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In vitro CYP1A activity in the zebrafish: temporal but low metabolite levels during organogenesis and lack of gender differences in the adult stage

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Abstract

The zebrafish (*Danio rerio*) is increasingly used as a screening model for acute, chronic and developmental toxicity. More specifically, the embryo is currently investigated as a replacement of *in vivo* developmental toxicity studies, although its biotransformation capacity remains a point of debate. As the cytochrome P450 1 (CYP1) family plays an important role in the biotransformation of several pollutants and drugs, a quantitative *in vitro* protocol was refined to assess gender- and age-related CYP1A activity in the zebrafish using the ethoxyresorufin-o-deethylase (EROD) assay. Microsomal protein fractions were prepared from livers of adult males and females, ovaries and whole embryo homogenates of different developmental stages. A large biological variation but no gender-related difference in CYP1A activity was observed in adult zebrafish. Embryos showed distinct temporal but low CYP1A activity during organogenesis. These *in vitro* data raise questions on the bioactivation capacity of zebrafish embryos in developmental toxicity studies.

Key words

Zebrafish, Embryo, Gender, CYP1A, EROD, Metabolism, Ontogeny, Microsomes

1. Introduction

The zebrafish embryo is gaining a lot of interest as an *in vitro* model to assess the teratogenic liability of drugs and chemicals. Initially intended as a screening tool [1], the focus on the zebrafish embryo is now more directed towards a reduction or even replacement of *in vivo* tests for developmental toxicity [2, 3]. The recent acceptance of this model as a validated alternative for acute toxicity testing of chemicals (Test Guideline 236) [4] also triggered its exploration as an alternative for other types of toxicity testing [5, 6]. As toxic and pharmacological effects may also be exerted by metabolites of the parent compound, knowledge on the bioactivation potential and overall biotransformation capacity is pivotal for a correct interpretation of toxicity studies in zebrafish. Differences in drug metabolism between zebrafish and man have been reported previously [7], and this has also been shown for other preclinical species, such as rat and dog [8]. However, the biotransformation capacity of zebrafish embryos and possible gender differences in adult zebrafish are still a point of debate and controversy [9, 10]. Gender-dependent differences in xenobiotic metabolism have been clearly observed in rats, which justify, for this species, the use of both genders in risk assessment. Differences have also been reported between women and men, but inter-individual variability is considered to be more important in humans [11, 12]. Therefore, knowledge on the presence or lack of gender-related biotransformation in the zebrafish may help in deciding whether one gender would be representative for the population or whether both genders are required in toxicity studies. Also for the zebrafish embryo, knowledge on its biotransformation capacity during organogenesis is key for developmental toxicity studies, as there is no maternal metabolism of the drug or chemical in contrast to *in vivo* studies in mammals. Zebrafish embryos are directly exposed to the compound via the medium

and as such a lack of or negligible bioactivation of the parent compound may lead to false negative results in the case of proteratogens, which impacts human risk assessment. The ultimate proof of lack or presence of bioactivation in the zebrafish embryo would be the absence or presence of malformations in embryos exposed to proteratogens. This has been done before [13]. However, the data are difficult to interpret, as one would need to be sure that only the metabolite(s) is/are teratogenic for the zebrafish embryo and not the parent compound itself. Additionally, other factors than bioactivation, such as absorption, distribution and excretion of the compound may complicate the results of a developmental toxicity study in the zebrafish. Therefore, we decided to first investigate the intrinsic biotransformation potential of zebrafish *in vitro*, i.e. by using microsomes of whole embryo homogenates and adult livers. These microsomes, which are subcellular fractions of endoplasmic reticulum obtained by (ultra)centrifugation steps, contain highly concentrated cytochrome P450 enzymes (CYPs) [14].

CYPs, and in particular the CYP1, CYP2 and CYP3 families are the most important groups of enzymes for bioactivation of xenobiotics [15]. For the zebrafish, the genetic features and synteny are well characterized for the different CYP isoforms, as is their expression in different organs. However, CYP activity data are either scarce or conflicting [7]. The CYP1 family is of a particular interest as it has a broad spectrum of substrates, including drugs and environmental pollutants [16]. The ethoxyresorufin-o-deethylase (EROD) assay is one of the most commonly used tests to assess CYP1 activity in humans and fish, including zebrafish [17, 18], especially for CYP1A1 and CYP1A, respectively [19, 20]. The high affinity of zebrafish CYP1A for ethoxyresorufin (ER) has been clearly demonstrated, as EROD activity was much lower in CYP1A morpholinos than in CYP1B1 morpholinos [21, 22].

However, differences in study design, including applied substrate concentration, gender, developmental stage, incubation temperature, etc. make the interpretation of the available EROD data for adult zebrafish and embryos very difficult (Table 1).

The aims of the present study were to set-up a standardized *in vitro* protocol to assess CYP1A activity in zebrafish and to apply this assay to different developmental stages during organogenesis to determine the biotransformation potential of zebrafish embryos during their critical window for teratogens. In addition, possible gender differences were assessed in adult zebrafish as this may explain discrepancies between studies and as such also help deciding whether both genders should be used in toxicity studies or not.

98 Table 1: Published *in vitro* studies on EROD kinetics (velocity) in adult zebrafish (liver or
99 whole body) and/or embryos, including number (n) of samples, gender (F: female, M: male),
100 incubation temperature, ethoxyresorufin (ER) concentration and (ultra)centrifugation
101 protocol (ND: not determined, ?: not known, hpf: hours post-fertilization, *: radius of rotation
102 not mentioned).

Study	**Velocity (pmol/min/mg protein)**		**Gender (Batches x n)**	**Temperature**	**ER concentration**	**(Ultra)centrifugation protocol**
	Adult liver	**Embryos**				
Troxel *et al*. (1997) [23]	23 ± 5	ND	3 x 2F	30°C	2 µM	1000 x g for 10 min
Mattingly and Toscano (2001) [24]	ND	≈ 0 at 24 and 48 hpf ≈ 0.5 at 72 hpf ≈ 0.5 at 96 hpf	ND	30°C	≈ 10 µM	2500 rpm* for 10 min
Arukwe *et al*. (2008) [25]	≈ 40 (whole body)	ND	? (3 x 1)	37°C	1.2 µM	12 000 x g for 20 min 100 000 x g for 60 min
Jonsson *et al*. (2009) [26]	260 ± 180	ND	? (3 x 5-7)	Room temperature	≈ 10 µM	10 000 x g for 15 min 105 000 x g for 60 min
Otte *et al*. (2010) [27]	ND	0.3 at 8 hpf 0.1 at 32 hpf 0.2 at 56 hpf 0.2 at 80 hpf 0.3 at 104 hpf 0.2 at 128 hpf	ND	Room temperature	≈ 11.4 µM	10 000 x g for 15 min
Halden *et al*. (2011) [9]	≈ 9 in F ≈ 10 in M	ND	4 x 2F 4 x 2M	Room temperature	0.5 µM	10 000 x g for 20 min
Pauka *et al*. (2011) [28]	≈ 18 (whole body – 2 weeks old)	ND	?	37°C	?	10 000 x g for 30 min

Gagnaire *et al.* (2013) [10]	5 in F 9 in M	ND	3 x 1F 3 x 1M	27±1°C	2 µM	9 000 x g for 10 min

2. Material and methods

2.1. Chemicals

Phenylmethanesulfonyl fluoride (PMSF), 1,10-phenanthroline (PHEN), Ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), Oil Red O, ER and resorufin (RS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). KH_2PO_4/K_2HPO_4 buffer was purchased from BD Gentest (Woburn, MA, USA). NADPH regenerating system solution A and B were obtained from Corning (Woburn, MA, USA). Total protein content was determined using the Pierce bicinchoninic acid assay (BCA Assay; Pierce Chemical, Rockford, IL, USA). This company also delivered the Halt Protease Inhibitor Cocktail®. Stock solutions of RS and ER were made by dissolving 1 mg in 50 ml and 5 ml DMSO, respectively. Aliquots were made and stored in dark reaction tubes (Greiner Bio-One GmbH, Frickenhausen, Germany) at -80°C. The absorbance was measured at 572 nm for RS and 482 nm for ER after diluting the stock solution 1:3 and 1:100 in 100 mM KH_2PO_4/K_2HPO_4 buffer. The exact concentrations were calculated using the extinction coefficient (ε RS =73.2 $M^{-1}.CM^{-1}$, ε ER =73.2 $M^{-1}.CM^{-1}$) [29, 30].

2.2. Animals and breeding

Adult (one to two years old) zebrafish (*Danio rerio,* in house wild type AB zebrafish line) were kept in glass aquaria of 60 L at a density of 1 fish/L, using a filtered system and day-night rhythm of 14/10 hours. The water parameters were 28 ± 1°C, 500 ± 40 $\mu S.cm^{-1}$ and 7.5 ± 0.3 of temperature, conductivity and pH. The water was

renewed at least once in a fortnight to keep the levels of ammonia, nitrite and nitrate below the detection limits. Fish were fed three times daily: twice daily with thawed food (*Artemia nauplii*, *Daphnia* or *Chironomidae* larvae) and once daily with granulated food (Biogran medium; Prodac International, Cittadella, Italy). For embryo collection, the fish were put in spawning tanks before the light was turned on. Embryos were collected 45 minutes after the start of spawning. Feces and coagulated eggs were removed and the embryos were gently washed using freshly prepared embryo solution, i.e. Instant Ocean® Sea Salt (Blacksburg, VA, USA) and sodium bicarbonate (VWR, Leuven, Belgium) dissolved in reverse osmosis water (conductivity 500 $\mu S.cm^{-1}$; pH 7.5). The embryos were kept in embryo solution under the same environmental conditions of light and temperature as for the adults. The embryo solution was renewed daily and dead embryos were removed. When the embryos reached the desired developmental stage, they were frozen in liquid nitrogen and stored at -80 °C to be used for the preparation of the embryo microsomal protein.

2.3. Tissue sampling

For the preparation of adult liver microsomes, each batch consisted of 10 females or 10 males. In total, five batches of each gender were prepared. Animals were fasted for 48 hours before they were euthanized by decapitation followed by a rapid destruction of the brains (Recommendation 2007/526/EC, Species Specific Section, Humane Killing Fish, p89). Livers were rinsed during the dissection process with pre-cooled washing buffer (100 mM KCl, 1 mM EDTA and 10 mM KH_2PO_4/K_2HPO_4 buffer at pH 7.4) to remove the remaining blood and avoid bile contamination. Additionally, both ovaries of 20 adult zebrafish were collected for microsomal protein preparation. For the embryo microsomal protein, five batches of embryos were collected for each of the six different developmental stages, i.e. 5, 24,

48, 72, 96 and 120 hpf (hours post-fertilization). In order to have a sufficient yield of microsomal protein, each batch consisted of at least 1500 embryos. The livers, ovaries and whole embryos were stored at -80°C until further use. The Ethical Committee of Animal Experimentation from the University of Antwerp (Belgium) approved the use of the animals in this study (ECD 2015-49).

2.4. Microsomal protein preparation

The protocol was adapted from Nilsen *et al*. (1998) [31]. All steps of the microsomal protein preparation were performed at 4°C. Homogenization buffer (100 mM KCl, 1 mM EDTA, 0.1 mM PMSF, 0.1 mM PHEN, 1 unit of protease inhibitor cocktail per 15 ml of the final volume and 100 mM KH_2PO_4/K_2HPO_4 buffer at pH 7.4) was added 1:1 v/v for all tissues, which were then homogenized using a Potter-Elvehjem Tissue Grinder (VWR, Radnor, USA) at 580 rpm. Ultrasonication was applied five times for five seconds with intervals of 10 seconds and 75% amplitude using Ultrasonic Processor VCX 130 (Sonics, Newtown, USA). Homogenates were centrifuged at 12,000 × g for 20 minutes and the supernatants were collected. During the first step of centrifugation of the liver homogenates, a high fat load was present on the surface of the supernatant, particularly in females. In the case of the embryos, a high load of melanophores contaminated the supernatant after the first centrifugation step, especially from 48 hpf onwards. Therefore, the protocol was adjusted with an additional centrifugation at 12,000 × g for 20 minutes to obtain more pure and clear supernatants. After removal of the remaining lipid layer from each sample, the supernatants were ultracentrifuged, using Optima™ MAX-TL ultracentrifuge (Beckman Coulter, Brea, USA), at 105,000 × g for 1 hour, after which the pellets were resuspended in the homogenization buffer. This ultracentrifugation step was repeated. The final microsomal pellets were suspended in a storage buffer

(homogenization buffer with 20% glycerol w/v), aliquoted and stored at -80ºC. The aliquots were used for EROD activity after determination of the protein concentration using the BCA Protein Assay. The reproducibility of this procedure was determined by evaluating different aliquots of one homogenate of liver tissues for protein content and EROD activity. Insect cell control Supersomes (BD Gentest, Woburn, MA, USA) and a pool of human liver microsomes (HLM) from 50 donors (HMMCPL-PL050B, Life Technologies, Thermo Fisher Scientific, Rockford, USA) were used as negative and positive controls, respectively.

2.5. Oil Red O staining

The livers of two males and two females were snap-frozen in liquid nitrogen. Cryosections were made and stained with Oil Red O to assess qualitatively possible gender differences in lipid content [32].

2.6. EROD assay

The incubation mixtures contained microsomal protein in 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4 with 5% and 1 % of NADPH regenerating system reagents A and B, respectively. Immediately after the start of the reaction that was induced by addition of the substrate (ER), fluorescence was measured at 28.5°C for 2 hours with intervals of 3 minutes using a Tecan GENios microplate reader (Tecan, Männedorf, Switzerland) at λ_{ex} 550 nm and λ_{em} 590 nm. A reaction temperature of 28.5°C was chosen as 26°C to 28.5°C is the optimal temperature range for zebrafish (embryos) [33]. For HLM, the EROD assay was performed at 28.5°C and 37°C to verify whether 28.5°C influenced the CYP activity, as 37°C is the body temperature for man. EROD activity is expressed as pmol of RS per mg microsomal protein per minute (pmol $RS.mg^{-1}.min^{-1}$). Standard concentrations of RS were made by diluting

199 the stock solution in 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4. Each experiment was
200 performed in triplicate at different days.

201 Based upon the papers from Mattingly and Toscano (2001) and Otte *et al*. (2010), an
202 initial concentration of 10 µM of ER was chosen to determine the optimal protein
203 concentration for the final experiments [24, 27]. This was conducted by incubation of
204 10 µM ER with 10 to 500 µg microsomal protein/ml (from 50 pooled livers of adult
205 male and female zebrafish). The enzyme kinetic parameters (K_m and V_{max}) of ER
206 biotransformation in adult zebrafish liver microsomes were determined using
207 different ER concentrations ranging from 0 to 40 µM.

208 *2.7. Data analysis*

209 The values of the negative control were subtracted at each time point for all samples.
210 Velocity of all reactions was calculated within the linear part of the reaction curve
211 and data are presented as mean ± SD. The limit of detection (LOD) was calculated
212 according to Armbuster and Pry (2008) [34]. The kinetic parameters of ER
213 biotransformation were determined using non-linear least-squares regression analysis
214 of untransformed data (GraphPad Software, La Jolla, CA, USA) and data were fit to
215 Michaelis Menten kinetics with an uncompetitive substrate inhibition model. The
216 estimated parameters were the maximum reaction velocity (V_{max}) and the substrate
217 concentration corresponding to 50% of the V_{max} (K_m). To assess normal distribution
218 of the data and homogeneity of variances, the one-sample Kolmogorov-Smirnov test
219 and Levene's test were used, respectively. A one-way ANOVA test (embryos) or *t*
220 test (adults) was applied for data that showed a normal distribution and no significant
221 differences among variances. When the data did not show a normal distribution, the
222 Kruskal-Wallis test for non-parametric data was applied and pairwise comparisons

were used to detect differences. Differences were considered significant when $p \leq 0.05$. All statistical analyses were performed by means of SPSS statistics version 20 (IBM, Armonk, NY, USA).

3. Results

3.1. Microsomal protein yield

The reproducibility of the preparation procedure for the microsomal protein from the same batch of zebrafish livers and its associated EROD activity was more than 90%. For the adult liver, which tended have larger volumes in females than in males, large inter-batch differences but no gender differences were present, i.e. 4.96 ± 2.84 and 4.57 ± 1.86 mg microsomal protein per 10 female and 10 male fish, respectively. For the ovaries, the microsomal protein yield was 1.07 ± 0.25 mg per fish. The yield of microsomal protein in the embryos increased significantly between 5 hpf and 72 hpf, after which it tended to slightly decrease again (Fig. 1).

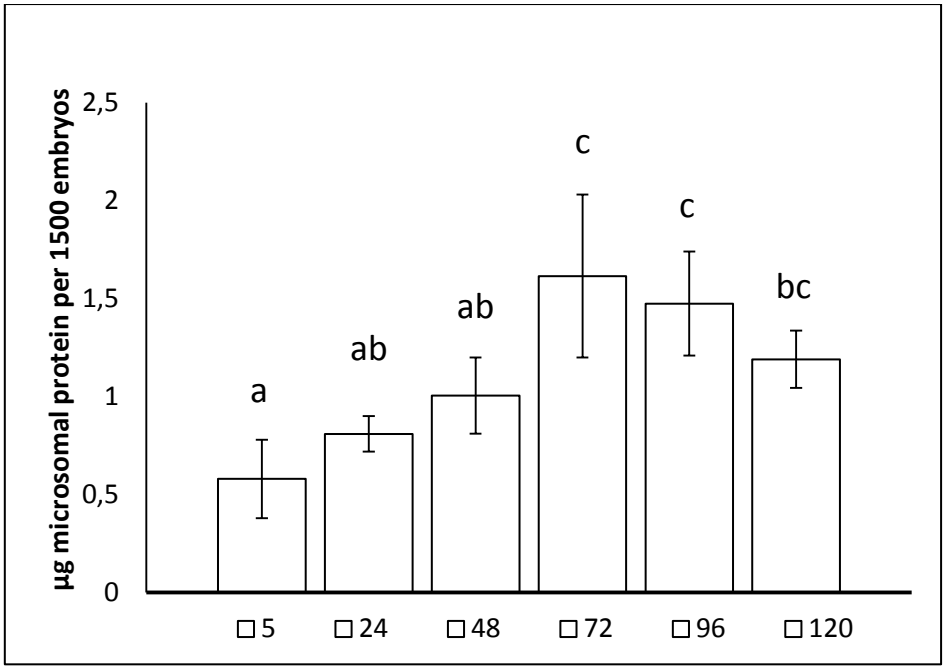

Figure 1: Microsomal protein yield of zebrafish embryos at different developmental stages (5, 24, 48, 72, 96 and 120 hpf). Bars represent the yield at each stage (mean ± SD, n = 5 batches of 1500 embryos). Significant differences ($p \leq 0.05$) between developmental stages are indicated by different letters.

3.2. Oil Red O staining

Female livers showed the presence of large lipid droplets in the hepatocytes whereas males showed a more condensed liver parenchyma (Fig. 2).

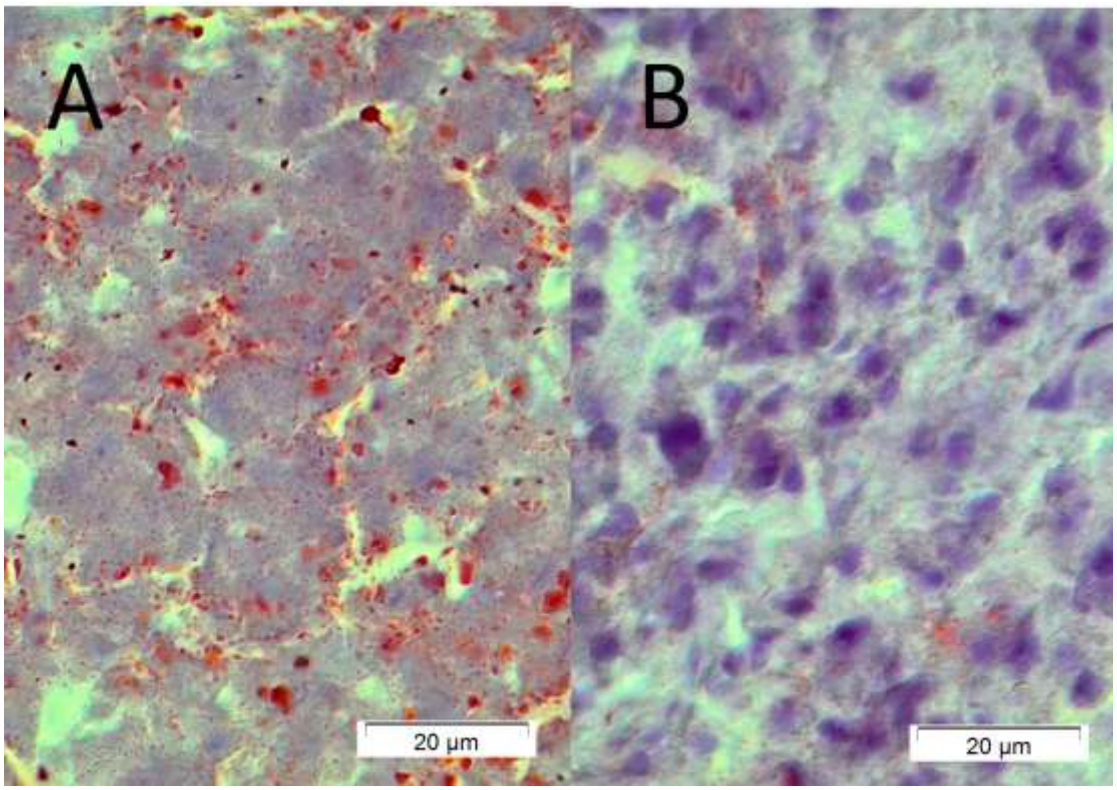

Figure 2: Oil Red O staining of adult zebrafish liver. A: Female liver tissue with high fat content (red stained dots). B: Male liver tissue with only scarce fat distribution and clear basophilic nuclei.

3.3. *EROD assay*

For the technical replicates, the repeatability of the EROD assay was more than 95% (Fig. 3). Additionally, HLM showed a similar EROD activity at 28.5°C and 37°C (difference less than 10%; data not shown). The LOD was 0.28 nM RS.

For the adult zebrafish liver microsomes, the optimal microsomal protein concentration was 100 µg/ml and enzyme kinetics showed a K_m of 0.63 ± 0.26 µM and a V_{max} 32.53 ± 5.04 pmol RS.mg^{-1}.min^{-1}. In view of the V_{max}, 1.2 µM ER (2-fold K_m) was used as substrate concentration for the final microsomal protein incubations of the different developmental stages, ovaries and different batches of adult female and male liver microsomes. EROD activity in the latter was 21.60 ± 4.70 and 17.60 ± 5.10 pmol RS.mg^{-1}.min^{-1} with inter-batch variations of 21.6% and 28.8%,

respectively. No gender differences were noted. HLM showed a lower EROD activity under the same conditions, i.e. 6.80 ± 0.31 pmol RS.mg^{-1}.min^{-1} (Fig. 3). The EROD activity in ovarian microsomes was 0.57 ± 0.33 pmol RS.mg^{-1}.min^{-1} with large inter-individual differences.

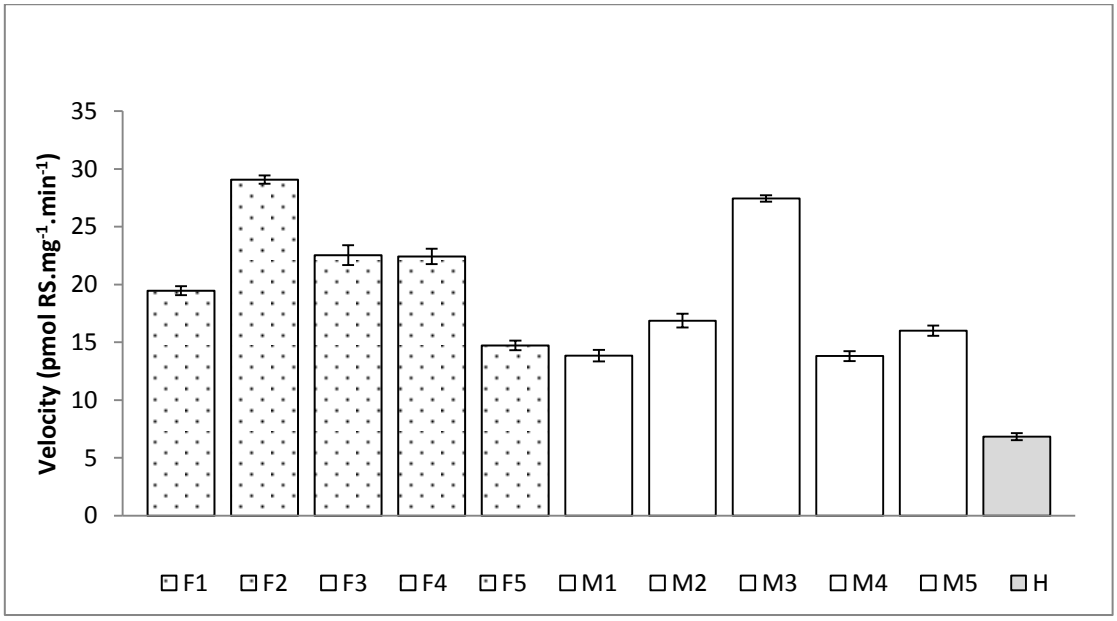

Figure 3: EROD activity in adult female (F) and male (M) zebrafish and human (H) liver microsomes as reference value. Bars represent the mean ± SD of 3 technical replicates of one batch (5 batches for each gender, 10 fish in each batch). No gender difference was observed.

The reaction velocity was much lower in the different developmental stages than in the adult zebrafish, but a temporal trend was present (Fig. 4). The highest EROD activity was detected at 5 hpf with a large inter-batch variation (1.50 ± 1.40 pmol RS.mg^{-1}.min^{-1}). At 24 hpf and 48 hpf, EROD activity dropped to negligible levels (0.33 ± 0.29 and 0.14 ± 0.15 pmol RS.mg^{-1}.min^{-1}), whereas EROD activity tended to increase again at 72 hpf (0.60 ± 0.50 pmol RS.mg^{-1}.min^{-1}) and even further at the end of organogenesis, i.e. 96 hpf (0.91 ± 0.47 pmol RS.mg^{-1}.min^{-1}). At 120 hpf, negligible EROD activity, i.e. 0.31 ± 0.20 pmol RS.mg^{-1}.min^{-1} was observed. EROD activity

was significantly lower at 48 hpf than at 5 hpf ($p \leq 0.05$) whereas 96 hpf showed a trend towards higher levels compared to 48 hpf ($p=0.068$).

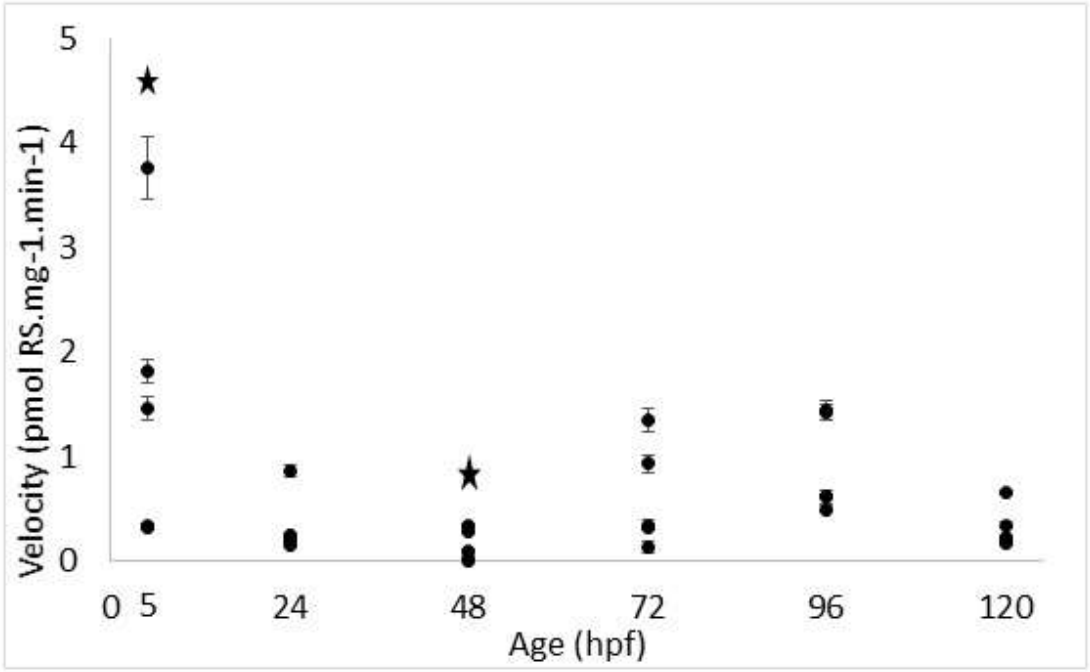

Figure 4: EROD activity of whole embryo microsomes at different developmental stages (5, 24, 48, 72, 96 and 120 hours post-fertilization (hpf)). Each developmental stage consists of 5 batches of pooled embryos (n= 1500/batch). The reaction velocity of each batch of embryos is plotted as a dot (i.e. mean ± SD of the 3 technical replicates). Stars represent significant differences ($p \leq 0.05$) between age groups.

4. Discussion

In vitro drug metabolism studies are part of the drug development process and can help in species selection for toxicity studies, i.e. the species with a metabolite profile the closest to man. However, other factors such as the expression of the target, pharmacokinetic data etc. are important as well. The most often used *in vitro* methods are: recombinant enzymes for reaction phenotyping, subcellular fractions such as microsomes or S9 fractions, and hepatocytes or liver slices. The use of recombinant

enzymes is an effective way to further unravel metabolic pathways but is too labor intensive and specific for a general screening of drug metabolism in several species. Cells and tissue slices of the liver are physiologically the most relevant samples to measure drug metabolism but enzymatic activities are not stable and obtaining and culturing cells from other organs can be very challenging [14]. Therefore, the most commonly used tools for *in vitro* drug metabolism studies are the S9 fraction and microsomes. The S9 fraction, which contains microsomes and cytosol, gives a more complete picture of drug metabolism, as not only CYPs but also a wide variety of other enzymes, including Phase II enzymes, flavin-monooxygenases etc. are present. However, for CYP activity studies microsomes are the preferred method as they contain almost exclusively CYPs [14]. This results in an approximately 4- to 5-fold higher CYP activity in the microsomal fraction than in the corresponding S9 fraction [14, 35]. Therefore, we also opted to use the microsomal protein fraction in our *in vitro* drug metabolism protocol and this also explains why lower EROD velocities were observed for the adult male and female liver [9, 10] and zebrafish embryos [27] in studies using the S9 fraction. As such, the S9 fraction may underestimate the CYP activity in zebrafish embryos and adults. Furthermore, our standardized protocol showed a high reproducibility (> 90%) for the preparation procedure of zebrafish microsomes, which makes it a very reliable technique to also investigate other CYP isoforms in this species.

Interestingly, the liver of male and female zebrafish showed a similar microsomal protein yield despite the fact that female livers tended to be larger. The latter was related to the higher lipid content compared to males, which became evident in the first centrifugation step after liver homogenization and was also confirmed histologically by the Oil Red O staining. These findings are in accordance with

317 Vliegenthart *et al.* (2014) who showed more vacuolated hepatocytes on paraffin
318 sections in female zebrafish than in males [36], and with Peute *et al*. (1978) who
319 studied the ultrastructure of female zebrafish livers and also found a high lipid
320 content, which they related to female reproductive function, i.e. vitellogenin synthesis
321 [37]. Besides the similar microsomal protein yield, we also noted similar EROD
322 activity in both genders, but with a large biological variation. The latter could explain
323 the large differences between earlier reported results on EROD activity in adult
324 zebrafish liver (Table 1). As these studies only used a small number of male and/or
325 female zebrafish, i.e. 3 to 4 groups of 2 to 3 adult fish [9, 10, 23], low or high EROD
326 activity in one fish will obviously influence the mean of the group. Large inter-
327 individual differences in CYP activity, often due to polymorphisms in CYP isoforms,
328 have also been reported in man [38] and are considered to be more important than
329 gender-related differences [11]. This is in accordance with our CYP1A data and as
330 such the zebrafish appears to be representative for human risk assessment, regardless
331 of the gender. However, this should be further substantiated for other CYP isoforms.
332 Furthermore, to avoid inter-individual variability as a confounder in EROD studies
333 with different treatment groups, we recommend using at least 5 batches of (livers
334 from) 10 fish (male, female or mixed gender) per group as we noted that the mean
335 EROD activity of these batches was in the same range of the Vmax that was
336 calculated from pooled liver microsomes of 50 male and female zebrafish.

337 For the embryos, the temporal increase in microsomal protein yield up to 72 hpf is
338 not surprising as the embryonic genome gets activated around gastrulation (5.25 hpf)
339 leading towards increased transcription and consecutive translation into proteins [39],
340 which are important for embryonic growth and development. However, the decrease
341 of the microsomal protein yield at later stages, i.e. 96 hpf and 120 hpf is intriguing.

342 Subfractionation (by using density gradients) of microsomes [40] from whole embryo
343 homogenates at different developmental stages could help to elucidate whether the
344 lower protein yield at 96 hpf and 120 hpf is due to a lower absolute amount of
345 microsomes or to a different composition of the microsomal fraction as microsomes
346 also contain phospholipids and RNA [41].

347 A temporal trend was also observed for CYP1A activity in the zebrafish embryos.
348 However, the activity was low during organogenesis, questioning the overall
349 biotransformation capacity of embryos in view of human risk assessment. Indeed,
350 when taking the inter-batch variation into account, the metabolite concentrations were
351 often just above the LOD in several of the developmental stages. Despite this fact,
352 subtle changes during development must be ongoing as Otte *et al.* (2010), who
353 investigated CYP1 activity directly in homogenate supernatants of a limited number
354 of zebrafish embryos (n = 25), showed a strikingly similar temporal trend for
355 embryos at 8, 32, 56, 80, 104 and 128 hpf [27]. The embryos at 8 hpf and 104 hpf had
356 the highest CYP1 activity, which corresponds with our 5 hpf and 96 hpf data. This
357 trend was further substantiated by strong EROD activity *in vivo* at these
358 developmental stages. Indeed, intense RS fluorescence was noted in the envelope
359 layer at 8 hpf and in the liver, intestine and circulatory system at 104 hpf, whereas a
360 much milder staining was observed in the other stages, especially at 32 and 56 hpf
361 [27]. The EROD activity in the earliest embryos is not surprising as it is mainly
362 maternally derived, which is further substantiated by the detection of CYP1A mRNA
363 in unfertilized eggs [42] and zebrafish ovaries, although CYP1 mRNA levels were
364 low in these latter [43]. This was also reflected by the low CYP1 ovarian activity that
365 was observed in our study, which may be due to a dilution effect by the stromal cells
366 in the ovary. The negligible CYP1 activity at 24 hpf and 48 hpf and the gradual

increase at 72 hpf and 96 hpf also corresponds well with the ontogeny of CYP1 mRNA in the zebrafish [43, 44]. In these studies, CYP1 mRNA was detected as early as 8 hpf with a decrease at 24 hpf and a peak at 72 hpf. These results are to some extent in accordance with the findings of Mattingly & Toscano (2001) who could not detect any CYP1A activity or proteins until 72 hpf, the time when the embryos hatched [24]. The higher CYP1 activity at 96 hpf, the end of organogenesis, and the clear drop at 120 hpf remains intriguing, as this was also observed *in vivo* by Otte *et al.* (2010). At 120 hpf the RS fluorescence was much less in the circulatory system (including the branchial arches, i.e. the future gills) and almost absent in the liver compared to the high intensity in these important metabolizing organs at 104 hpf [27]. The reason for this drop in CYP1A activity remains unclear and it is also in contrast with a study of Goldstone *et al.* (2009) who showed a peak of CYP1A mRNA at 120 hpf [45]. Furthermore, it is unknown when exactly zebrafish larvae attain adult CYP1A activity. From Pauka *et al.* (2011) we know that 2-week old zebrafish showed EROD activity that was similar to our adult data, whereas Alderton *et al.* (2010) showed only very low metabolite levels for several CYP substrates in 7-day old larvae [28, 46]. Hence, we can cautiously assume that CYP1A activity is fully mature in the zebrafish by 2 weeks of age.

In conclusion, our study showed that there is no gender difference in CYP1A activity in adult zebrafish but that large biological variability occurs both in adults and in embryos during organogenesis. Although a temporal trend in CYP1A activity was present, biotransformation remained low during organogenesis, which questions the bioactivation capacity of zebrafish embryos in developmental toxicity studies.

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6. References

[1] Chapin R, Augustine-Rauch K, Beyer B, Daston G, Finnell R, Flynn T, et al. State of the art in developmental toxicity screening methods and a way forward: a meeting report addressing embryonic stem cells, whole embryo culture, and zebrafish. Birth defects research Part B, Developmental and reproductive toxicology. 2008;83:446-56.
[2] Panzica-Kelly JM, Zhang CX, Augustine-Rauch K. Zebrafish embryo developmental toxicology assay. Methods in molecular biology. 2012;889:25-50.
[3] Beker van WA, Wolterbeek A, Te BL, Snel C, Menke A, Rubingh C, et al. A category approach to predicting the developmental (neuro) toxicity of organotin compounds: the value of the zebrafish (Danio rerio) embryotoxicity test (ZET). Reprod Toxicol. 2013;41:35-44.
[4] OECD. Test No. 236: Fish Embryo Acute Toxicity (FET) Test: OECD Publishing.
[5] Wang WD, Chen GT, Hsu HJ, Wu CY. Aryl hydrocarbon receptor 2 mediates the toxicity of Paclobutrazol on the digestive system of zebrafish embryos. Aquatic toxicology. 2015;159:13-22.
[6] Di Paolo C, Groh KJ, Zennegg M, Vermeirssen EL, Murk AJ, Eggen RI, et al. Early life exposure to PCB126 results in delayed mortality and growth impairment in the zebrafish larvae. Aquatic toxicology. 2015;169:168-78.
[7] Saad M, Cavanaugh K, Verbueken E, Pype C, Casteleyn C, Ginneken CV, et al. Xenobiotic metabolism in the zebrafish: a review of the spatiotemporal distribution, modulation and activity of Cytochrome P450 families 1 to 3. The Journal of toxicological sciences. 2016;41:1-11.
[8] Martignoni M, Groothuis GM, de Kanter R. Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. Expert opinion on drug metabolism & toxicology. 2006;2:875-94.
[9] Halden AN, Arnoldsson K, Haglund P, Mattsson A, Ulleras E, Sturve J, et al. Retention and maternal transfer of brominated dioxins in zebrafish (Danio rerio) and effects on reproduction, aryl hydrocarbon receptor-regulated genes, and ethoxyresorufin-O-deethylase (EROD) activity. Aquatic toxicology. 2011;102:150-61.
[10] Gagnaire B, Cavalie I, Camilleri V, Adam-Guillermin C. Effects of depleted uranium on oxidative stress, detoxification, and defence parameters of zebrafish Danio rerio. Archives of environmental contamination and toxicology. 2013;64:140-50.
[11] Mugford CA, Kedderis GL. Sex-dependent metabolism of xenobiotics. Drug Metab Rev. 1998;30:441-98.
[12] Waxman DJ, Holloway MG. Sex differences in the expression of hepatic drug metabolizing enzymes. Molecular pharmacology. 2009;76:215-28.
[13] Weigt S, Huebler N, Strecker R, Braunbeck T, Broschard TH. Zebrafish (Danio rerio) embryos as a model for testing proteratogens. Toxicology. 2011;281:25-36.

[14] Jia L, Liu X. The conduct of drug metabolism studies considered good practice (II): in vitro experiments. Current drug metabolism. 2007;8:822-9.
[15] Guengerich FP. Cytochrome P450 and chemical toxicology. Chem Res Toxicol. 2008;21:70-83.
[16] Zanger UM, Schwab M. Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. Pharmacology & therapeutics. 2013;138:103-41.
[17] Hegelund T, Ottosson K, Radinger M, Tomberg P, Celander MC. Effects of the antifungal imidazole ketoconazole on CYP1A and CYP3A in rainbow trout and killifish. Environmental toxicology and chemistry / SETAC. 2004;23:1326-34.
[18] Shimada T, Yamazaki H, Foroozesh M, Hopkins NE, Alworth WL, Guengerich FP. Selectivity of polycyclic inhibitors for human cytochrome P450s 1A1, 1A2, and 1B1. Chem Res Toxicol. 1998;11:1048-56.
[19] Scornaienchi ML, Thornton C, Willett KL, Wilson JY. Functional differences in the cytochrome P450 1 family enzymes from zebrafish (Danio rerio) using heterologously expressed proteins. Arch Biochem Biophys. 2010;502:17-22.
[20] Shimada T, Gillam EM, Sutter TR, Strickland PT, Guengerich FP, Yamazaki H. Oxidation of xenobiotics by recombinant human cytochrome P450 1B1. Drug Metab Dispos. 1997;25:617-22.
[21] Carney SA, Peterson RE, Heideman W. 2,3,7,8-Tetrachlorodibenzo-p-dioxin activation of the aryl hydrocarbon receptor/aryl hydrocarbon receptor nuclear translocator pathway causes developmental toxicity through a CYP1A-independent mechanism in zebrafish. Mol Pharmacol. 2004;66:512-21.
[22] Timme-Laragy AR, Noyes PD, Buhler DR, Di Giulio RT. CYP1B1 knockdown does not alter synergistic developmental toxicity of polycyclic aromatic hydrocarbons in zebrafish (Danio rerio). Mar Environ Res. 2008;66:85-7.
[23] Troxel CM, Buhler DR, Hendricks JD, Bailey GS. CYP1A induction by beta-naphthoflavone, Aroclor 1254, and 2,3,7,8-tetrachlorodibenzo-p-dioxin and its influence on aflatoxin B1 metabolism and DNA adduction in zebrafish. Toxicol Appl Pharmacol. 1997;146:69-78.
[24] Mattingly CJ, Toscano WA. Posttranscriptional silencing of cytochrome P4501A1 (CYP1A1) during zebrafish (Danio rerio) development. Dev Dyn. 2001;222:645-54.
[25] Arukwe A, Nordtug T, Kortner TM, Mortensen AS, Brakstad OG. Modulation of steroidogenesis and xenobiotic biotransformation responses in zebrafish (Danio rerio) exposed to water-soluble fraction of crude oil. Environmental research. 2008;107:362-70.
[26] Jonsson ME, Brunstrom B, Brandt I. The zebrafish gill model: induction of CYP1A, EROD and PAH adduct formation. Aquatic toxicology. 2009;91:62-70.
[27] Otte JC, Schmidt AD, Hollert H, Braunbeck T. Spatio-temporal development of CYP1 activity in early life-stages of zebrafish (Danio rerio). Aquat Toxicol. 2010;100:38-50.
[28] Pauka LM, Maceno M, Rossi SC, Silva de Assis HC. Embryotoxicity and biotransformation responses in zebrafish exposed to water-soluble fraction of crude oil. Bulletin of environmental contamination and toxicology. 2011;86:389-93.
[29] Klotz AV, Stegeman JJ, Walsh C. An alternative 7-ethoxyresorufin O-deethylase activity assay: a continuous visible spectrophotometric method for measurement of cytochrome P-450 monooxygenase activity. Analytical biochemistry. 1984;140:138-45.
[30] Prough RA, Burke MD, Mayer RT. Direct fluorometric methods for measuring mixed function oxidase activity. Methods in enzymology. 1978;52:372-7.
[31] Nilsen BM, Berg K, Goksoyr A. Induction of cytochrome P450 1A (CYP1A) in fish. A biomarker for environmental pollution. Methods in molecular biology. 1998;107:423-38.
[32] Penney DP, Powers JM, Frank M, Willis C, Churukian C. Analysis and testing of biological stains--the Biological Stain Commission Procedures. Biotechnic & histochemistry : official

publication of the Biological Stain Commission. 2002;77:237-75.
[33] Matthews M, Trevarrow B, Matthews J. A virtual tour of the Guide for zebrafish users. Lab animal. 2002;31:34-40.
[34] Armbruster DA, Pry T. Limit of blank, limit of detection and limit of quantitation. The Clinical biochemist Reviews / Australian Association of Clinical Biochemists. 2008;29 Suppl 1:S49-52.
[35] Zhang D, Luo G, Ding X, Lu C. Preclinical experimental models of drug metabolism and disposition in drug discovery and development. Acta Pharmaceutica Sinica B. 2012;2:549-61.
[36] Vliegenthart AD, Tucker CS, Del Pozo J, Dear JW. Zebrafish as model organisms for studying drug-induced liver injury. British journal of clinical pharmacology. 2014;78:1217-27.
[37] Peute J, van der Gaag MA, Lambert JG. Ultrastructure and lipid content of the liver of the zebrafish, Brachydanio rerio, related to vitellogenin synthesis. Cell and tissue research. 1978;186:297-308.
[38] Marinkovic N, Pasalic D, Potocki S. Polymorphisms of genes involved in polycyclic aromatic hydrocarbons' biotransformation and atherosclerosis. Biochemia medica. 2013;23:255-65.
[39] Giraldez AJ, Mishima Y, Rihel J, Grocock RJ, Van Dongen S, Inoue K, et al. Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. Science. 2006;312:75-9.
[40] Beaufay H, Amar-Costesec A, Thines-Sempoux D, Wibo M, Robbi M, Berthet J. Analytical study of microsomes and isolated subcellular membranes from rat liver. 3. Subfractionation of the microsomal fraction by isopycnic and differential centrifugation in density gradients. The Journal of cell biology. 1974;61:213-31.
[41] Amar-Costesec A, Beaufay H, Wibo M, Thines-Sempoux D, Feytmans E, Robbi M, et al. Analytical study of microsomes and isolated subcellular membranes from rat liver. II. Preparation and composition of the microsomal fraction. The Journal of cell biology. 1974;61:201-12.
[42] Goldstone JV, McArthur AG, Kubota A, Zanette J, Parente T, Jonsson ME, et al. Identification and developmental expression of the full complement of Cytochrome P450 genes in Zebrafish. BMC Genomics. 2010;11:643.
[43] Jonsson ME, Orrego R, Woodin BR, Goldstone JV, Stegeman JJ. Basal and 3,3',4,4',5-pentachlorobiphenyl-induced expression of cytochrome P450 1A, 1B and 1C genes in zebrafish. Toxicol Appl Pharmacol. 2007;221:29-41.
[44] Jonsson ME, Jenny MJ, Woodin BR, Hahn ME, Stegeman JJ. Role of AHR2 in the expression of novel cytochrome P450 1 family genes, cell cycle genes, and morphological defects in developing zebra fish exposed to 3,3',4,4',5-pentachlorobiphenyl or 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicol Sci. 2007;100:180-93.
[45] Goldstone JV, Jonsson ME, Behrendt L, Woodin BR, Jenny MJ, Nelson DR, et al. Cytochrome P450 1D1: a novel CYP1A-related gene that is not transcriptionally activated by PCB126 or TCDD. Arch Biochem Biophys. 2009;482:7-16.
[46] Alderton W, Berghmans S, Butler P, Chassaing H, Fleming A, Golder Z, et al. Accumulation and metabolism of drugs and CYP probe substrates in zebrafish larvae. Xenobiotica. 2010;40:547-57.