higher concentration of 1-OH BaP in the females *versus* males was observed after the injection of 5 mg/kg BaP. In contrast, higher levels of 7,8D BaP, 1-OH BaP and 3-OH BaP in males than females following injection of 25 mg/kg BaP are consistent with Vuorinen et al. (2006) who reported higher biliary concentrations of 1-OH phenanthrene in male than female eelpout (*Zoarces viviparus*), perch (*Perca fluviatilis*), and *P. flesus* at some stations in the Baltic Sea. The difference in the concentrations of the FACs between sexes across different doses of BaP might suggest sex-specific sensitivity of biotransformation enzyme genes in *C. gariepinus* to high dose of BaP, or E_2-dependent modulation of enzymatic activities.

As for biliary FAC concentrations, GST activity did not differ significantly between diploids and triploids. As well, GST activity did not differ between males and females in the present study which is consistent with some previous studies on the effects of BaP on GST activity in *C. gariepinus* (Mdegela et al., 2006a,2006b; Karami et al., 2011a). Gender differences in GST response have, however, been reported in some other species; male *O. niloticus* exposed to paraquat showed higher levels of GST and superoxide dismutase than females (Figueiredo-Fernandes et al., 2006). Considering a wider dose range than used in the present study may reveal significant effects on EROD and GST activities (*e.g.*, Karami et al., 2012a).

Despite the differences in biliary FACs between males and females, our results did not show gender-dependent EROD or GST activities as has been shown elsewhere. EROD activity of male mummichogs (*Fundulus heteroclitus*) caught from Newark Bay, USA was higher than females (McArdle et al., 2004). In another study, hepatocytes of female *P. flesus* showed lower capacity of non-enzymatic antioxidant defenses following BaP exposure than was observed in males (Winzer et al., 2002b). CYP1A expression in β-naphthoflavone-treated channel catfish (*Ictalurus punctatus*) was twice as high in males as females (Perkins and Schlenk, 1998). Different trends of changes in biliary FACs between males and females across doses of BaP injection, in the absence of different EROD activities between the genders, may suggest the involvement of different gender-dependent CYP1 subfamilies in biotransformation of BaP in *C. gariepinus*. Similar results have been reported in other fish species; Telli-Karakoç et al. (2002) found formation of hepatic DNA adducts and biliary 7,8D BaP in *S. maximus* in the absence of EROD induction. In *I. punctatus*, for example, induction of CYP1B mRNA expression was noted by 20 mg/kg i.p. injection of BaP (Willett et al., 2006). In another study, BaP i.p.-injected *F. heteroclitus* showed CYP1C1 and CYP1A mRNA expression inductions (Wang et al., 2006). 3,3′,4,4′,5-pentachlorobiphenyl (PCB126) induced four cytochrome P450 1 (CYP1) genes (CYP1A, CYP1B1, CYP1C1, and CYP1C2) in *Danio rerio* embryos (Jönsson et al., 2007).

4. Conclusions

This study was undertaken to assess the effects of gender, ploidy and dose of BaP injection on biliary FAC concentrations, and EROD and GST activities. This work demonstrated that different fish biomarkers are influenced by different factors. Biliary FAC concentrations were unaffected by ploidy whereas EROD activity was significantly different between diploids and triploids at different dose of injection. Biliary FAC concentrations were different between males and females at the tested doses of injection. Nevertheless, GST activity was not influenced by any of the tested factors. Further studies on hormonal levels and enzymatic activities of diploid and triploid male and female *C. garieinus* are required to understand the reasons behind the changes on biliary FACs, and EROD and GST activities following environmental pollutant exposure.

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