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# *p*-Nitrophenol and glutathione response in medaka (*Oryzias latipes*) exposed to MX, a drinking water carcinogen

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**Abstract**

When chlorine is introduced into public drinking water for disinfection, it can react with organic compounds in surface waters to form toxic by-products such as 3-chloro-4-(dichloromethyl)-5-hydroxy-2[5*H*]-furanone (MX). We investigated the effect of exposure to MX on cytochrome P450 2E1 (CYP2E1)-like activity and total glutathione (GSH) in the liver of the small fish model, medaka (*Oryzias latipes*). The multi-site carcinogen methylazoxymethanol acetate (MAMAc) was the positive control compound. Both medaka liver microsome preparations and S-9 fractions catalyzed the hydroxylation of *p*-nitrophenol (PNP), suggesting CYP2E1-like activity in the medaka. Male medaka exposed for 96 h to the CYP2E1 inducers ethanol and acetone under fasted conditions showed significant increases in PNP-hydroxylation activity. Furthermore, total reduced hepatic GSH was reduced in fish fasted for 96 h, indicating that normal feeding is a factor in maintaining xenobiotic defenses. Exposure to MX and MAMAc induced significant increases in hepatic CYP2E1-like activity, however MX exposure did not alter hepatic GSH levels. These data strengthen the role of the medaka as a suitable species for examining cytochrome P450 and GSH detoxification processes and the role these systems play in chemical carcinogenesis.
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## 1. Introduction

Over the past century, chlorination of public drinking water has dramatically decreased infectious waterborne diseases and is a cornerstone of American public health policy (see DeAngelo et al., 1991). Chlorine disinfection of drinking water, however, can produce mutagenic by-products, of which 3-chloro-4-(dichloromethyl)-5-hydroxy-2[5*H*]-furanone (MX) accounts for 30–50% of that mutagenicity (Kronberg and Vartiainen, 1988). Concern over health effects from chronic exposure to MX led to studies that showed it to be highly mutagenic to mammalian cells in vitro (Chang et al., 1991; Jannson and Hyttinen, 1994;

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Matsumura et al., 1994) and in vivo (Furihata et al., 1992; Jannson et al., 1993) as well as being a liver and multi-site carcinogen in rats (Komulainen et al., 1997, 2000). A review by the State of California designated MX a carcinogen (California EPA, 2000). Metabolism of MX appears to be mediated by the cytochrome P450 family of xenobiotic metabolizing enzymes. Mammalian studies of P450 enzyme activity show that MX exposure decreased ethoxyresorufin-*O*-deethylase (EROD) and pentoxyresorufin-*O*-deethylase (PROD) activity, suggesting inhibition of both CYP1A1 (PAH-inducible) and the CYP2B1 and CYP2B2 (phenobarbital-inducible) forms (Heiskanen et al., 1995). The phase II substrate glutathione (GSH) also appears to be involved in both the spontaneous and glutathione *S*-transferase (GST)-catalyzed detoxification of MX (Ishiguro et al., 1987; Watanabe et al., 1994; Ubom et al., 1994; Clark and Chipman, 1995).

Understanding xenobiotic detoxification mechanisms is integral to developing risk assessment strategies for regulating drinking water (Weisburger and Williams, 1981; Law et al., 1998). Non-rodent animal models including fishes might help these investigations. One such model, the medaka (*Oryzias latipes*), has been used in testing environmental carcinogens (Hoover, 1984; Hawkins et al., 1988; Bunton, 1996) including drinking water contaminants (Law et al., 1998; Teuschler et al., 2000; Toussaint et al., 2001a,b). The low spontaneous tumor rate and the opportunity to use large numbers in experiments make the medaka an especially attractive model. Nevertheless, relatively little is known about medaka detoxification pathways that are prominent in other models.

In the present study we examined the role of cytochrome P450 2E1 (CYP2E1) in MX metabolism in medaka liver by measuring MX-induced CYP2E1 activity using the CYP2E1 specific substrate *p*-nitrophenol (PNP). CYP2E1 is the principal catalyst in the metabolism of low-molecular-weight, volatile organic pollutants (Peter et al., 1990) including low-dose drinking water disinfection by-products (Allis et al., 2001; Zhao and Allis, 2002). In mammals, hydroxylation of PNP to 4-nitrocatechol (hereafter referred to as hydroxylated PNP) reflects CYP2E1 activity (Dicker et al., 1990; Duescher and Elfarra, 1993; Mishin et al., 1996). A second objective was to investigate changes in medaka liver GSH related to fasting during and following MX exposure. For both study components, tissues from medaka exposed to methylazoxymethanol acetate (MAMAc), a mutagen and carcinogen in rodents (Zedeck, 1984) and medaka (Couch and Harshbarger, 1985; Hawkins et al., 1985) served as a positive control. In mammals, MAMAc is known to be metabolized by CYP2E1 (Sohn et al., 1991) and believed to involve GSH in its detoxification (Vos and Van Bladeren, 1990); however, little is known of MAMAc metabolism in the medaka.

## 2. Materials and methods

### 2.1. Chemicals

*p*-Nitrophenol (PNP), 4-nitrocatechol, reduced glutathione (GSH), diethyldithiocarbamate (DDC), diallyl sulfide (DAS), troleandomycin (TAO), monobromobimane (mBrB), glutathione disulfide (GSSG) and tris-(2-carboxyethyl)-phosphine (TCEP) were purchased from Fisher Chemical (Pittsburgh, PA). CD1 mouse liver S-9 was obtained from XenoTech, LLC (Kansas City, KS). NADPH was purchased from Sigma Chemical Co (St. Louis, MO). 3-Chloro-4-(dichloromethyl)-5-hydroxy-2[5*H*]-furanone (MX) was generously provided by Dr Doug Wolf, Environmental Carcinogenesis Division, US Environmental Protection Agency, RTP, NC. Methylazoxymethanol acetate (MAMAc) was obtained from Ash-Stevens (Detroit, MI). All other reagents used were of the highest grade commercially available.

### 2.2. Medaka gender and cytochrome induction/inhibition investigations

The first step of this investigation was to determine whether medaka liver S-9 and microsomal protein could hydroxylate PNP, indicating CYP2E1-like activity. Although PNP hydroxylation is specific for CYP2E1 activity in mammals, CYP3A is reported to contribute slightly to PNP metabolism (Zerilli et al., 1997). To determine involvement of CYP3A, reactions were run using a known inhibitor, troleandomycin (TAO). Furthermore, to test whether PNP metabolism was catalyzed by soluble peroxidases in the medaka S-9 fraction, glutathione (GSH) was added to the incubation buffer and compared to control reactions without GSH. Gender differences in CYP2E1 activity were noted in mammalian studies (Dekant et al., 1995) and also investigated in this study.

Table 1
Experimental design of cytochrome P450 2E1 activity in medaka

| Treatment | Number of fish specimens at each sampling time | | | |
|---|---|---|---|---|
| | 0 h | 24 h | 48 h | 96 h |
| Untreated controls | 8/8 | 0/0 | 0/0 | 8/8 |
| Fasting 96 h | 8/8 | 0/0 | 0/0 | 8/8 |
| Ethanol (0.5%) + feed | 8/8 | 0/0 | 0/0 | 8/8 |
| Ethanol (0.5%) + fasting | 8/8 | 0/0 | 0/0 | 8/8 |
| Acetone (0.6%) + feed | 8/8 | 0/0 | 0/0 | 8/8 |
| Acetone (0.6%) + fasting | 8/8 | 0/0 | 0/0 | 8/8 |
| MX (30.0 mg/l) + feed | 8/0 | 8/0 | 8/0 | 8/0 |
| MAMAc (1.0 mg/l) + feed | 8/0 | 8/0 | 8/0 | 8/0 |

Results are given as male/female ratio. MX, 3-chloro-4-(dichloromethyl)-5-hydroxy-2[5*H*]-furanone; MAMAc, methylazoxymethanol-acetate. Ethanol, acetone, MX and MAMAc exposure were conducted under static renewal conditions for 96 h. Exposure media for both fed and fasting treatments were renewed 1 h following twice-daily feeding.

CYP2E1 induction and inhibition experiments were conducted to compare mammalian CYP2E1 activity to medaka. Medaka (8 months old) were obtained from a culture maintained at the Gulf Coast Research Laboratory, Ocean Springs, MS. Fish were separated by sex and fasted or treated with ethanol (0.5%) or acetone (0.6%) for 96 h, all known CYP2E1 inducers (Table 1). Exposures were conducted in 4-l Pyrex® beakers with eight fish per treatment in 3 l of toxicant media at $26 \pm 1$ °C in a recirculating water bath. Media of fed and fed plus ethanol or acetone treatments were changed 1 h after twice-daily feedings. Media of fasting and fasting plus ethanol or acetone treatments were changed at the same time as the fed group.

Inhibition assays were conducted on mouse and medaka S-9 and medaka microsomes using 50 μM of the CYP2E1 specific inhibitors diethyldithiocarbamate (DDC) and diallyl sulfide (DAS). To test whether PNP metabolism was catalyzed by soluble peroxidases, 0.5 mM glutathione (GSH) was added to the incubation buffer. In order to assess possible PNP metabolism from CYP3A, medaka S-9 control samples were run with 50 μM of the CYP3A specific inhibitor, troleandomycin (TAO).

### 2.3. Medaka MX and MAMAc cytochrome P450 investigations

Toxicity tests to determine lethal MX concentrations (0.01–1000 ppm or mg/l) in medaka yielded a 96-h $LC_{50}$ of 60 ppm for adult fish. The 96-h MAMAc exposure was 1.0 ppm, which was the maximum tolerable adult dose that did not cause mortality. Male medaka (8 months old) were exposed statically to MX and MAMAc for 96 h at 0.0 and 30.0 and 0.0, and 1.0 ppm, respectively. Exposures were conducted in 1-l Pyrex® beakers, with each compound having three replicates of eight fish in 1 l of toxicant media at $26 \pm 1$ °C. Individual beakers were placed in a recirculating water bath and mildly aerated to maintain adequate dissolved oxygen levels. Media in each treatment and control beaker was changed 1 h after twice-daily feedings. Carcinogen exposures were conducted in a single-pass, negative-pressure exposure suite using a 16:8 light/dark cycle. Eight fish each were sampled at 0, 24, 48 and 96 h.

Medaka were killed by an overdose of tricane methanesulfate (MS222; Crescent Research, Phoenix, AZ) and individual livers excised and weighed. S-9 fractions (post-mitochondrial supernatant) were prepared by homogenizing livers (1:40 w/v) in 0.25 M sucrose, 10 mM potassium phosphate buffer, pH 7.4, followed by centrifugation at $10\,000 \times g$ for 20 min at 4 °C. The supernatant, which was designated the S-9 fraction, was aliquotted and either used immediately or stored at −20 °C for less than 24 h. Microsomes were prepared from pooled control medaka livers that were homogenized and the S-9 fraction prepared as described above. The S-9 was centrifuged again for 60 min at $105\,000 \times g$ to produce the microsomal fraction. Washed microsomes were then resuspended in a 10 mM Tris–HCl buffer (pH 7.4) containing 1 mM EDTA and 20% (v/v) glycerol. Microsomes were immediately frozen in liquid nitrogen and stored at −80 °C. Protein concentrations were determined by the Bradford method according to the procedure recommended by the supplier, with bovine serum albumin used as the standard (Pierce Chemical Co, Rockford, IL).

To determine optimal assay conditions, medaka microsomal and S-9 protein fractions were added to ice-cold 100 mM potassium phosphate buffer, pH 6.8, containing 2.0 mM NADPH in a volume of 235 μl in 1.5-ml Eppendorf vials. The mixtures were preincubated at 22, 27, 32 and 37 °C for 5 min and the reactions were initiated by adding 240 μl of 100 mM potassium phosphate buffer (pH 6.8) containing 0.2 mM PNP and 5 mM magnesium chloride. The reactions proceeded for 30, 60

or 90 min and were terminated by adding 25 μl of trifluoroacetic acid, bringing the final volume to 500 μl. The samples were vortexed immediately and centrifuged for 15 min at 12 900×*g* in an Eppendorf centrifuge. A 50-μl aliquot of supernatant was analyzed for hydroxylated PNP. Blank measurements were performed by omitting NADPH from the incubation buffer. Hepatic S-9 samples (50 μg) from CD-1 mice were run as positive controls for the medaka S-9 reactions.

Hydroxylated PNP was measured by HPLC with a Hewlett Packard Zorbex XDB-C8 5-μm (4.6 mm×150 mm) column connected to a Beckman 126 solvent module coupled to a Beckman Gold 168 photodiode array detector. Ultraviolet absorbance was monitored at 345 nm. The assay was conducted using an isocratic mobile phase of solvent A (25% acetonitrile and 0.1% trifluoroacetic acid), followed by a column refresh gradient phase of solvent B (90% acetonitrile and 0.1% trifluoroacetic acid) pumped at 1.0 ml/min. The time program was 100% A for 10 min, 100% A to 100% B in 30 s, held at 100% B for 3.5 min, 100% B to 100% A in 30 s and held at 100% A for 5.5 min. The injection volume was 50 μl and the total run time was 20 min. The concentration of hydroxylated PNP was determined from a standard curve ($r^2=0.999$) constructed from known amounts of hydroxylated PNP run under normal assay conditions minus NADPH. The detection limit of 16 pmol in 50 μl corresponded to the reported limit for UV detection (Duescher and Elfarra, 1993).

### 2.4. Medaka treatment and assay for glutathione investigations

Medaka (1 year old) were exposed statically for 96 h at 0.0, 10.0 and 50.0 ppm MX and 0.0 and 1.0 ppm MAMAc. Exposures were performed for 96 h as described above. Three fish per sample period (0, 24, 48, 96, 144, 240 and 480 h) were examined. Following exposure, the remaining fish were placed in flow-through 5-l tanks and returned to a regular diet. For all exposures, water quality parameters including temperature, pH and dissolved oxygen were measured daily. Exposed and control medaka were killed by an overdose of tricane methanesulfate. Liver tissue was weighed, homogenized in 1:10 (w/v) 5% sulfosalicylic acid and clarified by centrifugation at 1200×*g* for 10 min at 4 °C. Samples were stored at −20 °C and were analyzed within 48 h. For the GSH labeling assay, 20 μl of sample was placed into an Eppendorf tube. Oxidized GSH (GSSG) in the sample was reduced by adding 20 μl of 7.5 mM tris(2-carboxyethyl)phosphine for 20 min at 22 °C. The mixture was buffered with 20 μl of 4 M *n*-ethylmorpholine (pH 8). The GSH in the sample was derivatized by adding 20 μl of 10 mM monobromobimane (in 1:1 acetonitrile/$H_2O$, by vol.) for 20 min at 22 °C in the dark. The reaction was stopped by adding 20 μl of 5.8 M perchloric acid for a total reaction volume of 100 μl.

Reaction products were analyzed on a Beckman 126 solvent module connected to a Jasco FP-920 fluorescence detector set for excitation at 360 nm and emission at 478 nm. Separations were achieved using a 150 mm×4.6 mm Hypersil ODS column equilibrated with an acetate buffer consisting of 0.25% acetic acid titrated to pH 4 with 1 M NaOH (solvent A). The products were eluted from the column with a 6-min gradient of acetonitrile (solvent B) (0–4 min, 0–30% B; 4–5 min, 30–100% B; 5–6 min, 100% B) at a flow rate of 1.5 ml/min. The column was then reconditioned for 6 min with A.

### 2.5. Analytical assay

Due to published reports on the volatility of MX, water samples were taken daily during exposure. MAMAc analytical analysis was not performed. Aqueous MX concentrations were measured by HPLC using a Hewlett Packard Zorbex XDB-C8 5-μm (4.6 mm×150 mm) column connected to a Beckman 126 solvent module coupled to a Beckman Gold 168 photodiode array detector. Ultraviolet absorbance was monitored at 224 nm. The assay was run using a gradient mobile phase of solvents A (20:80 methanol/water) and B (70:30 methanol/water) both with 10 mM pentanesulfonic acid (PSA) and pH adjusted to 3.0 with $H_3PO_4$. The time program was 100% A for 8 min to 100% B in 7 min and held at 100% B for 10 min at a flow rate of 1.0 ml/min. Exposure media were directly injected at a volume of 10 μl and the total run time was 25 min.

### 2.6. Statistical analysis

Linear regression analyses were performed using SIGMAPLOT version 6.0 software (SPSS Inc, Chicago, IL). Results are expressed as mean±S.E.

Comparisons between control and treated groups were performed using a one-way analysis of variance followed by Dunnett's $T$-test at $P<0.05$. Data that failed the normality test (populations with unequal variances) were analyzed using a Kruskal–Wallis test. Male cytochrome induction studies were analyzed using the Mann–Whitney test with a Bonferroni-adjusted $P$ value of 0.01. Analysis was performed using SPSS version 10.1 software (SPSS Inc, Chicago, IL).

## 3. Results

### 3.1. Water quality and analytical chemistry

Temperature, pH and dissolved oxygen levels during the experiments were within accepted ranges. MX concentrations in water declined by an average of 23% over 96 h in static non-renewal exposures and were not significantly affected by mild aeration for 24 h (data not shown).

### 3.2. Characterization, induction and inhibition of PNP metabolism

Both medaka liver microsome preparations and S-9 fractions were capable of hydroxylating PNP. In several pooled S-9 protein fractions, enzymatic reaction times and temperatures were tested to optimize enzymatic incubations. A liver from a single adult medaka liver weighing over 4.25 mg reliably yielded at least 125 μg of S-9 protein. The combination of 100 μg of S-9 protein and a 30-min reaction at 27 °C gave the greatest formation of hydroxylated PNP. PNP metabolism was linearly related to medaka microsomal ($r^2=0.994$, 10–80 μg) and S-9 ($r^2=0.989$, 25–200 μg) protein at the concentrations used and was dependent on NADPH.

Using the optimal enzymatic conditions, PNP metabolism in S-9 fractions from 8-month-old male medaka controls was almost double that observed in the females, 20.70±1.33 and 11.45±1.57 pmol/mg min, respectively ($n=8$) (Fig. 1). Hepatic S-9 samples (50 μg) from CD-1 mice were run as positive controls for the medaka S-9 reactions. Mouse PNP metabolism at 37 °C was 366.1±8.18 pmol/mg min ($n=8$), one order of magnitude higher than medaka male controls run at 27 and 37 °C (8.41±0.54 pmol/mg min). Medaka female control activity at 37 °C was below detection.

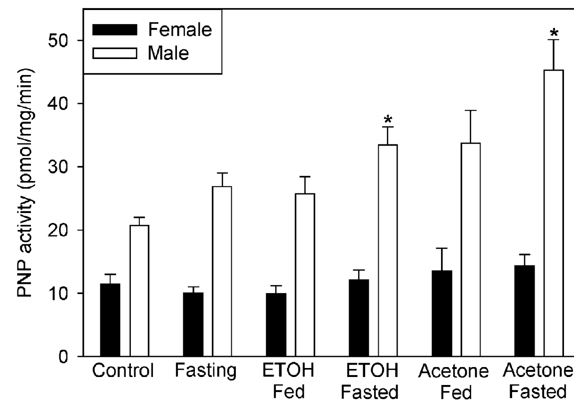


Fig. 1. Determination of $p$-nitrophenol (PNP) metabolism at 27 °C from male and female medaka S-9 protein (100 μg) treated with the known CYP2E1 inducers fasting, ethanol or acetone for 96 h. Also shown is the activity for medaka fasted plus ethanol or acetone. Values represent means±S.E. of eight fish per group. *Significantly different from control, $P<0.01$.

Effects on PNP metabolism of fasting, ethanol, ethanol plus fasting, acetone, and acetone plus fasting in male and female medaka are shown in Fig. 1. The combination of fasting with either ethanol or acetone treatment produced significant increases in PNP metabolism in males, but not in females. Inhibition by CYP2E1 specific inhibitors DDC and DAS reduced CD-1 mouse hepatic S-9 PNP metabolism to below detection limits (data not shown). Both compounds significantly reduced CYP2E1 activity in medaka S-9 (Fig. 2a) but only DAS produced a significant enzymatic reduction in medaka microsomes (Fig. 2b). S-9 from male medaka exposed to MX and MAMAc showed significant increases in PNP activity at 96 h, and 48 and 96 h, respectively (Fig. 3). GSH added to the incubation buffer of control S-9 fraction resulted in no difference in activity between control and GSH treatment, 20.600±2.366 and 19.800±1.939 pmol/mg min, respectively ($n=5$). In addition, the CYP3A specific inhibitor TAO showed no difference in PNP activity between control and TAO treatment, 19.780±0.615 and 18.850±1.108 pmol/mg min, respectively ($n=6$).

### 3.3. Total GSH levels in fasted and exposed medaka

The glutathione-bimate peak corresponded with the retention time from known amounts of pure GSH assayed for the development of a standard

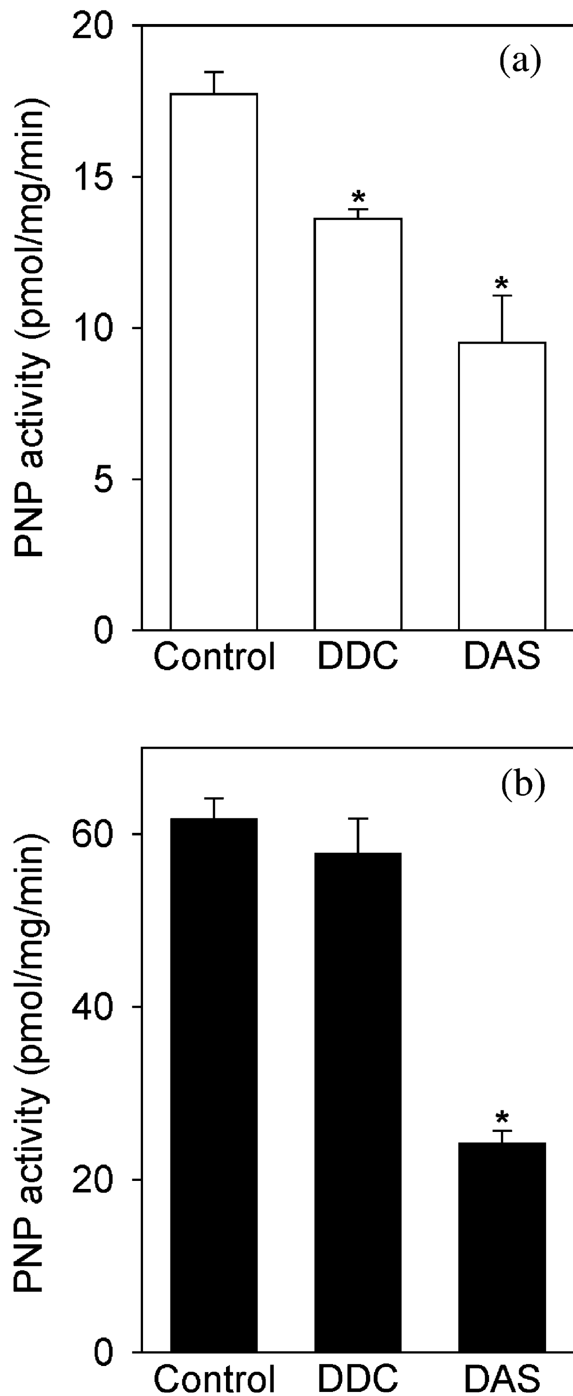


Fig. 2. Inhibition of medaka (a) liver S-9 (100 μg) and (b) microsome (25 μg) *p*-nitrophenol (PNP) metabolism with 50 μM of the CYP2E1 chemical inhibitors diethyldithiocarbamate (DDC) and diallyl sulfide (DAS). Values represent means±S.E. of six fish per group. *Significantly different from control, $P<0.05$.

curve ($r^2=0.9954$). The control level of GSH in medaka at time 0, prior to exposure and fasting, was 1.415±0.134 mM GSH ($n=10$). Levels of GSH from fed and 96-h fasted controls are shown in Fig. 4. No significant difference was found between values of fed fish during the study. These values were pooled and used to compare levels from fasted fish. A significant reduction in GSH was observed at 96 h in the fasted group. Once these fish were returned to a regular diet, GSH biosynthesis apparently occurred rapidly, producing a significant increase 48 h later.

Effects on GSH response during and following 96-h exposure to 0.0, 10.0 and 50.0 ppm MX and 0.0 and 1.0 ppm MAMAc are shown in Fig. 5a,b,c, respectively. Excessive mortality for 50.0 ppm MX following exposure precluded sampling of fish from that group at 480 h. We suspect a delayed effect following exposure to be responsible for the

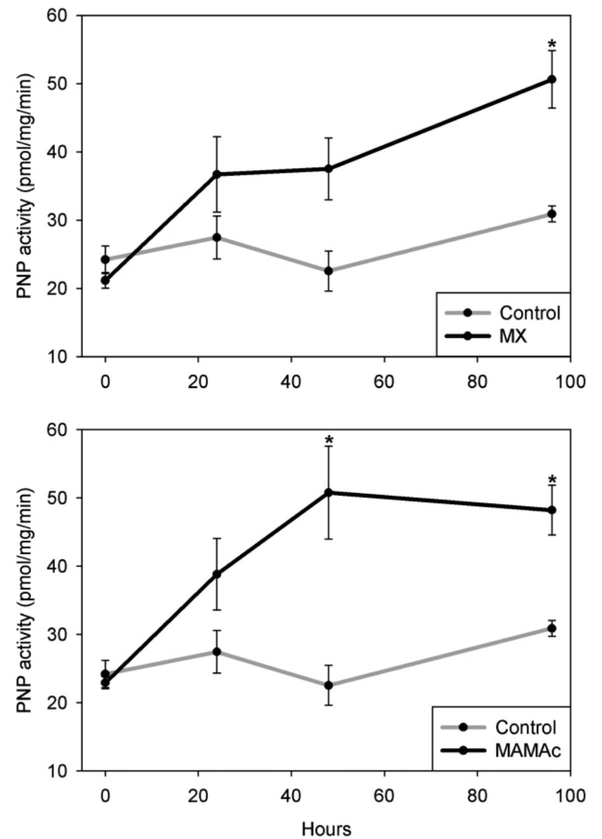


Fig. 3. Induction of *p*-nitrophenol (PNP) metabolism in male medaka S-9 during exposure to MX (30.0 ppm) and MAMAc (1.0 ppm). Values represent means±S.E. from eight fish per group. *Significantly different from control, $P<0.05$.

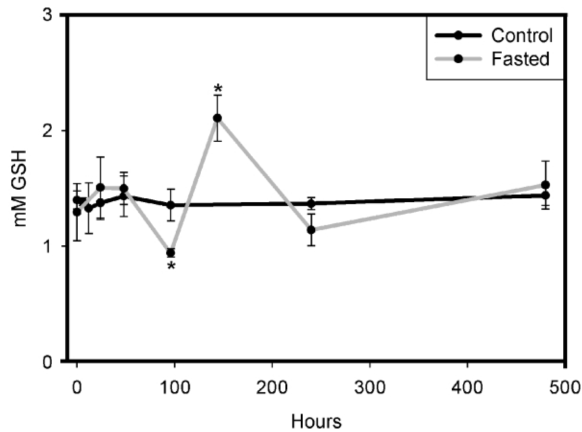


Fig. 4. Effects of fasting on liver glutathione (GSH) levels in medaka. Medaka were fasted for 96 h then returned to a regular diet. Control fed fish were maintained on a regular diet during the study. Values represent means ± S.E. from three fish per group. *Significantly different from control, $P < 0.05$.

mortality observed. Overall, both MX treatments closely paralleled control levels, with no significant changes occurring during the study. In the 1.0 ppm MAMAc treatment, significantly elevated GSH levels were observed at 48 and 96 h.

## 4. Discussion

Fish used in acute toxicity tests are typically fasted before and during exposure to ensure good water quality and reduce interaction of the test compound with waste materials (US EPA, 1996; ASTM, 2001). In the present study, fasting affected both CYP2E1-like activity and GSH levels in the male medaka. Fasting induces CYP2E1 activity in both rodents (Soh et al., 1996; Fry et al., 1999) and fish (Hong et al., 1987; Wall and Crivello, 1999). To avoid fasting effects on CYP2E1-like activity in medaka, we performed 96-h carcinogen exposures using twice-daily feedings, with exposure media renewal after each feeding. However, in GSH studies, fish were fasted to assess the response under standard 96-h exposure conditions.

Conducting a 96-h continuous exposure with fish under fed and renewal conditions vs. fasted and static conditions presented some difficulties. For example, increased fish handling results in elevated stress to the fish, and increased toxicant handling leads to a more hazardous work environment and increases the amount of test material required. The latter may be an important factor if limited amounts of the test compound are available.

The combination of fasting-induced CYP2E1 activity and decreased GSH may affect pharmacological or toxicological experiments. From these data, we recommend that fish studies employing compounds known or suspected to affect CYP2E1 or GSH activity should consider normal feeding vs. fasting during acute exposure in order to maintain the integrity of xenobiotic defenses. Fasting produces elevated CYP2E1 activity, probably by increasing CYP2E1 gene expression (Longo et al., 2000). Regardless of the mechanism, elevated CYP2E1 activity may affect the metabolism of some low-molecular-weight compounds that

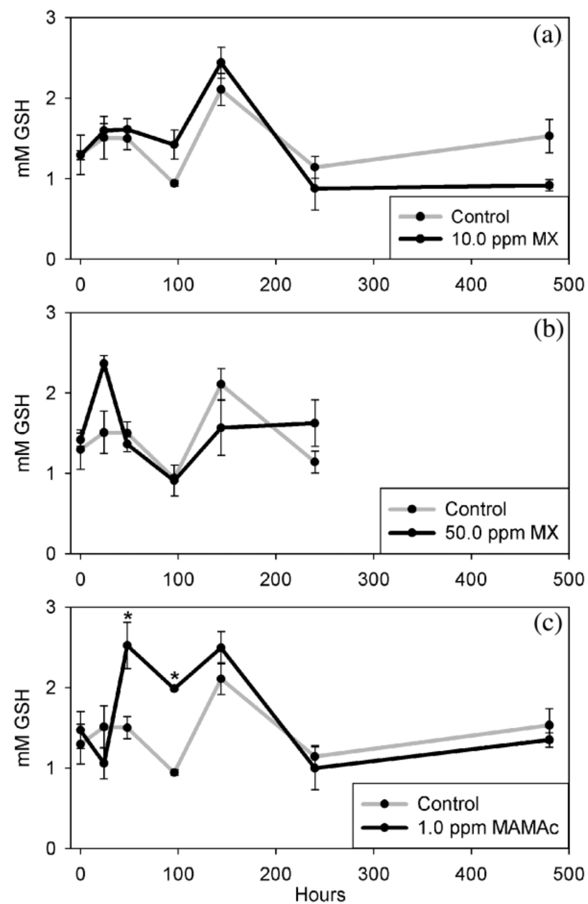


Fig. 5. Glutathione (GSH) levels in liver from medaka during and following 96-h exposure to (a) 10.0 ppm MX, (b) 50.0 ppm MX and (c) 1.0 ppm MAMAc. Fish were fasted during the 96-h exposure. Excess mortality precluded sampling of the 50.0 ppm treatment at 480 h. Values represent means ± S.E. from three fish per group. *Significantly different from control, $P < 0.05$.

require activation by CYP2E1 to exert their toxic or carcinogenic effect (Yang et al., 1990; Yoo et al., 1990; Guengerich et al., 1991; Raucy et al., 1993). In addition, fasting-mediated decreases in hepatic GSH have been implicated in impairment of GSH-dependent detoxification processes (Godwin and Wohaieb, 1988).

In our study, medaka hepatic S-9 fraction hydroxylated PNP, resulting in the formation of a single detectable metabolite, hydroxylated PNP (4-nitrocatechol). Hepatic S-9 activity in control CD1 mice correlated with published values of microsomal PNP metabolism of 1400 nmol/mg min (Gentest Corp, MA), assuming that 20–25% of S-9 protein is of microsomal origin (Duescher and Elfarra, 1993). In mammals, PNP metabolism is specific for CYP2E1 activity in liver microsomes (Dicker et al., 1990; Ryan and Levin, 1990; Koop, 1992; Jeong and Park, 1998). In medaka, however, the small amount of liver tissue available from each animal makes the preparation of microsomes from individual fish difficult. Use of hepatic S-9 fractions, however, is an effective alternative to microsomes when tissue volumes are limited (Mishin et al., 1996). Employing medaka hepatic S-9 fractions allowed for the assay of individual fish ($\geq$4.25 mg liver wet weight) rather than having to pool livers from several specimens for isolation of microsomes. Although PNP metabolism occurred in both male and female medaka hepatic S-9 fractions, male control activity was almost double that of female activity. This gender difference was also observed in mammals (Dekant et al., 1995) and the use of male rats for investigating CYP2E1 activity has been suggested (Morel et al., 1999).

In male, but not female medaka, CYP2E1-like activity was significantly increased in fasted fish that were treated with either ethanol or acetone, conditions known to induce CYP2E1 activity in mammals (Kraner et al., 1993; Song, 1995; Fry et al., 1999). Ethanol and acetone are often used as solvents for aquatic exposures, and in light of the CYP2E1 induction shown by these compounds in this and in mammalian studies, care should be taken when using organic solvents during aquatic exposures to minimize artificial elevation of CYP2E1 activity.

Cytochrome P450 2E1 activity in female control medaka was approximately half that observed from male controls, and treatments with CYP2E1 inducers were ineffective in females. The low PNP metabolism and lack of induction in females as compared to males may be attributed to lower levels of testosterone in females of reproductive age. Related studies in rodents show a link between testosterone, female CYP2E1 activity and nitrosamine activity, demonstrating that CYP2E1 was responsible for the bioactivation of selected nitrosamines and that testosterone post-transcriptionally regulated CYP2E1 protein expression (Pan et al., 1992; Kazakoff et al., 1994). In rodents, 1,2-dichlorobenzene, a compound that induces higher CYP2E1 activity in males than in females, is carcinogenic only to males (Nedelcheva et al., 1998). This may indicate a link for increased male medaka susceptibility to compounds that are bioactivated by CYP2E1. Although livers of control females had half the activity of males, the average liver weight of the female medaka was almost twice that of males ($9.49 \pm 0.60$ and $4.48 \pm 0.23$ mg wet weight, respectively, $n = 64$). This may denote equal CYP2E1-like enzymatic activity between the two sexes, with male medaka having a greater induction response due to testosterone-regulated CYP2E1 protein expression. However, CYP2E1 regulation is complex, involving transcriptional, pretranslational, translational and post-translational components (Song, 1995).

The addition of 50 μM of the CYP3A inhibitor TAO had no effect on PNP metabolism in control medaka S-9, indicating that CYP3A does not significantly contribute to the metabolism of PNP in these fish. In a similar experiment with winter flounder, microsomes pretreated with 80 μM TAO also showed no effect on the CYP2E1-specific substrate chlorzoxazone (CZX) metabolism (Wall and Crivello, 1998). Based on studies examining PNP metabolism in mammals, it was presumed that activity was primarily contributed by CYP2E and to a lesser extent by CYP3A (Zerilli et al., 1997). Prior studies in mammals have shown that a dose of 20 μM TAO is sufficient to inhibit 75–90% of CYP3A activity, yet have no effect on CYP2E activity (Chang et al., 1994).

Inhibition experiments using 50 μM of the CYP2E1 specific inhibitors DDC and DAS resulted in a significant reduction in medaka S-9 CYP2E1 by both compounds and in microsome samples by DAS only. In contrast, the same concentrations of DDC and DAS reduced CD-1 mouse S-9 CYP2E1 activity below detection limits. These data indicate that a medaka cytosolic enzyme may contribute to PNP metabolism, or the enzyme

responsible for PNP metabolism may not be identical to mammalian CYP2E1. This observation is supported by the lack of mammalian CYP2E1 antibody cross-reactivity in medaka microsomes (Lipscomb et al., 1997). Although the medaka cytochrome involved in this study and its genetic relationship to those of other species is currently unknown, this study shows that CYP2E1-like activity occurs in medaka liver.

Both MX and MAMAc induced PNP metabolism during the course of the 96-h exposure. MX induced a gradual increase in activity, with a significant increase noted at 96 h, whereas MAMAc induced significant increases at 48 and 96 h. The MAMAc 96-h sample activity was slightly less than that observed at 48 h. The highest PNP metabolism observed in medaka S-9 during the exposure to MX and MAMAc was 52.18 and 50.76 pmol/mg min, respectively. These levels are greater than the increases observed in fasting plus ethanol or acetone exposure, supporting the utility of the medaka PNP assay in detecting induction resulting from exposure to selected carcinogenic compounds.

GSH levels during and following exposure to MX usually paralleled that in control specimens. Control GSH levels were similar to those observed in other teleost species (Wallace, 1989; Gallagher et al., 1992; Almar et al., 1998). Increases in GSH are believed to be a compensatory response to xenobiotic exposure (Spector, 1991). This is consistent with the conjugation of GSH with reactive xenobiotic metabolites and reactive oxygen species (DeLeve and Kaplowitz, 1991). Fish exposed to xenobiotics, such as MX in pulp and paper mill effluents, show elevated levels of GSH. The lack of GSH involvement observed from MX-exposed fish in this study may imply that the liver is not the primary site of toxic involvement, that GSH is not involved in MX detoxification, or that the fish effectively cope with the compound under these experimental conditions. Rat and mouse hepatocytes exposed to MX underwent promotion of unscheduled DNA synthesis in vitro, but not in vivo, showing that the liver was not necessarily the target organ (Nunn et al., 1997).

In MAMAc exposure, GSH levels increased significantly at 48 and 96 h. Fish exposed to hydrocarbons (Thomas and Wofford, 1984a,b), pesticides (Davies, 1985; Gallagher et al., 1992), complex xenobiotic mixtures (Lindstrom-Seppa and Oikari, 1990; Stein et al., 1992; Nishimoto et al., 1995) and pulp and paper mill effluents (Oikari et al., 1988) have also shown elevated GSH levels. Once the fish were removed from the MAMAc exposure media and returned to a normal diet, levels returned to near that of controls and remained so until the end of the study. This indicates that the medaka recovered quickly from the toxicant challenge. Channel catfish also recovered quickly following an injection of diethyl maleate, with hepatic GSH levels significantly reduced at 6 h, followed by complete recovery at 24 h (Gallagher et al., 1992).

In summary, medaka liver microsome preparations and S-9 fractions catalyzed the hydroxylation of PNP, indicating CYP2E1-like activity. PNP metabolism in non-exposed male medaka was almost twice that of female activity, and fasted male medaka exposed to ethanol and acetone showed significant increases in activity. Also, fasting for 96 h significantly reduced total hepatic GSH. We recommend, therefore, that teleost studies investigating compounds with known or suspected CYP2E1 or GSH involvement should consider normal feeding vs. fasting during acute exposure in order to maintain the integrity of xenobiotic defenses. In addition, care should be taken when using ethanol or acetone as solvents for aquatic exposures, since both have CYP2E1-inducing properties. The CYP2E1 specific inhibitors DDC and DAS significantly reduced medaka PNP metabolism activity, but the same dose drastically reduced mouse control activity below detection limits, suggesting that the enzyme responsible for PNP metabolism in medaka is not identical to mammalian CYP2E1. Although the medaka cytochrome involved in this study and its genetic relationship to those of other species is currently unknown, this study confirms that CYP2E1-like activity occurs in medaka liver. Exposure to the carcinogens MX and MAMAc induced significant increases in hepatic CYP2E1-like activity, indicating that the enzyme may be involved in the biotransformation of both compounds. Only MAMAc produced a significant effect on total hepatic GSH. The results of this study strengthen the role of the medaka as a suitable species for examining cytochrome P450 and GSH detoxification processes and the role these systems play in chemical carcinogenesis.

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