

# Cytochrome P450 1 Genes in Birds: Evolutionary Relationships and Transcription Profiles in Chicken and Japanese Quail Embryos

Maria E. Jönsson<sup>1,2\*</sup>, Bruce R. Woodin<sup>2</sup>, John J. Stegeman<sup>2</sup>, Björn Brunström<sup>1</sup>

<sup>1</sup> Department of Environmental Toxicology, Uppsala University, Uppsala, Sweden, <sup>2</sup> Biology Department, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts, United States of America

## Abstract

**Background:** Cytochrome P450 1 (*CYP1*) genes are biomarkers for aryl hydrocarbon receptor (AHR) agonists and may be involved in some of their toxic effects. *CYP1*s other than the *CYP1A*s are poorly studied in birds. Here we characterize avian *CYP1B* and *CYP1C* genes and the expression of the identified *CYP1* genes and *AHR1*, comparing basal and induced levels in chicken and quail embryos.

**Methodology/Principal Findings:** We cloned cDNAs of chicken *CYP1C1* and quail *CYP1B1* and *AHR1*. *CYP1C*s occur in several bird genomes, but we found no *CYP1C* gene in quail. The *CYP1C* genomic region is highly conserved among vertebrates. This region also shares some synteny with the *CYP1B* region, consistent with *CYP1B* and *CYP1C* genes deriving from duplication of a common ancestor gene. Real-time RT-PCR analyses revealed similar tissue distribution patterns for *CYP1A4*, *CYP1A5*, *CYP1B1*, and *AHR1* mRNA in chicken and quail embryos, with the highest basal expression of the *CYP1A*s in liver, and of *CYP1B1* in eye, brain, and heart. Chicken *CYP1C1* mRNA levels were appreciable in eye and heart but relatively low in other organs. Basal transcript levels of the *CYP1A*s were higher in quail than in chicken, while *CYP1B1* levels were similar in the two species. 3,3',4,5,5'-Pentachlorobiphenyl induced all *CYP1*s in chicken; in quail a 1000-fold higher dose induced the *CYP1A*s, but not *CYP1B1*.

**Conclusions/Significance:** The apparent absence of *CYP1C1* in quail, and weak expression and induction of *CYP1C1* in chicken suggest that *CYP1C*s have diminishing roles in tetrapods; similar tissue expression suggests that such roles may be met by *CYP1B1*. Tissue distribution of *CYP1B* and *CYP1C* transcripts in birds resembles that previously found in zebrafish, suggesting that these genes serve similar functions in diverse vertebrates. Determining *CYP1* catalytic functions in different species should indicate the evolving roles of these duplicated genes in physiological and toxicological processes.

**Citation:** Jönsson ME, Woodin BR, Stegeman JJ, Brunström B (2011) Cytochrome P450 1 Genes in Birds: Evolutionary Relationships and Transcription Profiles in Chicken and Japanese Quail Embryos. PLoS ONE 6(12): e28257. doi:10.1371/journal.pone.0028257

**Editor:** John A. Craft, Glasgow Caledonian University, United Kingdom

**Received:** October 12, 2011; **Accepted:** November 4, 2011; **Published:** December 2, 2011

**Copyright:** © 2011 Jönsson et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** Funding to MEJ and BB was from the Carl Tryggers Stiftelse and The Swedish Research Council Formas. Funding for BRW and JJS was from the United States National Institutes of Health (National Institute of Environmental Health Sciences), grants R01ES015912 and P42ES007381 to JJS. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

\* E-mail: maria.jonsson@ebc.uu.se

## Introduction

Members of the cytochrome P450 (CYP) superfamily of enzymes are present in most organisms, including bacteria, archaea, plants, fungi, and animals. They catalyze oxidative metabolism of various endogenous and exogenous compounds. Endogenous substrates include eicosanoids, cholesterol, bile acids, steroids, biogenic amines, vitamin D3, and retinoids [1,2]. Enzymes in the *CYP1*, *CYP2*, *CYP3*, and *CYP4* families also metabolize exogenous compounds, such as plant or fungal secondary metabolites, environmental pollutants, and pharmaceuticals [3,4]. The *CYP1* enzymes have been studied extensively because they can generate reactive and sometimes carcinogenic metabolites from environmental pollutants (e.g., polycyclic aromatic hydrocarbons, PAHs), but the interest in their endogenous functions is growing [e.g., [5]].

Genes in four *CYP1* subfamilies - *CYP1A*, *CYP1B*, *CYP1C*, and *CYP1D* - are expressed in fish and amphibians, while mammalian species express *CYP1A*, *CYP1B*, and in some cases *CYP1D* genes

[6,7,8,9,10,11,12]. In fish and the frog *Xenopus tropicalis*, expression of *CYP1A*, *CYP1B* and *CYP1C* genes is induced by exposure to agonists of the aryl hydrocarbon receptor (AHR), among the most potent being 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 3,3',4,4',5-pentachlorobiphenyl (PCB126); *CYP1D* genes do not seem to be inducible by AHR agonists [6,7,10,13].

Avian species vary substantially in sensitivity to embryo toxicity of halogenated aromatic hydrocarbons that activate the AHR [14,15]. Chicken embryos are particularly sensitive to these compounds and the effects of exposure *in ovo* include reduced hatchability, developmental abnormalities, and induction of *CYP1A*-catalyzed enzyme activity [16,17,18]. Japanese quail embryos are considerably less sensitive than chicken embryos to TCDD and PCB126, both in terms of embryo toxicity and ethoxyresorufin *O*-deethylase (EROD) induction [19,20,21]. The difference in sensitivity has been attributed to variations in a few amino acid residues in the AHR [15,22].

Birds have two *CYP1A* genes, *CYP1A4* and *CYP1A5*, which are orthologous to mammalian *CYP1A1* and *CYP1A2* [23] and which are inducible by AHR agonists [9,24]. At least some bird species also express *CYP1B1*; the constitutive localization of *CYP1B1* mRNA has been determined in embryonic chicken (*Gallus gallus*) and quail (*Coturnix coturnix japonica*) [8], but the inducibility of *CYP1B1* in birds has not been reported. A *CYP1C* gene was identified recently in the chicken genome [25], and *CYP1Cs* also appear in the Ensembl databases on the turkey (*Meleagris gallopavo*) and the mallard duck (*Anas platyrhynchos*) genomes. Expression of bird *CYP1Cs* at the transcript or protein level has not been studied at all.

The objectives of this work were to define some features of *CYP1* genes in birds, particularly the *CYP1Bs* and *CYP1Cs*. We cloned cDNAs of quail *CYP1B1*, chicken *CYP1C1*, and quail *AHR1*, and determined basal mRNA expression profiles of the full complement of *CYP1s* and *AHR1* in chicken and quail embryos. Induction of *CYP1s* was studied in early embryos and yolk sac membranes after *in ovo* exposure to PCB126. We also compared syntenies around *CYP1B* and *CYP1C* genes in birds to those in other vertebrate species. The results indicate remarkable conservation of some features of *CYP1* genes among vertebrates, although differences were also found among birds, and between birds and other vertebrates.

## Results

### Cloning and sequence comparisons

Using primers targeting the predicted chicken *CYP1C1* [25] we cloned and determined the sequence of a cDNA covering the full coding region (1637 bp) of the transcript (GenBank: JN656933). The cloned chicken *CYP1C1* nucleotide and deduced amino acid sequences showed 99.6% and 99.0% sequence identity to the predicted transcript and protein, respectively. Figure 1 shows the deduced amino acid sequence of chicken *CYP1C1* aligned with *X. tropicalis* *CYP1C1*, and zebrafish (*Danio rerio*) *CYP1C1* and *CYP1C2* [7]. *CYP1C*-like sequences found in the genomes of the turkey, mallard duck, and anole lizard (*Anolis carolinensis*) are also included in Fig. 1. Chicken *CYP1C1* showed 93% and 87% amino acid sequence identity with the corresponding regions of the turkey and mallard *CYP1Cs*, and the identity was higher in the substrate recognition site (SRS) regions (94% and 93% for turkey and mallard *CYP1C*). Compared with *X. tropicalis* *CYP1C1*, chicken *CYP1C1* exhibited 57% and 68% sequence identity in the full length protein and SRS regions, respectively. The anole and chicken *CYP1Cs* showed 54% and 69% sequence identity in the full length protein and SRS regions, respectively. Zebrafish *CYP1C1* and *CYP1C2* showed only 51% and 47% identity with chicken *CYP1C1* in the full protein whereas slightly higher identities were observed in the SRS regions (54% for *CYP1C1* and 49% for *CYP1C2*).

Extensive cloning efforts did not uncover a *CYP1C* ortholog in quail. PCR was performed with combinations of 12 forward and 10 reverse primers targeting *CYP1C1* regions that are conserved between the chicken, turkey, and mallard duck. In the reactions we used quail cDNA from whole embryos and from tissues which have a high *CYP1C1* expression in zebrafish (eye, brain, and heart [7]), or genomic DNA from a 4-day-old whole quail embryo. Amplification of quail cDNA using the quantitative real-time RT-PCR primers designed for chicken *CYP1C1* did yield a product, but that product was part of *CYP1B1*.

A cDNA resembling *CYP1B1* was cloned from quail (GenBank: JN656934), and a sequence with close similarity to *CYP1B1* was identified also in the zebra finch (*Taeniopygia guttata*) genome. The

cloned quail *CYP1B1* sequence was 950 bp long, corresponding to approximately 60% of a complete coding *CYP1B1* sequence, and the predicted protein included SRS 2–6 (Fig. 2). The deduced amino acid sequence of quail *CYP1B1* showed 99% identity with the corresponding region of the known chicken *CYP1B1*, and the SRS regions available in both predicted proteins (SRS 3–6) were identical. The quail *CYP1B1* and the predicted zebra finch *CYP1B1* showed 96% amino acid identity for the cloned segment and for the SRS regions. Quail *CYP1B1* also showed 69%, 60%, and 58% sequence identity with same region of *CYP1B1s* in human, *X. tropicalis*, and zebrafish, and higher degrees of identity in the SRS regions (75%, 65%, and 69%, respectively).

We also cloned a cDNA for quail *AHR1* (corresponding to amino acid numbers 231–395 of chicken *AHR1*), which includes most of the AHR ligand-binding domain (GenBank: JN656935). Figure 3 shows the translated cloned quail *AHR1* sequence aligned with AHR proteins in seven birds, *X. tropicalis*, human, mouse, and zebrafish. Two clades of AHR proteins have been identified in fish and birds, the *AHR1s* and *AHR2s*. The quail *AHR1* showed 99% sequence identity with *AHR1* in other birds, while lower identities (62–72%) were obtained when compared to the *AHR2* in chicken, albatross, and cormorant. Quail *AHR1* showed 82% sequence identity to a third predicted AHR (*AHR1B*-like) protein found in the chicken genome (located next to *AHR2* on chromosome 7). The quail *AHR1* sequence showed 70%, 84%, and 75% identity to the *AHR1A*, *AHR1B*, and *AHR2* proteins in zebrafish, respectively. (Accession numbers of all *CYP1C1*, *CYP1B1*, and *AHR* genes mentioned here are shown in Table 1).

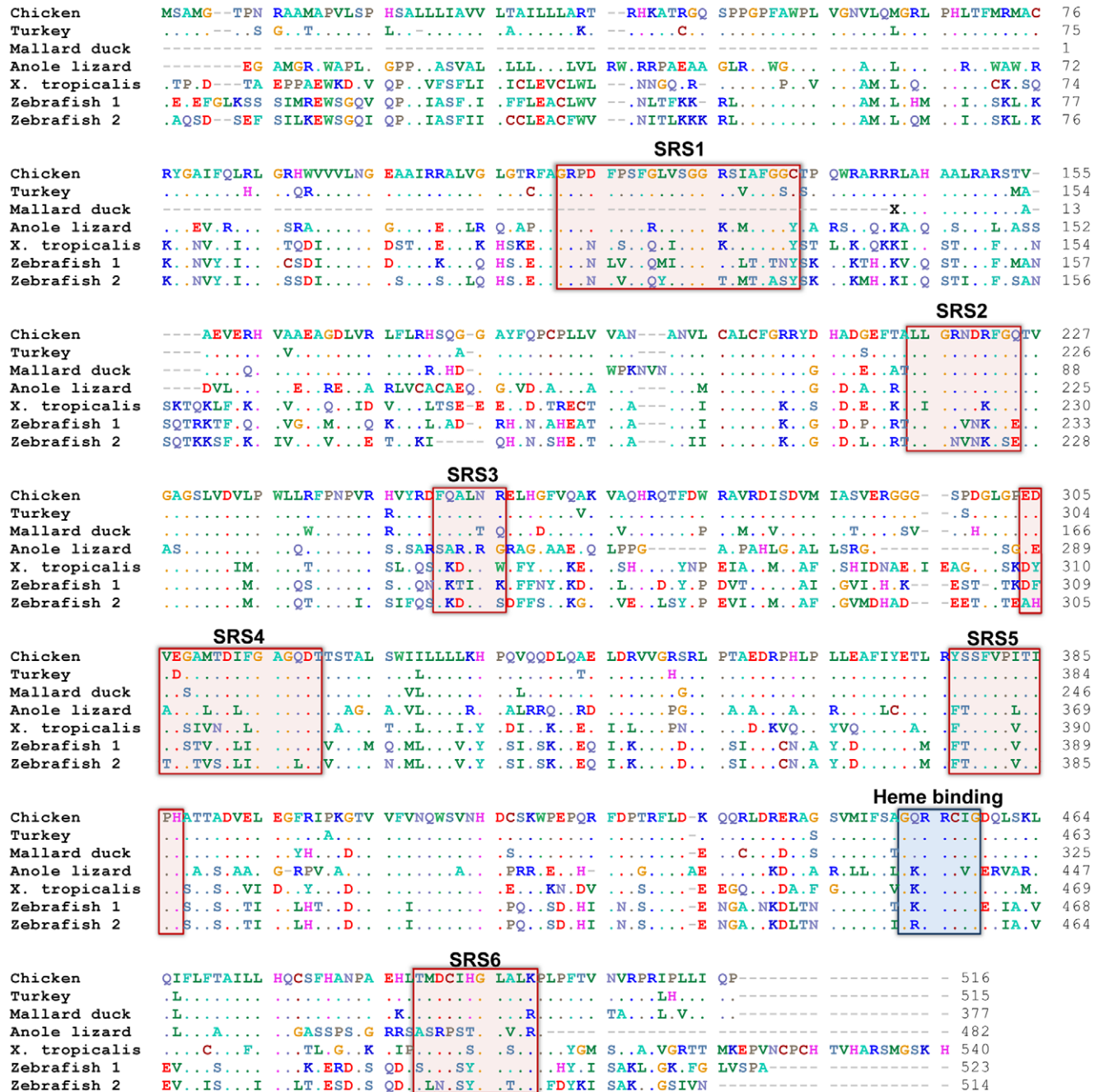
### Dioxin response elements

Putative dioxin response elements (DREs) were sought in the promoter regions of the chicken, turkey, and mallard *CYP1C* genes, using the sequence 5'-(T/G)NGCGTG-3' [26,27]. Within 10 kb upstream from the start codons of the *CYP1C* genes two putative DREs were found in chicken (at –458 and –1671 bp) and one was found in turkey (at –9193 bp). In the mallard duck genome database (version 1, Pre Ensembl) a fragment in the beginning of the *CYP1C* gene is unidentified (including approximately the first 140 nucleotides downstream from the start codon); putative DREs were found located at about 3600, 5500, and 5800 bp upstream from the 5'-edge of the unidentified region.

### Syteny

In order to examine the degree of conservation of the genomic region around the *CYP1C* locus we identified the three genes closest on either side of *CYP1C1* in chicken (*RPUSD2* - *CASC5* - *RAD51* - [*CYP1C1*] - *FAM82A2* - *GCHFR* - *DNAJC17*), and localized the genomic position of orthologs to these genes in various species (Fig. 4). Our results indicate that all seven genes have the same order in *X. tropicalis*, anole, chicken, and mallard, while *CASC5* is absent in turkey (Fig. 4). Mouse and human have the same arrangement of these genes, except that *CYP1C1* is missing (Fig. 4). In the zebra finch genome, a segment including *RPUSD2*, *CASC5*, and *RAD51* was found located 900 kb downstream from *FAM82A2*, *GCHFR*, and *DNAJC17* on chromosome 5 (and *CYP1C1* was missing). In zebrafish, *RAD51* and *FAM82A2* were found next to each other on chromosome 20 whereas the two *CYP1C* paralogs are arranged in tandem on another chromosome [7] (the zebrafish *CYP1Cs* were mapped to chromosome 17 in previous zebrafish genome assemblies, but this mapping has not been confirmed in Zv9 as yet). No shared syteny with chicken is found near upstream from the two zebrafish *CYP1Cs*, but orthologs to *GCHFR* and *DNAJC17* are located

# CYP1C



**Figure 1. Cloned chicken CYP1C1 deduced amino acid sequence aligned with orthologous proteins in other species.** Accession numbers are shown in Table 1. doi:10.1371/journal.pone.0028257.g001

downstream from the *CYP1Cs* (Fig. 4). Zebrafish *RPUSD2* and *CASC5* were found on chromosomes 17 and 1, respectively. Three-spined stickleback showed a syntenic arrangement similar to that of zebrafish for these genes (in the stickleback *RPUSD2* and the *CYP1Cs* are located on the same chromosome 3.3 Mb apart).

In all species examined here, *CYP1B1* was found adjacent to a *FAM82A2* paralog, *FAM82A1* (or *FAM82A*; Fig. 4).

In chicken and zebra finch, no *CYP1D1* ortholog was found in the region of *TMCI*, *ALDH1A1*, and *ANAX1*, which is the location

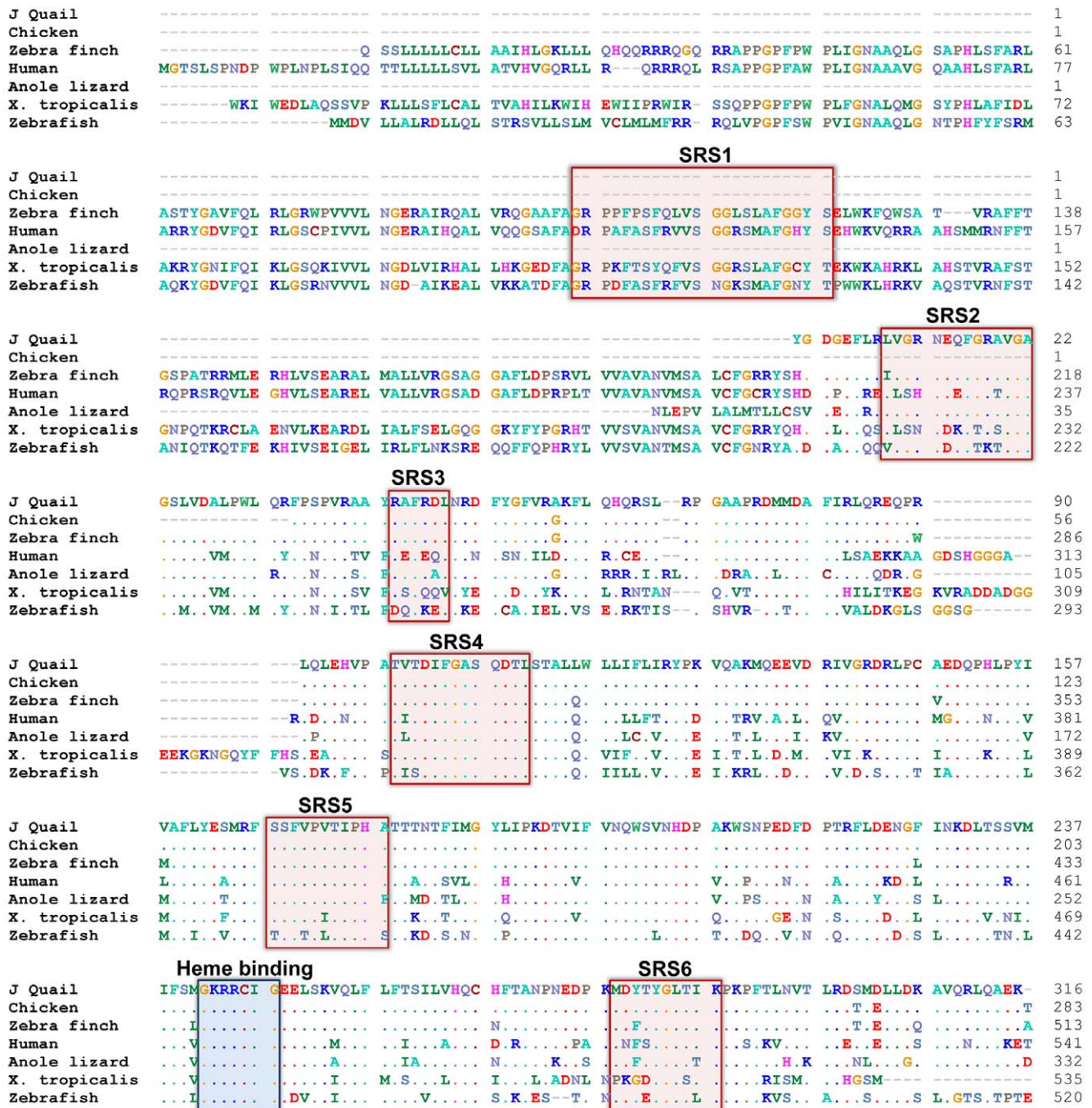
for *CYP1D1* in rhesus monkey, zebrafish, anole lizard, *X. tropicalis*, and other species [12,28]. Neither was *CYP1D1* found by blast searches in the chicken and zebra finch genomes.

## Tissue distribution patterns of *CYP1* and *AHR1* mRNA

Basal levels of *CYP1* and *AHR1* expression were determined in liver, chorioallantoic membrane (CAM), eye, brain, heart, and yolk sac membrane (YSM) in chicken and quail sampled on incubation day 13 and 11, respectively (equivalent developmental



# CYP1B1



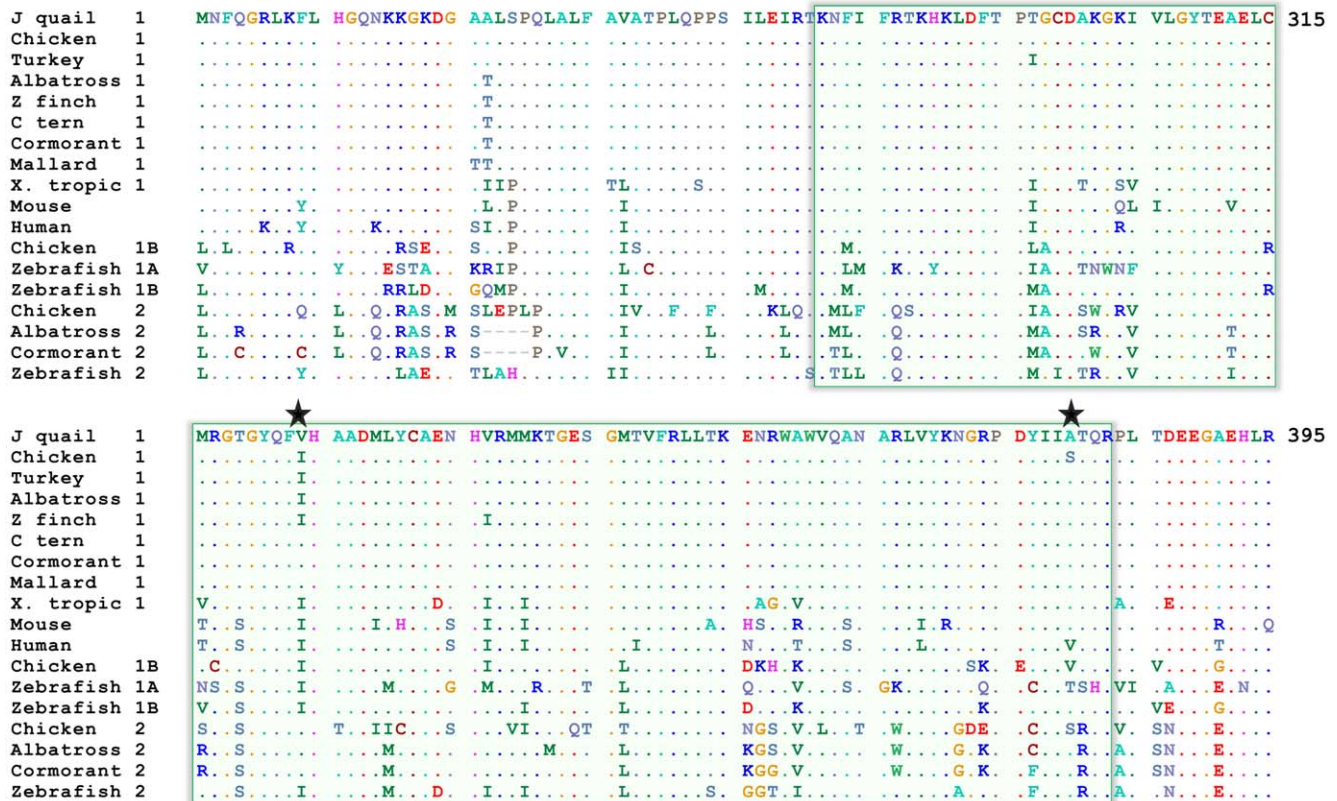
**Figure 2. Cloned Japanese quail CYP1B1 deduced amino acid sequence aligned with orthologous proteins in other species.** Accession numbers are shown in Table 1. doi:10.1371/journal.pone.0028257.g002

stages). Overall, the distribution patterns were very similar in the two species. In both chicken and quail, the liver showed the strongest expression of *CYP1A4* followed by CAM and eye (Fig. 5). *CYP1A5* was considerably more strongly expressed in liver than in other tissues and *CYP1B1* was strongly expressed in eye, brain, and heart in both species (Fig. 5). However, our results suggest the levels of *CYP1A4* and *CYP1A5* mRNA were much higher in quail than in chicken, whereas *CYP1B1* was expressed at roughly similar

levels in the two species. The expression levels of *AHR1* mRNA were fairly similar in liver, CAM, eye, brain, and heart in chicken while a somewhat larger variation was observed for *AHR1* expression among these tissues in quail (Fig. 5). YSM showed the lowest *AHR1* expression in both species (Fig. 5). We also analyzed *CYP1C1* mRNA expression in chicken; the eye showed the highest level followed by heart while other organs showed relatively low levels (Fig. 5). The reference gene, elongation factor

### AHR ligand binding domain

### PAS B



**Figure 3. Amino acid sequence alignment of AHR ligand binding domains in Japanese quail and other species.** In the figure “1”, “1A”, “1B”, and “2”, denote AHR1, AHR1A, AHR1B, and AHR2. Abbreviations: J quail = Japanese quail, C tern = common tern, Z finch = zebra finch, and X. tropic = *Xenopus tropicalis*. Accession numbers are shown in Table 1. doi:10.1371/journal.pone.0028257.g003

1 alpha (*EF1A*), seemed to be expressed at a similar level in chicken and quail, and in both species the six different tissues showed only small variations in *EF1A* mRNA expression levels (Fig. 5).

The expression patterns of the various *CYP1s* within a tissue were compared in chicken and quail (Fig. 6). The results suggest that the *CYP1As* and *CYP1B1* were expressed to a roughly similar level in chicken liver whereas the *CYP1As* were much more strongly expressed than *CYP1B1* in quail liver (Fig. 6). In both species *CYP1A4* appeared to be the most strongly expressed and *CYP1A5* the most weakly expressed of the *CYP1s* in CAM, whereas *CYP1B1* seemed to be the dominant *CYP1* transcript in eye, brain, and heart (Fig. 6). *CYP1C1* was rather strongly expressed in the eye (in chicken). The expression patterns in YSM varied between the two species, *CYP1B1* looking more strongly expressed than the *CYP1As* in chicken YSM, whereas the opposite was observed in quail.

#### Expression patterns of *CYP1* and *AHR1* mRNA during early chicken embryo development

Expression of the four *CYP1s* and *AHR1* was determined in whole-body samples of unexposed chicken embryos collected on incubation days 1, 2, 3, 5, and 7 (Table 2). We found that all transcripts were expressed at all sampling time points. During the whole 7-day period studied a similar pattern appeared: *CYP1B1* was the most strongly expressed and *CYP1A5* was the second most strongly expressed of the *CYP1s*, while expression of *CYP1A4* and *CYP1C1* was considerably weaker than that of *CYP1B1* (Table 2).

The time course of *CYP1* mRNA expression in whole-body samples from incubation day 3 to 7 is shown in Figure 7. The day 1 and day 2 embryos gave small total RNA yields, and less RNA was used in the assay for these samples than for older embryos. The embryos from the first two sampling times were therefore not included in the time course analysis. Within the study period *CYP1B1*, *CYP1C1*, and *AHR1* expression levels were relatively stable, while expression of *CYP1A4* and *CYP1A5* showed an increase from day 5 to day 7 (Fig. 7).

Table 3 shows the levels of *CYP1* and *AHR1* expression in whole-body, YSM, and CAM samples on day 7. While *CYP1B1* was the most strongly expressed of the *CYP1s* in the whole-body samples and CAM, *CYP1A5* showed the strongest expression in YSM (Table 3). In CAM the *AHR1* was more strongly expressed than the *CYP1s*.

#### *CYP1* mRNA induction by PCB126

The effect of PCB126 on *CYP1* mRNA expression was determined in YSM and whole-body of chicken and quail embryos sampled 24 hours after injection. The control levels in whole-body and YSM were roughly similar in chicken and quail except for the level of *CYP1A4* in YSM, which was much higher in quail than in chicken. Chicken and quail eggs were injected with 0.2 and 200 µg PCB126 kg<sup>-1</sup>, respectively, doses that were shown by Brunström and Halldin [19] to induce hepatic EROD activity to a similar level in embryos of the two species. In chicken the PCB126 exposure induced expression of all four *CYP1s* in both



**Table 1.** GenBank or Ensembl accession numbers of the studied transcripts.

Species	Gene	Number
<b>Chicken</b>	<b><i>CYP1C1</i></b>	<b>JN656933 (cloned)</b>
<b>Turkey</b>	<i>CYP1C1</i> -like	ENSMGAG00000015774
<b>Mallard duck</b>	<i>CYP1C1</i> -like	ENSAPLG00000001387
<b>Anole lizard</b>	<i>CYP1C1</i> -like	ENSACAG00000013750
<i>Xenopus tropicalis</i>	<i>CYP1C1</i>	HQ018042
<b>Zebrafish</b>	<i>CYP1C1</i>	NM001020610
<b>Zebrafish</b>	<i>CYP1C2</i>	NM001114849
<b>Japanese quail</b>	<b><i>CYP1B1</i></b>	<b>JN656934 (cloned)</b>
<b>Chicken</b>	<i>CYP1B1</i>	XP419515
<b>Zebra finch</b>	<i>CYP1B1</i> -like	XP002191325
<b>Human</b>	<i>CYP1B1</i>	NP000095
<b>Anole lizard</b>	<i>CYP1B1</i> -like	XP003216002
<i>Xenopus tropicalis</i>	<i>CYP1B1</i>	HQ018041
<b>Japanese quail</b>	<b><i>AHR1</i></b>	<b>HM053555, JN656935 (cloned)</b>
<b>Chicken</b>	<i>AHR1</i>	AAF70373
<b>Turkey</b>	<i>AHR1</i>	XP003207170
<b>Albatross</b>	<i>AHR1</i>	BAC87795
<b>Zebra finch</b>	<i>AHR1</i>	XP002188964
<b>Common tern</b>	<i>AHR1</i>	AF192503
<b>Cormorant</b>	<i>AHR1</i>	BAD01477
<b>Mallard duck</b>	<i>AHR1</i>	AF192501
<i>Xenopus tropicalis</i>	<i>AHR1</i>	CX900378
<b>Mouse</b>	<i>AHR</i>	NM013464
<b>Human</b>	<i>AHR</i>	AAH70080
<b>Chicken</b>	<i>AHR1B</i> -like	ENSGALG00000004322
<b>Zebrafish</b>	<i>AHR1A</i>	NP571103
<b>Zebrafish</b>	<i>AHR1B</i>	AAY42958
<b>Chicken</b>	<i>AHR2</i>	XP421887
<b>Albatross</b>	<i>AHR2</i>	BAC87796
<b>Cormorant</b>	<i>AHR2</i>	BAF64245
<b>Zebrafish</b>	<i>AHR2</i>	CAK11168
<b>Japanese quail</b>	<b><i>EF1A</i></b>	<b>JN656936 (cloned)</b>

doi:10.1371/journal.pone.0028257.t001

YSM and whole-embryos (Fig. 8). Chicken *CYP1C1* was induced 2-fold compared with the control in both sample types, but the control level of this transcript was 10-fold higher in the whole-embryos than in the YSM. In quail, exposure to the 1000 times higher dose of PCB126 resulted in a significant induction of *CYP1A4* in both YSM and whole-embryos; *CYP1A5* showed induction in whole-embryos and a tendency for induction in YSM, whereas *CYP1B1* expression was not significantly affected by the PCB126 exposure in quail (Fig. 8).

## Discussion

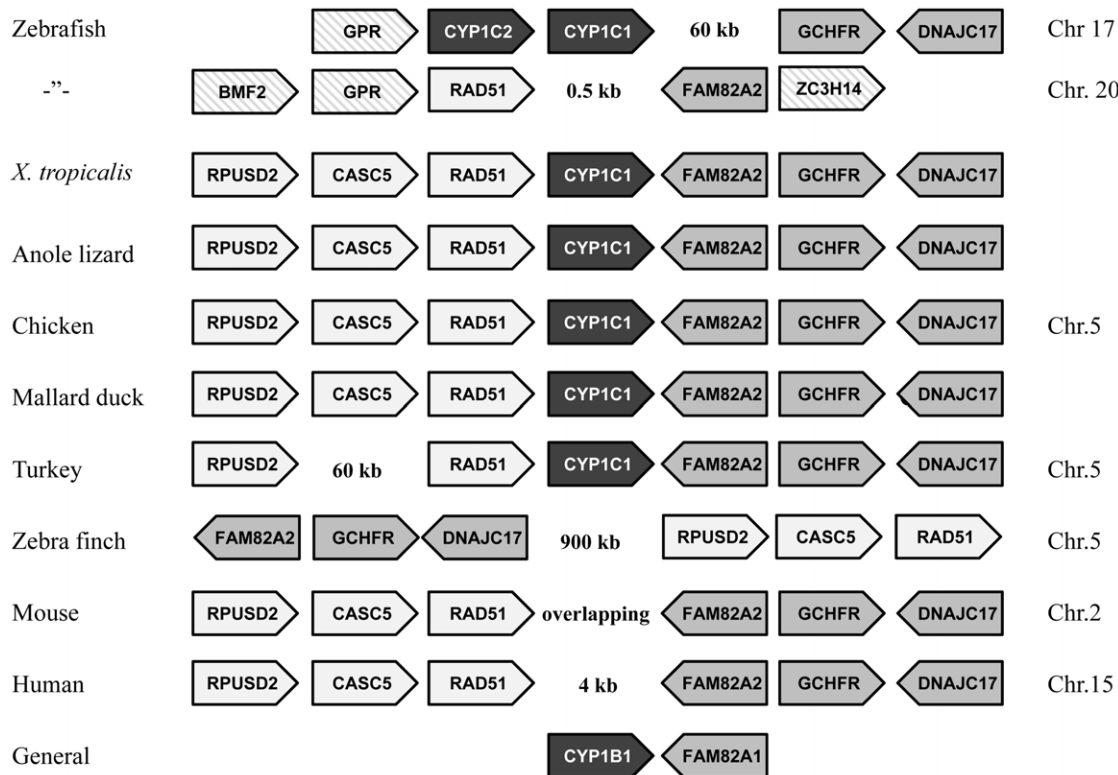
This study deals with *CYP1* genes and their expression in birds, focusing particularly on members of the *CYP1B* and *CYP1C* subfamilies. Phylogenetic analyses identify two major subclades in the vertebrate *CYP1* family, one comprising the *CYP1A*s and *CYP1D*s and the other comprising the *CYP1B*s and *CYP1C*s [13,29]. Our results establish that the *CYP1B/1C* subclade, as well

as the *CYP1A/1D* subclade, occurs in birds (although *CYP1D* genes seem to be missing). Genes related to the vertebrate *CYP1* genes have been found in the sea urchin *Strongylocentrotus purpuratus* (*CYP1*-like genes) and in the sea squirts *Ciona intestinalis* and *Ciona savignyi* (*CYP1E1* and *CYP1F1-CYP1F4*; Fig. 9), suggesting that *CYP1*-like genes were present in animals even before the Cambrian explosion [25,30]. Goldstone et al. [25] found that the sea squirt genes can be assigned to either the *CYP1A/1D* subclade (*CYP1E1*) or the *CYP1B/1C* subclade (*CYP1F*s) whereas the sea urchin genes do not fall into either of these subclades. This suggests that the two *CYP1* subclades were established in early chordates (Fig. 9) [25].

The two subclades, multiple subfamilies, and several pairs of paralogs indicate that the *CYP1* gene family has undergone several duplication events. Duplication of whole genomes, genomic segments, or single genes are believed to be important for evolution of new functions [31]. Genes are also lost over time of evolution, supposedly as they become superfluous. *CYP1D1* is expressed in fish, *X. tropicalis*, and the cynomolgus monkey (*Macaca fascicularis*), while it is a pseudogene in human and some other mammals [6,12,13,28]. Surprisingly, *CYP1D* has yet to be found in any bird species; whether it was lost early in the avian line is an important question. The *CYP1C*s appear to have been lost in mammals generally [29].

Our findings hint that the role of *CYP1C*s is weakening also in birds. Chicken, quail, turkey, and mallard duck belong to the superorder Galloanserae (orders Galliformes and Anseriformes), while zebra finch belongs to the superorder Neoaves (order Passeriformes), which appears to have undergone a rapidly radiating evolution, comprising 95% of extant species [32]. Among the species studied, there was no clear evolutionary trend in the presence/absence of *CYP1C1*, i.e., it was found in chicken, turkey, and mallard, but not in their close relative quail. Furthermore, no hit for *CYP1C* was obtained in blast searches of the zebra finch genome database. Turkey *CYP1C* was predicted to be a pseudogene in Ensembl, having one small intron (5'-CCCC-3'). This could be an inaccurate prediction due to sequencing error since removal of one cytosine and use of the intron as a codon results in a translated protein sequence highly similar to the chicken and mallard *CYP1C*s (Fig. 1). However, only one putative DRE was found within 10 kb upstream from turkey *CYP1C* (at about 9 kb upstream from the start codon), which raises a question about the inducibility of this gene via the AHR. The mallard *CYP1C* gene had three putative DREs in the upstream promoter region, but gene prediction is uncertain since part of the promoter sequence (and the start codon) was unidentified. In the zebra finch two *CYP*s that are not *CYP1C* orthologs were found at other places on chromosome 5. These genes were *CYP2R1* (vitamin D 25-hydroxylase) and *CYP46A1* (cholesterol 24-hydroxylase) and the regions around these *CYP*s exhibited a high degree of shared synteny in zebra finch, chicken, and turkey. In the zebra finch, genomic rearrangement appears to have occurred precisely at the location equivalent to that of *CYP1C1* in the other birds (Fig. 4). Whether this represents true genomic rearrangement rather than misassembly is not clear at present. That some *CYP1C1* roles may be diminishing in birds is suggested also by the relatively low basal level of *CYP1C1* mRNA expression in chicken embryos (except in the eye and heart) and that *CYP1C1* is only slightly inducible by PCB126 in birds, while *CYP1C1* is relatively strongly inducible in fish [7,10,33,34,35]. To resolve the evolutionary fate of *CYP1C1* in birds, this gene needs to be studied in a larger number of species.

The region around the *CYP1C* locus shows a high degree of shared synteny in many vertebrate species, including the anole lizard. The genes next to *CYP1C1*, *RAD51* and *FAM82A2*, code for



**Figure 4. Synteny of *CYP1C* and *CYP1B1* regions in various species.** Data were collected from the current assembly versions of the genome databases in Ensembl (<http://www.ensembl.org/info/about/species.html>): Zebrafish (version Zv9), *X. tropicalis* (version JGI 4.2, scaffolds: GL173137 and GL173263), anole lizard (version AnoCar2.0, scaffold GL343264.1), chicken (version 2.1, Chr. 5), mallard duck (version 1.0, scaffold 2370), turkey (version UMD2, Chr. 5), zebra finch (version taeGut3.2.4, Chr. 5), mouse (version NCBIM37, Chr. 2), and human (version GRCh37, Chr. 15). Zebrafish *CYP1C1* and *CYP1C2* have been mapped to chromosome 17 in previous zebrafish genome assemblies [7], but this mapping has not been confirmed in Zv9 as yet. The synteny of *CYP1B1* is shared by all species shown here. Chr = chromosome. doi:10.1371/journal.pone.0028257.g004

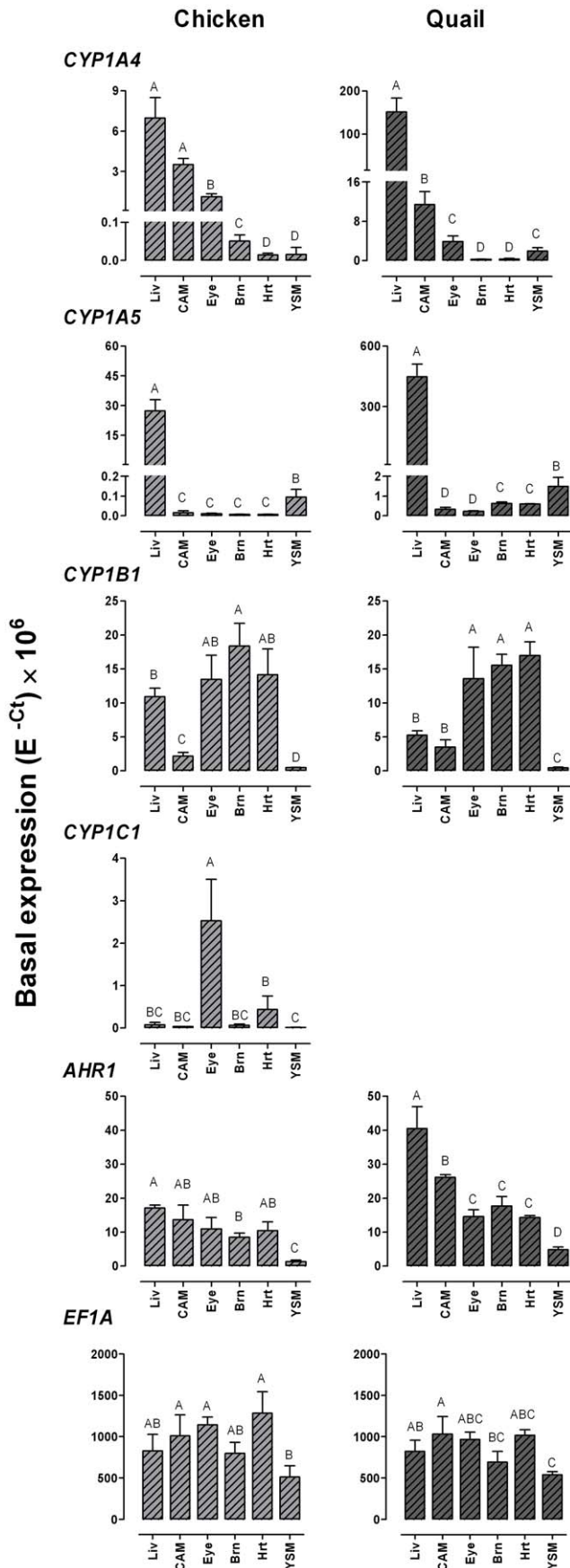
highly conserved proteins with developmental functions, i.e., DNA repair (*RAD51*) and differentiation and apoptosis (*FAM82A2*). These two genes contain several putative DREs in their promoter regions (within about 2 kb upstream from the start site) in zebrafish, chicken, *X. tropicalis* and human. Interestingly, an ortholog of *FAM82A2*, *FAM82A1* (*FAM82A*) was found located next to *CYP1B1* on the chromosome in all species examined here (Fig. 4). This could mean that the regions of *CYP1B1* and *CYP1C1* derive from two copies generated by genome duplication in early vertebrates.

### Constitutive expression

The tissue distribution profiles for basal levels of *CYP1A*, *CYP1B*, and *CYP1C* mRNA in chicken and quail embryos (Fig. 5) were astonishingly similar to those in adult zebrafish. In both fish and birds there are distinct differences in mRNA expression patterns between the subclades, with *CYP1A* (and *CYP1D* in fish) being more highly expressed in the liver and *CYP1B/CYP1C* being more highly expressed in eye, heart, and brain [7,13]. Expression of *CYP1A4*, *CYP1A5*, and *AHRI* has been determined also in the cormorant (*Phalacrocorax carbo*), and the mRNA expression profiles of these genes in liver, heart, and brain were almost identical to those in chicken and quail embryos [36]. The tissue distribution of the two bird *CYP1As* also showed some similarity to the distribution of the two *CYP1As* in mammals, in which *CYP1A1* (ortholog of *CYP1A4*) is widely expressed whereas *CYP1A2* (ortholog of *CYP1A5*) is expressed strongly only in the liver [37].

Regarding distribution of *CYP1B1* in mammals, heart and brain show high expression levels in mice, while in human *CYP1B1* is highly expressed in heart and weakly expressed in brain [38]. Unlike the quite strong *CYP1B1* expression in the bird embryo liver, *CYP1B1* is weakly expressed in the mammalian liver [38]. It is a curious finding that the eye is the tissue where *CYP1C1* was most highly expressed in the 13-day chicken embryo. Notably the two *CYP1Cs* show a high expression also in the adult zebrafish eye [7]. The roles of *CYP1C* in the eye are not known. In mammals, *CYP1B1* is critical for normal eye development [39,40]. *CYP1Cs* might share this role in other vertebrates.

The relatively high level of *CYP1B1* expression over the course of development in most tissues (excluding liver) in both chicken and quail embryos suggests that it plays a role in developing birds. *CYP1B1* mRNA has been localized (by *in situ* hybridization) to the developing eye, neural system, somites, and other structures in chicken embryos [8]. In embryonic zebrafish the basal level of *CYP1B1* mRNA expression peaks at approximately 32 hours after fertilization, in a period of organogenesis and differentiation, while *CYP1A* expression peaks about three weeks after fertilization, and expression of the *CYP1Cs* peaks just after hatching [33]. Choudhary et al. [41] found that in developing mice *CYP1B1* is expressed during neural patterning and somitogenesis, organogenesis, and later fetal stages, whereas *CYP1A1* is expressed during gastrulation only, while *CYP1A2* expression was not detected at all. Thus *CYP1B1* appears to be important in early development in zebrafish, chickens, and mice.



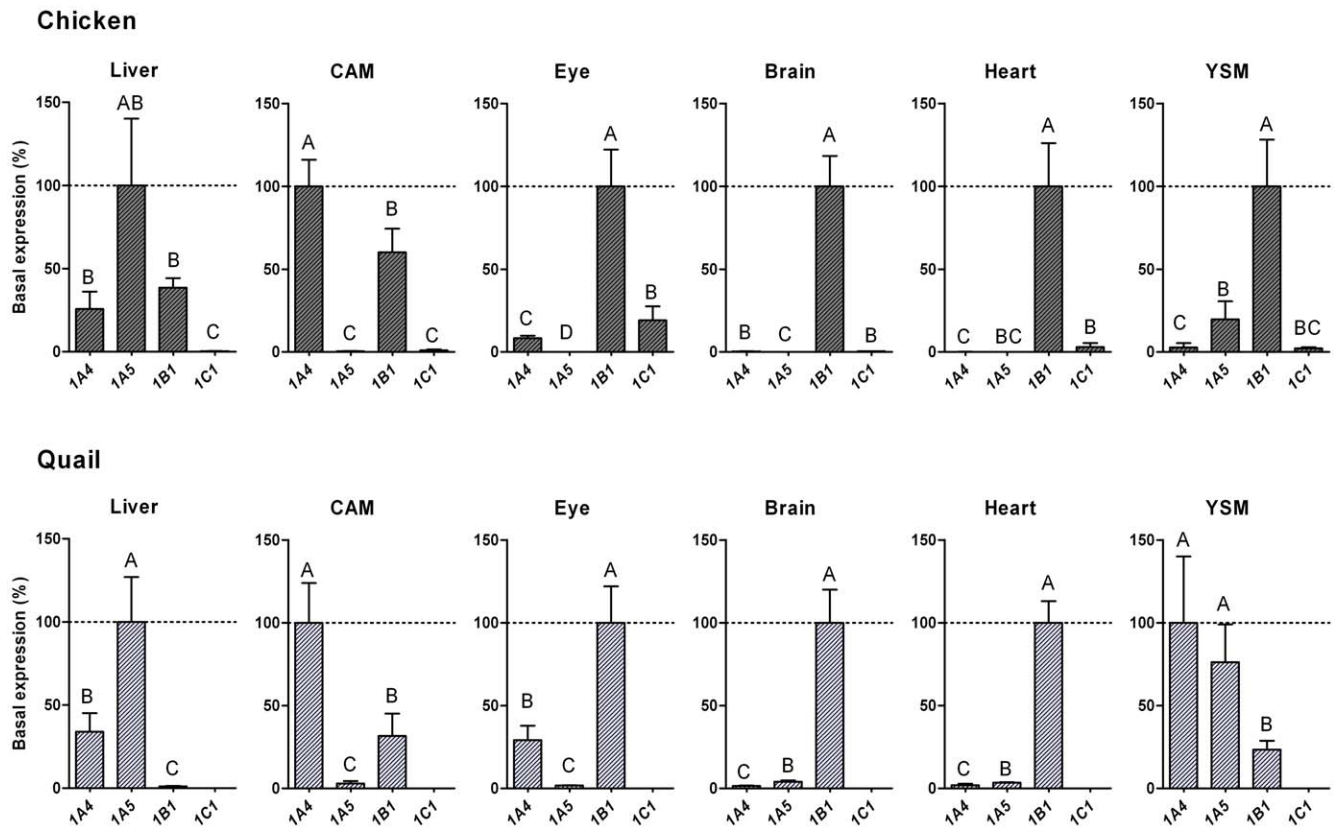
**Figure 5. Tissue distribution of *CYP1* and *AHR1* mRNA in unexposed embryonic chicken and Japanese quail.** Samples were collected at a similar developmental stage in chicken and quail (on incubation day 13 and 11, respectively). Levels of mRNA expression for *CYP1A4*, *CYP1A5*, *CYP1B1*, *AHR1*, and *EF1A* in chicken and Japanese quail, and for *CYP1C1* mRNA in chicken were determined by real-time RT-PCR. Results are shown as non-normalized data ( $E^{-Ct} \times 10^6$ ; mean  $\pm$  SD). Statistical differences in transcript levels among tissues were determined by one-way ANOVA followed by Tukey's multiple comparisons test and are shown by different letters ( $p < 0.05$ );  $n = 3-4$  for chicken and  $n = 3$  for quail. Abbreviations: Liv = liver, CAM = chorioallantoic membrane, Brn = brain, Hrt = heart, and YSM = yolk sac membrane. doi:10.1371/journal.pone.0028257.g005

Endogenous substrates for CYP1 enzymes include various eicosanoids, estradiol, retinoids, and uroporphyrinogen and melatonin (reviewed by [1,4,5]). Chambers et al. [8] found that CYP1B1 can catalyze a step in the formation of retinoic acid, and suggested it is involved in retinoid-mediated patterning. The CYP1s also have been suggested to metabolize endogenous AHR ligands that could play roles in development and differentiation [42]. One molecule which could have this function is the tryptophan photoproduct 6-formylindolo[3,2-b]carbazole (FICZ) [43]. FICZ activates the AHR at hormonal levels and is metabolized by human CYP1A1, CYP1A2, and CYP1B1 with an extraordinarily high efficiency [44]. In conclusion, the CYP1 enzyme functions may include synthesis and degradation of endogenous AHR agonists and other signaling molecules.

### The *AHR* and *CYP1* mRNA induction

Induction of *CYP1A*, *CYP1B*, and *CYP1C* genes and most toxic effects of TCDD are mediated via the AHR. The *AHR* genes are divided into two clades, *AHR1* and *AHR2*. Mammals have a single *AHR1* gene and no *AHR2* gene, while fish and birds have both *AHR1* and *AHR2* genes [36,45]. In zebrafish, AHR-dependent toxicity and *CYP1* induction are mediated principally via *AHR2*, whereas in birds *AHR1* seems to be prominent in these roles [27,36,45]. We found that *AHR1* mRNA was expressed at relatively high levels in a variety of tissues in both quail and chicken (Fig. 5). In the cormorant, *AHR1* mRNA expression shows a wider distribution and higher level than *AHR2* mRNA expression [36]. Features of the AHR may explain differences in sensitivity to dioxin-like compounds in certain mouse strains [46], and in birds, where for instance turkey and quail are much less sensitive than chicken [19,47]. Frogs show a low sensitivity to dioxin toxicity and have an AHR with low dioxin affinity [48,49]. The differing sensitivities to dioxin of mouse strains were shown to be due to differences in specific amino acid residues in the AHR ligand binding domain [46]. Similarly, differences in two amino acid positions in the ligand binding domain were shown to distinguish common tern (resistant) and chicken (susceptible) *AHR1*s [22]. The identity of the amino acids at these two positions predicted the sensitivity in a wide range of bird species [15]. Our results confirm those of Head and co-workers [15], showing that quail *AHR1* has valine in position 324 and alanine in position 380 (the same as seen in the tern *AHR1*), while these positions have isoleucine and serine in chicken (Fig. 3). The turkey *AHR1* has isoleucine and alanine in these positions, but also differs from the chicken and common tern *AHR1* by having an isoleucine instead of threonine in position 297; the mallard *AHR1* has two threonines instead of two alanines in the positions 256–257, but otherwise is identical to the quail *AHR1* in the ligand binding domain (Fig. 3 and [15]). In addition to features associated with a resistant AHR, i.e., a low sensitivity to developmental toxicity of dioxins [50] and weak EROD response to PCB126 observed in





**Figure 6. Expression patterns for *CYP1* mRNA in various tissues in unexposed embryonic chicken and Japanese quail.** Samples were collected at a similar developmental stage in chicken and quail (on incubation day 13 and 11, respectively). Levels of mRNA expression for *CYP1A4*, *CYP1A5*, and *CYP1B1* in chicken and Japanese quail, and for *CYP1C1* mRNA in chicken were determined by real-time RT-PCR. Data were normalized (calculated by  $E^{-\Delta Ct}$ ) and results are shown as percentage of the gene with the highest level of expression within a tissue (mean  $\pm$  SD). Statistical differences among transcript levels within a tissue were determined by one-way ANOVA followed by Tukey's multiple comparisons test and are shown by different letters ( $p < 0.05$ );  $n = 3-4$  for chicken and  $n = 3$  for quail. doi:10.1371/journal.pone.0028257.g006

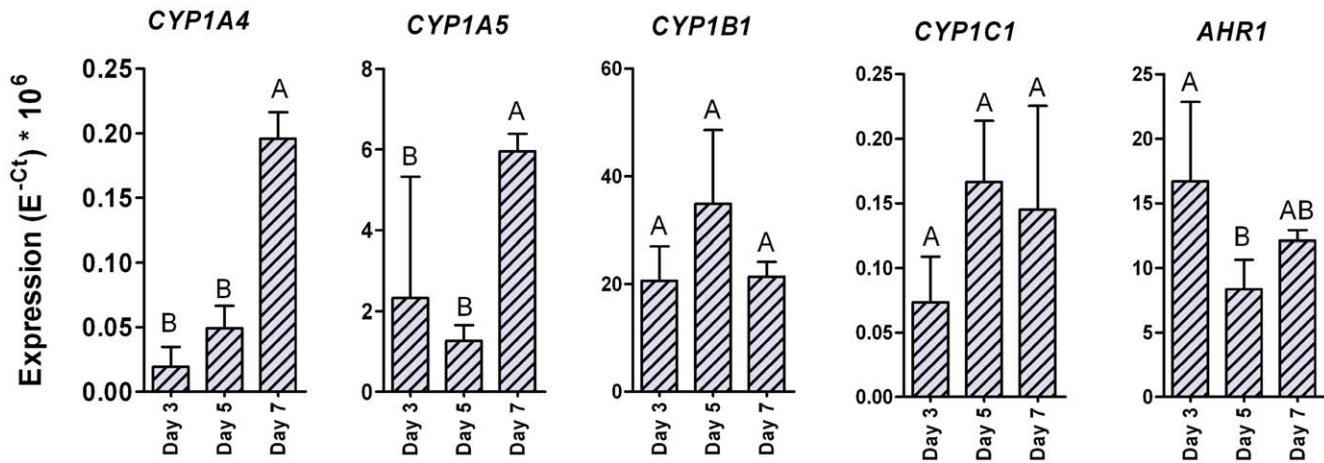
**Table 2. Relative levels of basal expression for *CYP1* and *AHR1* mRNA in early chicken embryos.**

Transcript	Percentage of <i>CYP1B1</i> expression (mean $\pm$ SD)				
	Day 1	Day 2	Day 3	Day 5	Day 7
<i>CYP1A4</i>	0.4 $\pm$ 0.5	0.2 $\pm$ 0.0	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1	0.9 $\pm$ 0.1
<i>CYP1A5</i>	45 $\pm$ 32	39 $\pm$ 22	11 $\pm$ 15	4 $\pm$ 1	28 $\pm$ 2
<i>CYP1B1</i>	<b>100<math>\pm</math>137</b>	<b>100<math>\pm</math>9</b>	<b>100<math>\pm</math>31</b>	<b>100<math>\pm</math>45</b>	<b>100<math>\pm</math>13</b>
<i>CYP1C1</i>	1.6 $\pm$ 1.4	2.3 $\pm$ 0.9	0.4 $\pm$ 0.2	0.5 $\pm$ 0.1	0.7 $\pm$ 0.4
<i>AHR1</i>	51 $\pm$ 56	158 $\pm$ 86	81 $\pm$ 30	24 $\pm$ 7	57 $\pm$ 4

Whole-body samples of unexposed chicken embryos were collected on developmental days 1, 2, 3, 5, and 7 and analyzed by real-time RT-PCR. Data were normalized (calculated by  $E^{-\Delta Ct}$ ) and results are shown as percentage of the *CYP1B1* mRNA level ( $n = 3$ ). The embryos analyzed were staged according to Hamburger and Hamilton [63] and [http://msucares.com/poultry/reproductions/poultry\\_chicks\\_embryo.html](http://msucares.com/poultry/reproductions/poultry_chicks_embryo.html): Day-1 samples were taken after 31 h of incubation (stage 9, seven somites). At this stage the nervous system, eye, and heart have begun to develop. On day two (sampled at 50 h: stage 16, 19–22 somites) heart beats can be observed. On day three (sampled at 74 h: stages 20–21, 40–43 somites) nose, legs, and wings begin to appear. On day five (stage 27) the beak and reproductive organs start to form and sex differentiation occurs. On day seven (stage 31) feather papillae begin to appear. doi:10.1371/journal.pone.0028257.t002

embryo liver [19], quail exhibited a weaker *CYP1* mRNA induction by PCB126 than chicken despite the 1000 times higher dose given to quail.

The basal levels of hepatic *CYP1A4* and *CYP1A5* expression were much higher in quail embryos than in chicken embryos at similar stages, whereas basal *CYP1B1* expression looked largely similar in the two species. The significance of this difference between the genes is not understood. However, assuming there is a maximal capacity to synthesize the transcript for a given *CYP1*, the relative induction level would be low when compared to a high basal level. A higher basal level in the quail could explain the “weaker” induction of *CYP1A4* in YSM of quail than of chicken. Hence, a lower “fold-induction” level of a *CYP1* gene could reflect a high constitutive level of expression rather than a low responsiveness. However this would not explain the weaker induction of *CYP1A5* in quail vs. chicken, and the induction of *CYP1B1* in chicken but not in quail (Fig. 8). Rather these differences could be related to differences in AHR affinity for PCB126 not compensated for by the higher dose given to quail. In addition to AHR affinity of the inducer, the level of *CYP1* induction depends on the number of functional DREs in the gene promoters, epigenetic factors, interaction of the AHR with nuclear receptors (e.g., estrogen receptor), cofactors, the AHR repressor, etc. [26,51,52,53,54,55]. Because the *CYP1*s are likely to be expressed in different cell types, it would be informative to study



**Figure 7. Basal levels of expression for *CYP1* and *AHR* mRNA during early development of chicken.** Basal levels of mRNA expression were determined for *CYP1A4*, *CYP1A5*, *CYP1B1*, *CYP1C1*, and *AHR1* in whole-body samples of embryonic chicken collected on developmental days 3, 4, and 7. The samples were analyzed by real-time RT-PCR. Results are shown as non-normalized data ( $E^{-Ct} \times 10^6$ ; mean  $\pm$  SD). Statistical differences in mRNA levels among development days were determined by one-way ANOVA followed by Tukey's multiple comparisons test and are shown by different letters ( $p < 0.05$ ),  $n = 3$ .

doi:10.1371/journal.pone.0028257.g007

cell-specific induction of the *CYP1s*, and *CYP1C1* in particular, in chicken.

We previously found that the patterns of induction of *CYP1A*, *CYP1B*, and *CYP1C* were similar in zebrafish and *X. tropicalis* after exposure to PCB126 [6,33]. In zebrafish embryos *CYP1A*, *1B1*, *1C1*, and *1C2* were induced 280-, 23-, 23-, and 40-fold versus the control and in tadpoles *CYP1A*, *1B1*, and *1C1* were induced up to 90-, 3-, and 8-fold versus the control [6,33]. Together with the present results (Fig. 8) these findings indicate the *CYP1A* genes are more responsive to PCB126 than the *CYP1B/CYP1C* genes in developing animals. Thus, a strong inducibility appears to be an evolutionarily conserved feature of the *CYP1As*, which could have to do with their functions.

## Conclusions

In this study we establish that *CYP1C1* is present in some birds. We show that *CYP1C1* mRNA is rather highly expressed in the chicken embryo eye. *CYP1B1* appears to have a high developmental expression in both chicken and quail. The similar

distribution patterns of *CYP1C* and *CYP1B* transcripts in chicken and zebrafish imply that these *CYP1s* may serve similar functions in diverse vertebrates. Together with the absence of *CYP1Cs* in mammals, the apparent absence of *CYP1C1* in quail, and weak expression and induction of *CYP1C1* mRNA in chicken suggests that *CYP1Cs* have diminishing roles in tetrapods, which may be met by *CYP1B1*. Determining catalytic functions of CYP1 proteins in different species should indicate the evolving roles of these duplicated genes in physiological and toxicological processes. The studies reported here expand our view of the likely history and role of *CYP1s*.

## Methods

### Eggs

Fertilized eggs from chicken (White Leghorn) and Japanese quail were obtained from local Swedish breeders (OVA Production AB, Vittinge, and Olstorps Konserverfabrik, Färgelanda, respectively). Eggs were incubated at 37.5°C and 60% relative humidity with automatic turning every 6 hours until sampled. The experiments of this study were approved by Uppsala Ethical Committee for Research on Animals (Uppsala district court; permit number C 282/9).

### Cloning and synteny

Complementary DNA of chicken *CYP1C1* was cloned using primers targeting the predicted full coding transcript. Total RNA was extracted from whole-body homogenate of chicken embryos (incubated for 5 days) using RNA STAT 60 (Tel. Test Inc. Friendswood, TX, USA). Subsequently mRNA was isolated from the total RNA using MicroPoly(A)Purist™ Kit (Ambion Inc., Austin, TX, USA), and cDNA was synthesized using the Omniscript reverse transcriptase kit with random hexamer primers (Eurofins MWG Operon, Huntsville, AL, USA). Amplification of cDNA was performed using the Advantage® 2 polymerase PCR kit according to instructions provided by the manufacturer (Clontech Laboratories Inc., Mountain View, CA, USA). The PCR products were resolved on a 1% agarose gel. A product of approximately 1600 bp was isolated and ligated into the pGEM-T Easy Vector (Promega, Madison, WI, USA), and the

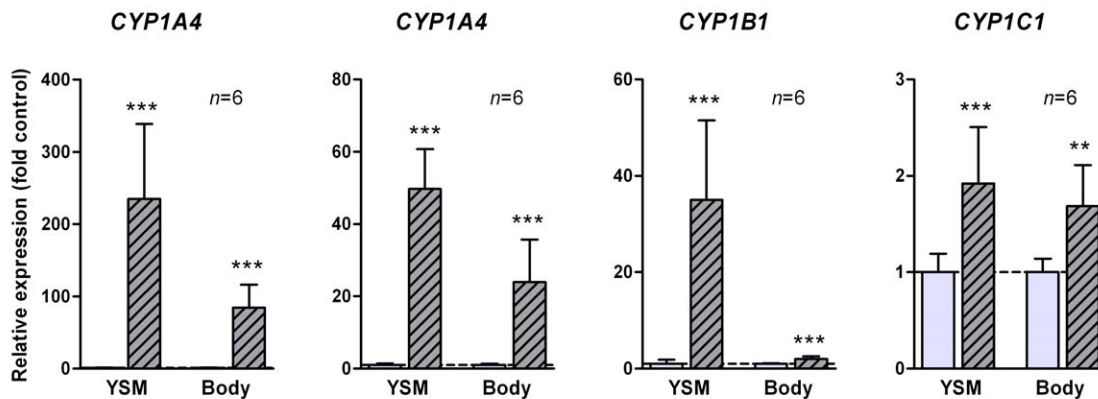
**Table 3. Basal levels of *CYP1* and *AHR1* mRNA expression in chicken embryos on developmental day 7.**

Transcript	Expression (mean $\pm$ SD)		
	Body	YSM	CAM
<i>CYP1A4</i>	0.20 $\pm$ 0.02	0.10 $\pm$ 0.02	0.2 $\pm$ 0.1
<i>CYP1A5</i>	6.0 $\pm$ 0.4	<b>18 <math>\pm</math> 6</b>	0.11 $\pm$ 0.04
<i>CYP1B1</i>	<b>21 <math>\pm</math> 3</b>	0.5 $\pm$ 0.4	<b>2 <math>\pm</math> 2</b>
<i>CYP1C1</i>	0.1 $\pm$ 0.1	0.04 $\pm$ 0.03	0.2 $\pm$ 0.1
<i>AHR1</i>	12 $\pm$ 1	7 $\pm$ 2	28 $\pm$ 11

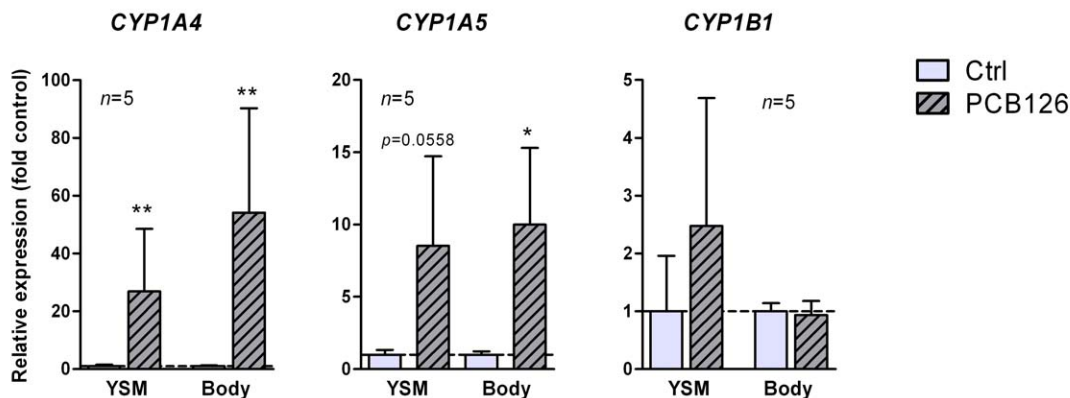
Samples of whole-body, yolk sac membrane (YSM) and chorioallantoic membrane (CAM) were collected from unexposed 7-day-old chicken embryos and analyzed by real-time RT-PCR ( $n = 3$ ). Results are shown as non-normalized data calculated by  $E^{-Ct} \times 10^6$ . Each sample contained cDNA prepared from 30 ng of total RNA. The highest values among the *CYP1s* in a sample type are shown in bold.

doi:10.1371/journal.pone.0028257.t003

## Chicken (day 5)



## Quail (day 4)



**Figure 8. Effect of PCB126 on *CYP1* mRNA expression in chicken and Japanese quail embryos.** Inducibility of the *CYP1*s was examined after exposure to PCB126 by egg injection on day 4 in chicken ( $n=6$ ) and on day 3 in quail ( $n=5$ ). Solutions of PCB126 dissolved in a peanut oil:water emulsion were injected into the yolks,  $0.2 \mu\text{g PCB126 kg}^{-1}$  to chicken and  $200 \mu\text{g PCB126 kg}^{-1}$  to quail. Controls were injected with peanut oil:water emulsion. After 24 hours of exposure yolk sac membrane ("YSM") and whole-body ("Body") samples were collected. The samples were analysed by real-time RT-PCR and relative expression levels determined by  $E^{-\Delta\Delta\text{Ct}}$ . Statistically significant differences between the control- and PCB126-exposed groups were determined with Student's *t* test. Welch's correction was used when data did not show normal distribution. Significance levels are shown by asterisks  $p<0.05$  (\*),  $p<0.01$  (\*\*), and  $p<0.001$  (\*\*\*). doi:10.1371/journal.pone.0028257.g008

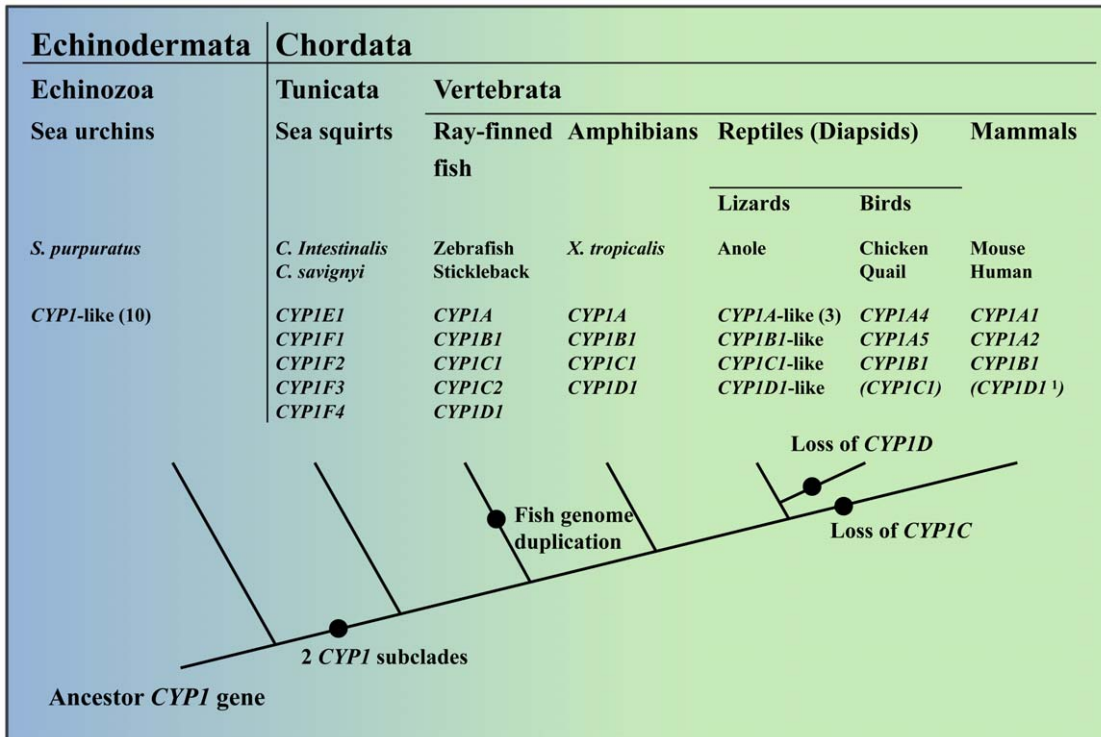
construct was transformed into *Escherichia coli* (TOP 10 Kit, Invitrogen, Carlsbad, CA, USA). Plasmids were purified from cultures of positive clones and sequenced (Eurofins MWG Operon). The sequences obtained were assembled using Sequencher® (Gene Codes Corporation, Ann Arbor, MI, USA), resulting in a consensus sequence corresponding to the full coding part of the predicted chicken *CYP1C1*.

We also cloned partial sequences of quail *CYP1B1*, *AHR1*, and *EFLA* using primers designed to target conserved regions of the chicken orthologs. Total RNA was prepared from whole-body homogenate of 4-day-old quail embryos using the Aurum™ total RNA fatty and fibrous tissue kit (Bio-Rad Laboratories Inc., Hercules CA, USA) and the RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad). Quail cDNAs were amplified using the gene-specific primers with Advantage® 2 polymerase PCR kit (Clontech Laboratories Inc.). The PCR products were sequenced by Uppsala Genome Center (Rudbeck

Laboratory, Uppsala) and sequences obtained were aligned using ClustalW in BioEdit [56]. The cloned sequences of chicken *CYP1C1* and quail *CYP1B1*, *AHR1*, and *EFLA* were assigned the following GenBank accession numbers: JN656933, JN656934, JN656935 and JN656936, respectively.

Seeking an ortholog for *CYP1C1* in quail we designed primers (12 forward and 10 reverse) targeting regions conserved between chicken *CYP1C1* and the *CYP1C1* predictions in turkey and mallard duck. In addition to cDNA from embryonic day 4 we used pooled cDNA from eye, brain, and heart (collected on embryonic day 11). A *CYP1C1* gene was also sought using genomic DNA isolated from quail whole-body homogenate (embryonic day 4) with DNeasy Blood & Tissue Kit (Qiagen). We also amplified quail cDNA using the quantitative real-time RT-PCR primers designed for chicken *CYP1C1* (Table 4), but the product obtained was of *CYP1B1*. Consequently, we did not find any ortholog for *CYP1C1* in quail neither in genomic DNA nor in cDNA made from total RNA.





**Figure 9. Evolutionary history of the *CYP1* family genes.** The panel shows the presence of *CYP1*-like genes and *CYP1A*, *CYP1B*, *CYP1C*, *CYP1D*, *CYP1E* and *CYP1F* subfamily genes in various echinozoan, tunicate, and vertebrate classes. Suggested events of gene duplication and gene loss are shown by bullets. Ten *CYP1*-like genes have been identified in the genome of the sea urchin *S. purpuratus* and three *CYP1A*-like genes in the genome of the anole lizard. These genes have not been further studied. Genes within brackets indicate known or suspected absence in at least one species within the taxon (birds, mammals). Data were collected from Godard et al. [29], Goldstone et al. [25], Jönsson et al. [7], Goldstone et al. [13], Gao et al. [35], Jönsson et al. [6], and the anole lizard genome database ([http://www.ensembl.org/Anolis\\_carolinensis/Info/Index](http://www.ensembl.org/Anolis_carolinensis/Info/Index)). Footnote: <sup>1</sup> *CYP1D1* is a pseudogene in human, is expressed in macaques [12,13], and appears to be absent in the mouse. doi:10.1371/journal.pone.0028257.g009

The deduced amino acid sequences of the cloned cDNA were aligned with homologous sequences in other species and sequence identities were examined after pair-wise alignments using BioEdit. The SRS regions were localized out from Lewis et

al. [57]. The synteny of *CYP1C* and *CYP1B* genes was determined in zebrafish, *X. tropicalis*, chicken, turkey, mallard duck, zebra finch, mouse, and human using the genome databases in Ensembl.

**Table 4.** Sequences of all real-time RT-PCR primers used in the experiments.

Species/Transcript	GenBank Acc. No.	Forward primer (5' to 3')	Reverse primer (5' to 3')	Product size
<b>Chicken</b>				
<i>CYP1A4</i>	NM205147.1	ACTGCCAGGAGAAAAGGACAG	TCAAAGCCTGCCCAAACAG	97
<i>CYP1A5</i>	NM205146.1	TTCACCATCCCGCACAGCA	GTTTCTCATCGTATTCACTTGCC	109
<i>CYP1B1</i> <sup>1</sup>	XM419515.2	CATCTTCTCATCAGGTATCCAAAAGT	GTACAGGAAAGCCACGATGTAG	130
<i>CYP1C1</i>	JN656933	TGTGCCCATCACCATTCCACAT	ACTGACCACTGGTTGACAAAGAC	99
<i>AHR1</i>	NM204118.1	GCTGTGATGCAAAAGGAAAGATTGTC	ATTCCACTCTACCCGTCTTC	148
<i>EF1A</i>	NM204157.2	GATGTCTACAAAATTGGTGGCATTGG	GCTTCATGGTGCATCTCAACAG	140
<b>Japanese quail</b>				
<i>CYP1A4</i>	GQ906939.1	GCAAGTGAACCACGATGAGAAGAT	ACCACTTTGTACCCTCTGTCC	111
<i>CYP1A5</i>	GQ906938.1	GCAAGTGAACCACGATGAGAACT	TTTCCCCAATGCACCTCCTT	126
<i>CYP1B1</i> <sup>1</sup>	JN656934	CATCTTCTCATCAGGTATCCAAAAGT	GTACAGGAAAGCCACGATGTAG	130
<i>AHR1</i>	JN656935	GCTGTGATGCAAAAGGAAAGATTGTC	CTCTCACCTGTCTTCATCATTCCG	142
<i>EF1A</i>	JN656936	CTACAAAATTGGTGGCATTGGTACTG	TGACAACCATGCCTGGCTTCA	77

<sup>1</sup>The same primer pair was used for chicken and quail *CYP1B1*. doi:10.1371/journal.pone.0028257.t004

### CYP1 mRNA expression in chicken and quail embryos

Basal levels of *CYP1* mRNA were determined in unexposed chicken embryos sampled after various times of incubation (1, 2, 3, 5, and 7 days). Whole embryos and YSM were sampled separately (day 3, 5, and 7). In addition, CAM was collected from chicken at embryonic day 7. Finally, liver, CAM, eye, brain, heart, and YSM were collected from chicken and quail at embryonic day 13 and 11, respectively. All samples were frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

Inducibility of the *CYP1*s was examined after exposure to PCB126 by egg injection. Injection solutions were prepared by dissolving PCB126 in a peanut oil:lecithin mixture (10:1, v:v) which was emulsified in water (1:1.5, v:v) by ultra-sonication. Equivalent peanut oil:lecithin:water emulsions without PCB126 were prepared for controls. The emulsions were injected into the yolks of embryonated eggs after 3 (quail) and 4 (chicken) days of incubation. The volumes injected were 20  $\mu\text{l}$  (quail) and 100  $\mu\text{l}$  (chicken) corresponding to 200  $\mu\text{g}$  PCB126/kg for quail and 0.2  $\mu\text{g}$  PCB126/kg for chicken, doses that are high enough to induce hepatic EROD activity [19]. After injection, the holes in the shells were sealed with melted paraffin wax and the eggs were returned to the incubator. The embryos were sampled 24 hours later. Whole embryos and YSM were sampled separately, frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ .

### Quantitative real-time RT-PCR

Total RNA was isolated and DNase-treated using the Aurum<sup>TM</sup> total RNA fatty and fibrous tissue kit (Bio-Rad) according to Bio-Rad's instructions. The purity and quantity of RNA were determined spectrophotometrically (260/280 and 260/230 nm ratios were generally 2 or above) using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). Total RNA was reverse transcribed using the iScript cDNA Synthesis kit (Bio-Rad).

Gene-specific quantitative real-time RT-PCR primers for chicken and quail *CYP1A4*, *CYP1A5*, *CYP1B1*, *AHR1*, and *EF1A*, and for chicken *CYP1C1*, were synthesized by Sigma-Aldrich (St. Louis, MO, USA) (Table 4). The predicted amplicon length was 75–150 bp. PCR was conducted using a Rotor Gene 6000 real-

time PCR machine (Qiagen, Hilden, Germany). The 20- $\mu\text{l}$  PCR reaction mixtures consisted of iQ SYBR Green Supermix (Bio-Rad), forward and reverse primers (5 pmoles of each; Table 4) and cDNA derived from 30 ng of total RNA. All samples were analyzed in duplicate with the following protocol:  $95^{\circ}\text{C}$  for 10 min followed by 30–40 cycles (cycle numbers varying with transcript levels) of  $95^{\circ}\text{C}$  for 15 s and  $62^{\circ}\text{C}$  for 45 s. At the end of each PCR run a melt curve analysis was performed in the range from  $55^{\circ}\text{C}$  to  $95^{\circ}\text{C}$ .

### Calculations and statistics

Finding a reference gene which is stable during development or which does not vary among tissues is difficult. Therefore, in some cases basal levels of *CYP1* and *AHR1* mRNA expression were calculated without normalization to an internal control (indicated in figure and table legends). In these calculations we used the equation  $E^{-CT}$  where  $E$  = PCR efficiency and  $CT$  = threshold cycle [58,59]. The effect of PCB126 on mRNA expression was determined after calculation of  $E^{-\Delta\Delta CT}$  [59]. *EF1A* was used as a reference gene for both quail and chicken; in neither of the two species *EF1A* was significantly affected by the PCB126 exposure. Mean values of  $E$  for within-experiment amplicon groups were determined by the LinRegPCR program using data within 10% of the group median [60,61]. The  $E$  values obtained ranged from 1.83 to 1.92. Outliers were excluded based on the Grubbs test [62]. Statistical analyses were performed using Prism 5 by GraphPad Software Inc. (San Diego, CA, USA) with log-transformed data. The statistical methods used were Student's  $t$  test and one-way ANOVA followed by Tukey's or Dunnett's post hoc tests. Data were log-transformed before statistical analysis when the variances differed between groups. In the figures data are shown as mean+SD. Numbers of biological replicates used ( $n$ ) are given in the figure legends.

### Author Contributions

Conceived and designed the experiments: MEJ JJS BB. Performed the experiments: MEJ BRW BB. Analyzed the data: MEJ. Contributed reagents/materials/analysis tools: MEJ JJS BB. Wrote the paper: MEJ JJS BB.

### References

- Choudhary D, Jansson I, Stoilov I, Sarfarazi M, Schenkman JB (2004) Metabolism of retinoids and arachidonic acid by human and mouse cytochrome P450 1b1. *Drug Metab Dispos* 32: 840–847.
- Nebert DW, Dalton TP (2006) The role of cytochrome P450 enzymes in endogenous signalling pathways and environmental carcinogenesis. *Nat Rev Cancer* 6: 947–960.
- Zhou SF, Zhou ZW, Yang LP, Cai JP (2009) Substrates, inducers, inhibitors and structure-activity relationships of human Cytochrome P450 2C9 and implications in drug development. *Curr Med Chem* 16: 3480–3675.
- Nebert DW, Russell DW (2002) Clinical importance of the cytochromes P450. *Lancet* 360: 1155–1162.
- Nebert DW, Karp CL (2008) Endogenous functions of the aryl hydrocarbon receptor (AHR): intersection of cytochrome P450 1 (CYP1)-metabolized eicosanoids and AHR biology. *J Biol Chem* 283: 36061–36065.
- Jönsson ME, Berg C, Goldstone JV, Stegeman JJ (2011) New CYP1 genes in the frog *Xenopus (Silurana) tropicalis*: Induction patterns and effects of AHR agonists during development. *Toxicol Appl Pharmacol*.
- Jönsson ME, Orrego R, Woodin BR, Goldstone JV, Stegeman JJ (2007) Basal and 3,3',4,4',5-Pentachlorobiphenyl-induced expression of Cytochrome P450 1A, 1B and 1C Genes in Zebrafish. *Toxicol Appl Pharmacol* 221: 29–41.
- Chambers D, Wilson L, Maden M, Lumsden A (2007) RALDH-independent generation of retinoic acid during vertebrate embryogenesis by CYP1B1. *Development* 134: 1369–1383.
- Gilday D, Bellward GD, Sanderson JT, Janz DM, Rifkind AB (1998) 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induces hepatic cytochrome P450-dependent arachidonic acid epoxidation in diverse avian orders: regioisomer selectivity and immunochemical comparison of the TCDD-induced P450s to CYP1A4 and 1A5. *Toxicol Appl Pharmacol* 150: 106–116.
- Zanette J, Jenny MJ, Goldstone JV, Woodin BR, Watka LA, et al. (2009) New cytochrome P450 1B1, 1C2 and 1D1 genes in the killifish *Fundulus heteroclitus*: Basal expression and response of five killifish CYP1s to the AHR agonist PCB126. *Aquat Toxicol* 93: 234–243.
- Shimada T, Sugie A, Shindo M, Nakajima T, Azuma E, et al. (2003) Tissue-specific induction of cytochromes P450 1A1 and 1B1 by polycyclic aromatic hydrocarbons and polychlorinated biphenyls in engineered C57BL/6J mice of arylhydrocarbon receptor gene. *Toxicol Appl Pharmacol* 187: 1–10.
- Uno Y, Uehara S, Murayama N, Yamazaki H (2011) CYP1D1, pseudogenized in human, is expressed and encodes a functional drug-metabolizing enzyme in cynomolgus monkey. *Biochem Pharmacol* 81: 442–450.
- Goldstone JV, Jönsson ME, Behrendt L, Woodin BR, Jenny MJ, et al. (2009) Cytochrome P450 1D1: a novel CYP1A-related gene that is not transcriptionally activated by PCB126 or TCDD. *Arch Biochem Biophys* 482: 7–16.
- Brunström B (1988) Sensitivity of embryos from duck, goose, herring gull, and various chicken breeds to 3,3',4,4'-tetrachlorobiphenyl. *Poultry science* 67: 52–57.
- Head J, Hahn ME, Kennedy SW (2008) Key amino acids in the aryl hydrocarbon receptor predict dioxin sensitivity in avian species. *Environ Sci Technol* 42: 7535–7541.
- Powell DC, Aulerich RJ, Meadows JC, Tillitt DE, Giesy JP, et al. (1996) Effects of 3,3',4,4',5-pentachlorobiphenyl (PCB 126) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) injected into the yolks of chicken (*Gallus domesticus*) eggs prior to incubation. *Arch Environ Contam Toxicol* 31: 404–409.
- Brunström B, Darnerud PO (1983) Toxicity and distribution in chick embryos of 3,3',4,4'-tetrachlorobiphenyl injected into the eggs. *Toxicology* 27: 103–110.
- Brunström B, Andersson L (1988) Toxicity and 7-ethoxyresorufin O-deethylase-inducing potency of coplanar polychlorinated biphenyls (PCBs) in chick embryos. *Arch Toxicol* 62: 263–266.

19. Brunström B, Halldin K (1998) EROD induction by environmental contaminants in avian embryo livers. *Comp Biochem Physiol C* 121: 213–219.
20. Cohen-Barnhouse AM, Zwiernik MJ, Link JE, Fitzgerald SD, Kennedy SW, et al. (2011) Sensitivity of Japanese quail (*Coturnix japonica*), Common pheasant (*Phasianus colchicus*), and White Leghorn chicken (*Gallus gallus domesticus*) embryos to in ovo exposure to TCDD, PeCDF, and TCDF. *Toxicol Sci* 119: 93–103.
21. Yang Y, Wiseman S, Cohen-Barnhouse AM, Wan Y, Jones PD, et al. (2010) Effects of in ovo exposure of white leghorn chicken, common pheasant, and Japanese quail to 2,3,7,8-tetrachlorodibenzo-p-dioxin and two chlorinated dibenzofurans on CYP1A induction. *Environmental toxicology and chemistry/SETAC* 29: 1490–1502.
22. Karchner SI, Franks DG, Kennedy SW, Hahn ME (2006) The molecular basis for differential dioxin sensitivity in birds: role of the aryl hydrocarbon receptor. *Proc Natl Acad Sci U S A* 103: 6252–6257.
23. Goldstone HM, Stegeman JJ (2006) A Revised Evolutionary History of the CYP1A Subfamily: Gene Duplication, Gene Conversion, and Positive Selection. *J Mol Evol* 62: 708–717.
24. Gilday D, Gannon M, Yutzey K, Bader D, Rifkind AB (1996) Molecular cloning and expression of two novel avian cytochrome P450 1A enzymes induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *J Biol Chem* 271: 33054–33059.
25. Goldstone JV, Goldstone HM, Morrison AM, Tarrant A, Kern SE, et al. (2007) Cytochrome P450 1 genes in early deuterostomes (tunicates and sea urchins) and vertebrates (chicken and frog): origin and diversification of the CYP1 gene family. *Mol Biol Evol* 24: 2619–2631.
26. Zeruth G, Pollenz RS (2007) Functional analysis of cis-regulatory regions within the dioxin-inducible CYP1A promoter/enhancer region from zebrafish (*Danio rerio*). *Chem Biol Interact* 170: 100–113.
27. Lee JS, Kim EY, Iwata H (2009) Dioxin activation of CYP1A5 promoter/enhancer regions from two avian species, common cormorant (*Phalacrocorax carbo*) and chicken (*Gallus gallus*): association with aryl hydrocarbon receptor 1 and 2 isoforms. *Toxicol Appl Pharmacol* 234: 1–13.
28. Kawai Y, Ikenaka Y, Fujita S, Ishizuka M (2010) The CYP1D subfamily of genes in mammals and other vertebrates. *Mamm Genome* 21: 320–329.
29. Godard CA, Goldstone JV, Said MR, Dickerson RL, Woodin BR, et al. (2005) The new vertebrate CYP1C family: cloning of new subfamily members and phylogenetic analysis. *Biochem Biophys Res Commun* 331: 1016–1024.
30. Blair JE, Hedges SB (2005) Molecular phylogeny and divergence times of deuterostome animals. *Mol Biol Evol* 22: 2275–2284.
31. Ohno S (1999) Gene duplication and the uniqueness of vertebrate genomes circa 1970–1999. *Semin Cell Dev Biol* 10: 517–522.
32. Hackett SJ, Kimball RT, Reddy S, Bowie RC, Braun EL, et al. (2008) A phylogenomic study of birds reveals their evolutionary history. *Science* 320: 1763–1768.
33. Jönsson ME, Jenny MJ, Woodin BR, Hahn ME, Stegeman JJ (2007) Role of AHR2 in the expression of novel cytochrome P450 1 family genes, cell cycle genes, and morphological defects in developing zebrafish exposed to 3,3',4,4',5-pentachlorobiphenyl or 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicol Sci* 100: 180–193.
34. Jönsson ME, Gao K, Olsson JA, Goldstone JV, Brandt I (2010) Induction patterns of new CYP1 genes in environmentally exposed rainbow trout. *Aquat Toxicol* 98: 311–321.
35. Gao K, Brandt I, Goldstone JV, Jönsson ME (2011) Cytochrome P450 1A, 1B, and 1C mRNA induction patterns in three-spined stickleback exposed to a transient and a persistent inducer. *Comp Biochem Physiol C* 154: 42–55.
36. Yasui T, Kim EY, Iwata H, Franks DG, Karchner SI, et al. (2007) Functional characterization and evolutionary history of two aryl hydrocarbon receptor isoforms (AhR1 and AhR2) from avian species. *Toxicol Sci* 99: 101–117.
37. Dey A, Jones JE, Nebert DW (1999) Tissue- and cell type-specific expression of cytochrome P450 1A1 and cytochrome P450 1A2 mRNA in the mouse localized in situ hybridization. *Biochem Pharmacol* 58: 525–537.
38. Choudhary D, Jansson I, Stoilov I, Sarfarazi M, Schenkman JB (2005) Expression patterns of mouse and human CYP orthologs (families 1–4) during development and in different adult tissues. *Arch Biochem Biophys* 436: 50–61.
39. Choudhary D, Jansson I, Rezaul K, Han DK, Sarfarazi M, et al. (2007) Cyp1b1 protein in the mouse eye during development: An immunohistochemical study. *Drug Metab Dispos* 35: 987–994.
40. Choudhary D, Jansson I, Sarfarazi M, Schenkman JB (2006) Physiological significance and expression of P450s in the developing eye. *Drug Metab Rev* 38: 337–352.
41. Choudhary D, Jansson I, Schenkman JB, Sarfarazi M, Stoilov I (2003) Comparative expression profiling of 40 mouse cytochrome P450 genes in embryonic and adult tissues. *Arch Biochem Biophys* 414: 91–100.
42. Chiaro CR, Patel RD, Marcus CB, Perdeu GH (2007) Evidence for an aryl hydrocarbon receptor-mediated cytochrome p450 autoregulatory pathway. *Mol Pharmacol* 72: 1369–1379.
43. Wei YD, Helleberg H, Rannug U, Rannug A (1998) Rapid and transient induction of CYP1A1 gene expression in human cells by the tryptophan photoproduct 6-formylindolo[3,2-b]carbazole. *Chem -Biol Interact* 110: 39–55.
44. Wincent E, Amini N, Luecke S, Glatt H, Bergman J, et al. (2009) The suggested physiologic aryl hydrocarbon receptor activator and cytochrome P450 substrate 6-formylindolo[3,2-b]carbazole is present in humans. *J Biol Chem* 284: 2690–2696.
45. Hahn ME, Karchner SI, Evans BR, Franks DG, Merson RR, et al. (2006) Unexpected diversity of aryl hydrocarbon receptors in non-mammalian vertebrates: insights from comparative genomics. *J Exp Zool* 305A: 693–706.
46. Poland A, Palen D, Glover E (1994) Analysis of the four alleles of the murine aryl hydrocarbon receptor. *Mol Pharmacol* 46: 915–921.
47. Brunström B, Lund J (1988) Differences between chick and turkey embryos in sensitivity to 3,3',4,4'-tetrachloro-biphenyl and in concentration/affinity of the hepatic receptor for 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Comp Biochem Physiol C* 91: 507–512.
48. Lavine JA, Rowatt AJ, Klimova T, Whittington AJ, Dengler E, et al. (2005) Aryl hydrocarbon receptors in the frog *Xenopus laevis*: two AhR1 paralogs exhibit low affinity for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). *Toxicol Sci* 88: 60–72.
49. Jung RE, Walker MK (1997) Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on development of anuran amphibians. *Environ Toxicol Chem* 16: 230–240.
50. Cohen-Barnhouse AM, Zwiernik MJ, Link JE, Fitzgerald SD, Kennedy SW, et al. (2011) Developmental and posthatch effects of in ovo exposure to 2,3,7,8-TCDD, 2,3,4,7,8-PECDF, and 2,3,7,8-TCDF in Japanese quail (*Coturnix japonica*), common pheasant (*Phasianus colchicus*), and white leghorn chicken (*Gallus gallus domesticus*) embryos. *Environ Toxicol Chem* 30: 1659–1668.
51. Matthews J, Gustafsson JÅ (2006) Estrogen receptor and aryl hydrocarbon receptor signaling pathways. *Nucl Recept Signal* 4: e016.
52. Hahn ME, Allan LL, Sherr DH (2009) Regulation of constitutive and inducible AHR signaling: complex interactions involving the AHR repressor. *Biochem Pharmacol* 77: 485–497.
53. Hankinson O (2005) Role of coactivators in transcriptional activation by the aryl hydrocarbon receptor. *Arch Biochem Biophys* 433: 379–386.
54. Zeruth G, Pollenz RS (2005) Isolation and characterization of a dioxin-inducible CYP1A1 promoter/enhancer region from zebrafish (*Danio rerio*). *Zebrafish* 2: 197–210.
55. Beedanagari SR, Taylor RT, Bui P, Wang F, Nickerson DW, et al. (2010) Role of epigenetic mechanisms in differential regulation of the dioxin-inducible human CYP1A1 and CYP1B1 genes. *Mol Pharmacol* 78: 608–616.
56. Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids Symp Ser* 41: 95–98.
57. Lewis DF, Gillam EM, Everett SA, Shimada T (2003) Molecular modelling of human CYP1B1 substrate interactions and investigation of allelic variant effects on metabolism. *Chem Biol Interact* 145: 281–295.
58. Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 3: 1101–1108.
59. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402–408.
60. Ramakers C, Ruijter JM, Deprez RH, Moorman AF (2003) Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci Lett* 339: 62–66.
61. Ruijter JM, Ramakers C, Hoogaars WMH, Karlen Y, Bakker O, et al. (2009) Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res* 37: e45.
62. Grubbs FE (1969) Procedures for detecting outlying observations in samples. *Technometrics* 11: 1–21.
63. Hamburger V, Hamilton HL (1951) A series of normal stages in the development of the chick embryo. *Journal of Morphology* 88: 49–92.