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Identification and Expression of Multiple *CYP1*-like and *CYP3*-like Genes in the Bivalve Mollusk *Mytilus edulis*

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Abstract

Various sequencing projects over the last several years have aided the discovery of previously uncharacterized invertebrate sequences, including new cytochrome P450 genes (CYPs). Here we present data on the identification and characterization of two *CYP1*-like and three *CYP3*-like genes from the bivalve mollusk *Mytilus edulis*, and assess their potential as biomarkers based on their responses to several known vertebrate aryl hydrocarbon receptor (AHR) agonists. Quantitative real-time PCR was used to measure *CYP* transcript levels in digestive gland, labial palps, adductor muscle, gill, foot, and different regions of the mantle. Levels of both *CYP1*-like genes were highest in digestive gland, whereas labial palps had the highest expression levels of the three *CYP3*-like genes followed by digestive gland and outer margin of the mantle. Mussels were exposed by injection to the AHR agonists, β -naphthoflavone (BNF; 25 $\mu\text{g.g}^{-1}$), 3,3',4,4',5-polychlorinated biphenyl (PCB126; 2 $\mu\text{g.g}^{-1}$), or 6-formylindolo[3,2-b]carbazole (FICZ; 0.1 $\mu\text{g.g}^{-1}$), or to Aroclor 1254 (a mixture of PCBs; 50 $\mu\text{g.g}^{-1}$) for 24 hours, followed by *CYP* expression analysis. There was no statistically significant change in expression of either of the *CYP1*-like genes after exposure to the various AHR agonists. The *CYP3*-like-1 gene was significantly up-regulated by BNF in gill tissues and the *CYP3*-like-2 gene was up-regulated in digestive gland by PCB126 and in gill tissue by BNF. These results suggest that distinct mechanisms of *CYP* gene activation could be present in *M. edulis*, although the importance of the *CYP1*-like and *CYP3*-like genes for xenobiotic and endogenous lipids biotransformation requires additional investigation.

Keywords

cytochrome P450; CYP; AHR; *Mytilus edulis*; blue mussel; pollution

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Introduction

The cytochrome P450s (CYPs) comprise one of the largest and most versatile protein families in living organisms, and they catalyze an extraordinary range of important biochemical reactions. Vertebrate CYPs are distributed in 18 families and a growing number of subfamilies, with for example, 57 genes arranged into 42 subfamilies in humans (Nelson et al., 2004). A large diversity of *CYP* genes also occurs in invertebrates, including members of the prebilateria such as the sea anemone (Goldstone, 2008), early-diverging deuterostomes (e.g., sea urchin (Goldstone et al., 2006)), and protostomes, including the ecdysozoa (e.g. *Daphnia pulex* (Baldwin et al., 2009) and *Caenorhabditis elegans* (Gotoh, 1998)), and the lophotrochozoa (e.g., the gastropod *Lottia gigantea* (Gotoh, 2012; Nelson, 2011; Nelson et al., 2012)). In all animals CYPs are thought to have roles in xenobiotic metabolism and bioactivation, and in development and maintenance of homeostasis, via regulation of signaling molecules (e.g. steroid hormones, eicosanoids and retinoic acid) (Nebert and Dalton, 2006; Nebert and Russell, 2002; Rewitz et al., 2006). However, even in vertebrates not all CYPs have known functions, and for most species many CYPs can be considered as “orphans”, enzymes for which the biological role is unknown.

In vertebrates, members of the CYP1 and CYP2 families (all in CYP Clan 2) and the CYP3 family (in CYP Clan 3) are known for the oxidative transformation of xenobiotics, e.g., drugs, pesticides and polycyclic aromatic hydrocarbons (PAHs) (Nebert and Russell, 2002). The induction of vertebrate *CYP1* genes occurs via the aryl-hydrocarbon receptor (AHR), activated by xenobiotic chemicals such as planar halogenated hydrocarbons and PAHs, and by dietary compounds such as flavonoids (Denison et al., 2002; Hahn, 2002). The expression of CYP1 family members, especially of the CYP1A subfamily in fish, birds and mammals, has been used for decades as a marker of environmental or experimental exposure to AHR agonists (Bucheli and Fent, 1995; Payne, 1976; Stegeman et al., 1986).

Bivalves have long been known to accumulate foreign organic chemicals (Stegeman and Teal, 1973) and metals (Huggett et al., 1973) and have been employed extensively as sentinels in monitoring programs, e.g., the Mussel Watch Program in the USA and the MEDPOL and BIOMAR programs in Europe (Dondero et al., 2006; Venier et al., 2006). Many studies have examined total microsomal cytochrome P450 content, levels of CYP enzyme activity, or CYP protein by immunoassay in bivalve mollusks exposed to chemicals (Canova et al., 1998; Livingstone, 1998; Livingstone et al., 1989; Monari et al., 2007; Monari et al., 2009; Shaw et al., 2002; Snyder et al., 2001; Sole and Livingstone, 2005; Wootton et al., 1995). The interpretation of those measurements has been complicated by low catalytic activity of microsomal enzymes with diagnostic substrates for CYP1A-like activity, such as ethoxyresorufin (ethoxyresorufin-O-deethylase activity, EROD) and benzo[a]pyrene (benzo[a]pyrene hydroxylase; BPH), and lack of specific cross-reactivity of antibodies for CYP1As, (Chaty et al., 2004; Jonsson et al., 2006a; Jonsson et al., 2006b). Thus, the use of CYP protein or activity as a marker of exposure to or effects of environmental contaminants in bivalves has met with limited success and weak inference at best, reflecting the limited characterization of *CYP* genes and proteins in mollusks.

The sequencing of the gastropod *Lottia gigantea* genome revealed numerous *CYP* genes, and bivalve EST libraries have provided a new basis of comparison into molluscan CYP diversity (Craft et al., 2010; Fiedler et al., 2010; Gotoh, 2012; Lockwood and Somero, 2011; Milan et al., 2011; Nelson, 2011; Nelson et al., 2012). We recently identified more than 50 unique *CYP* sequences in *Mytilus californianus* (Zanette et al., 2010) from previously sequenced EST libraries (Connor and Gracey, 2011). Some of these sequences clustered with *CYP1* sequences of vertebrates and with *CYP1*-like genes in *Lottia gigantea* (Zanette et al., 2010). In the present study we focused on *M. edulis* CYP sequences, seeking possible

homologs of vertebrate CYPs that respond to chemical exposure. We cloned full-length sequences for multiple *CYP* genes, and found them related to genes in vertebrate CYP1 and CYP3 families. We examined organ distribution of expression and the responses to selected AHR agonists, including 3,3',4,4',5-polychlorinated biphenyl congener 126 (PCB126), a mixture of PCBs (Aroclor 1254), and the synthetic compound β -naphthoflavone (BNF). Notably, we also tested the endogenous tryptophan metabolite 6-formylindolo[3,2-b]carbazole (FICZ), a highly potent and rapidly metabolized AHR agonist suggested to be a physiological ligand for AHR (Wincent et al., 2009). The results are foundational for studies of *CYP1*- and *CYP3*-like genes in bivalves.

Methods

Animal sampling and laboratory care

Mussel *Mytilus edulis* of both sexes, 6–8 cm in shell length, were sampled from a clean reference site (Scorton Creek, Massachusetts, USA) in August and September 2008, transferred to the laboratory and acclimated at 20°C in flowing seawater for one week. Food was given twice a day as a mixture of microalgae *Thalassiosira weissflogii*, *Tetraselmis chuii* and *Isochrysis sp.*, cultured for this purpose.

Cloning of *CYP1*-like and *CYP3*-like transcripts in *Mytilus edulis*

Digestive gland was dissected from one randomly selected untreated mussel. Total RNA was isolated using the AurumTM Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad Laboratories Inc., Hercules, CA), which eliminates genomic DNA by DNase treatment. RNA quantity and quality were determined spectrophotometrically (Nanodrop ND 1000; NanoDrop Technologies, Wilmington, DE). cDNA was synthesized from 2 μ g of total RNA, using the Omniscript Reverse Transcriptase kit (Qiagen Inc., Valencia, CA), anchored oligo(dT) primer (MWG Biotech, Inc., High Point, NC) and RNasin RNase inhibitor (Promega Corp., Madison, WI).

Primers used in the identification of the *M. edulis CYP1-like-1*, *CYP1-like-2* and *CYP3-like-3* sequences were designed with Primer3 (Rozen and Skaletsky, 2000). The primers were designed based on the *CYP1*-like and *CYP3*-like transcripts from the mussel *M. californianus*, previously identified by phylogenetic analysis (Zanette et al., 2010), and were to regions of the transcripts expected to be highly conserved. *CYP1*-like-1 primers were designed based on one *M. californianus* contig obtained from EST sequences in the GenBank with accession numbers ES401002, ES398625, ES407183, ES400274, ES401422, ES400520, ES403691, ES405860, ES401376, ES402773, ES404163, ES398150 and ES407922. Primers for *CYP1*-like-2 and *CYP3*-like-3 were designed based on *M. californianus* EST sequences with the accession numbers ES399754 and ES400411, respectively. Primer sequences are shown in Table 1. The full-length sequences of the two other *M. edulis CYP3*-like genes analyzed in the present study were found in Genbank, accession numbers AB479540 and AB479539. We did not clone those sequences but used them to design qPCR primers (below). (A paper describing these latter two *M. edulis CYP3*-like genes was published recently (Cubero-Leon et al., 2012), after our study was completed. The sequences we refer to as *CYP3*-like-1 and *CYP3*-like-2 are referred to as *CYP3A-like isoform 2* and *CYP3A-like isoform 1*, respectively, in that paper.)

The PCR amplified products for the new *CYP* sequences were resolved on 1.5 % agarose gel, isolated and ligated in pGEM-T Easy Vector (Promega), and transformed into *E. coli* (TOP 10 Kit, Invitrogen). Plasmids were purified from cultures of positive clones (QiaPrepTM, Qiagen) and sequenced (MWG Biotech). The 5' and 3' ends of those transcripts were obtained by rapid amplification of cDNA ends (RACE) with the BD SmartTM RACE

cDNA Amplification Kit (Clontech) and gene-specific primers described in Table 2. Gel-purified 3' and 5' RACE products were cloned and sequenced as described above.

Phylogenetic analysis of *Mytilus edulis* CYPs

Nucleotide sequences for *CYP1*-like-1, *CYP1*-like-2, *CYP3*-like-1, *CYP3*-like-2 and *CYP3*-like-3 were translated and aligned with other CYP sequences obtained from various databases and publications (*Mytilus californianus* (Connor and Gracey, 2011), *Lottia gigantea*, JGI; *Crassostrea gigas* (Zhang et al., 2012)) using Muscle [v3.8.31; (Edgar, 2004)]. Phylogenetic trees were constructed by analyzing predicted amino acid sequences using maximum likelihood [RAxML 7.2.6; (Stamatakis, 2006a; Stamatakis et al., 2008)]. Regions of uncertain alignment were masked using a custom script. The WAG-CAT model of amino acid substitution with a gamma distribution of substitution rates (PROTMIXWAG) was used in all likelihood analyses, based on likelihood tests using RAxML (Stamatakis, 2006b).

Experimental treatment

A small hole, 1.5 mm diameter, was notched between the mussel valves, one day prior to injection of the chemicals (treated groups) or vehicle (vegetable oil, control group). Mussels collected in August 2008 were injected in the adductor muscle with 100 µl of the PCB mixture (Aroclor 1254; final dose 50 µg per g of mussel tissue), 6-formylindolo[3,2-b]carbazole (FICZ; 0.1 µg/g) or pure vegetable oil (Pure Vegetable Oil, Crisco®, J. M. Smucker Company, Orrville, OH, 44667, USA). Mussels collected in September 2008 were injected with 100 µl of BNF (β-naphthoflavone; 25 µg/g), PCB126 (3,3',4,4',5-pentachlorobiphenyl; 2 µg/g) or vegetable oil. Animals were allowed to rest on the bench for 30 min prior to being distributed in 10 L aerated aquaria (n=8 mussels per group). The doses employed in the injection of all four chemicals have been shown to strongly induce *CYP1A* gene expression or catalytic activity in fish (Chaty et al., 2004; Jonsson et al., 2009; Jonsson et al., 2007; Zanette et al., 2009). The dose calculation in this experiment was done assuming an average whole meat weight of 16 g per mussel, based on the biometry of five mussels from the same sets of samples.

At 24 h after injection, control mussels from the August 2008 experiment were sampled and digestive gland, gonad, labial palps, mantle margin, adductor muscle, gills and foot of individual mussels were dissected for use in the organ-specific gene expression analysis. The digestive glands of mussels treated with Aroclor 1254 and FICZ were dissected as well. In the September 2008 experiment, digestive glands and gills were dissected 24 h after injection with vegetable oil (control), PCB126 or BNF. The dissected organs were immediately placed in RNAlater (Ambion), held for 24 h at 4 °C, then stored at -20 °C.

Quantification of *CYP1*-like and *CYP3*-like transcripts

Total RNA was extracted and cDNA was synthesized as described before. Real-time PCR primers were designed to the new *M. edulis* *CYP1*-like and *CYP3*-like sequences, and to the 28S gene (based on alignments from AF120587, Z29550, and AF339512). Primers were obtained from MWG Biotech (Primer sequences are shown in Table 3). Real-time PCR was performed using iQ SYBR Green Supermix (according to the manufacturer's instructions) and the iQ Real-Time PCR Detection System (Bio-Rad). For each sample, gene expression was analyzed in triplicate with the following protocol: 95°C for 3 min and 40 cycles of 95 °C for 15 s and 62 °C for 1 min. Melt curve analysis was performed on the PCR products at the end of each run to ensure that a single product was amplified. Quantitative assessment of mRNA or rRNA transcript molecule numbers was calculated using the standard curves generated from dilutions (10^2 – 10^9 molecules) of plasmid (pGEM-T Easy Vector) containing 150–1200 bp of a given target transcript. *CYP* transcript numbers were calculated

by determining the mean 28S rRNA transcript molecule numbers from all samples for a given tissue and using that value to adjust all individual samples by using a normalization factor (28 S mol. number for sample)/(28S mol. number average in the organ) to calculate the relative transcript numbers per 1 μ L of cDNA (equivalent to 100 ng of total RNA). All real-time quantitative-PCR results for *CYP* transcripts represent the relative number of *CYP* transcript molecules per 100 ng of total RNA.

Statistics

Equality of variance was tested, accepting $P > 0.05$ (Bartlett's test) (Prism 4, GraphPad Software Inc., San Diego, CA). Data were logarithmically transformed to improve equality of variances. The differences among transcript levels for *CYP*s in different organs, or among control and treated groups, were evaluated using one-way analysis of variance (ANOVA, $P < 0.05$), followed by Tukey's test for multiple comparisons (Prism 4).

Results

Identification, cloning and analysis of *M. edulis* CYP sequences

Using non-specific primers designed from *M. californianus* *CYP* sequences, *M. edulis* nucleotide sequences of 1125, 831 and 662 bp were amplified from randomly primed cDNA by PCR, cloned, and sequenced. These were preliminarily designated as two new *CYP1*-like sequences and one new *CYP3*-like sequence, respectively. Sequencing of 5' and 3' ends yielded transcripts that were 1683, 1433 and 1614 base pairs in length and that coded for predicted proteins with 502, 461 and 468 amino acids, respectively (accession numbers JX885878, JX885879, JX885882) (Figure 1 and Figure 2). In addition, sequences identified as *CYP3*-like transcripts in *M. californianus* ESTs (Zanette et al., 2010), were searched against *M. edulis* sequences in GenBank. This search revealed two full-length *M. edulis* *CYP3*-like sequences (accession number AB479540 and AB479539) with predicted proteins of 503 and 515 amino acids, respectively, which were 51% identical to one another (Table 4). As noted above, those two *CYP3*-like sequences were recently described elsewhere (Cubero-Leon et al., 2012).

Phylogenetic analysis of the five *CYP*s showed that the inferred amino acid sequences clustered with known vertebrate *CYP1* and *CYP3* sequences (Figure 3). Accordingly, we have designated these genes as *CYP1*-like-1 and *CYP1*-like-2, and *CYP3*-like-1, *CYP3*-like-2, and *CYP3*-like-3, based on their phylogenetic relationships to the *CYP1*s and *CYP3*s. Initial alignments of the predicted protein sequences suggested that *CYP1*-like-2 and *CYP3*-like-3 are missing 35–40 amino acids at the amino terminal end (Figure 1 and Figure 2). All sequences were masked to remove uncertainly aligned regions prior to phylogenetic analysis, including most of these missing sections. The *CYP1*-like sequences clustered closer to vertebrate *CYP1* sequences than to other families in Clan 2 (i.e., *CYP2*s, *CYP17*s and *CYP21*s). Figure 3 also shows that the *M. edulis* *CYP1*-like sequences clustered with the *CYP1*-like genes from the owl limpet, *Lottia gigantea*. The *M. edulis* *CYP3*-like sequences clustered in Clan 3 with *CYP*s from a variety of other molluscan species, in a sister clade to the vertebrate *CYP3*s.

The new *M. edulis* *CYP1*-like-1 and *CYP1*-like-2 predicted proteins share 33–34 % and 28–29 % identity with the human *CYP1*As, respectively, and 46 % to one another (Table 4). The new *M. edulis* *CYP3*-like-3 predicted protein exhibits 54 % and 43 % identity with the previously identified *M. edulis* *CYP3*-like-1 and *CYP3*-like-2 sequences, respectively (Table 5). *CYP3*-like-1, *CYP3*-like-2 and *CYP3*-like-3 predicted proteins also respectively share 38 %, 39 % and 36 % identity with human *CYP3A4*, respectively.

The inferred protein sequences of these new CYPs all have the classic conserved structural motifs, including the helix-C (WxxxR), helix-I (GxE/DTT), helix-K (ExxR), PERF (PxxFxPE/DRF) and the heme binding domain (xFxxGxRxCxG) (Figures 1 and 2). Sequences in putative substrate recognition sites (SRS) were examined (Gotoh, 1992; Kirischian et al., 2011). SRS 2, SRS 3 and SRS 6, showed less similarity with these SRS sequences of vertebrate CYPs in family 1 or 3 than did other SRSs. In contrast, SRS4 and SRS5 showed the highest similarity comparing the mussel CYP1-like or CYP3-like sequences, with the presumed homologous vertebrate CYP1 or CYP3 sequences (Figures 1 and 2, respectively).

CYP1-like and CYP3-like gene expression in *Mytilus edulis* organs

The two newly identified *M. edulis* CYP1-like genes showed similar patterns of organ-specific expression, with higher levels of transcripts observed in digestive gland, compared to other organs. Levels of CYP1-like-1 expression were intermediate in the gonad and labial palps, and very low in mantle margin, adductor muscle and foot. Levels of CYP1-like-2 expression were markedly higher in the digestive gland compared to the other six organs (Figure 4).

Similar to the CYP1-like genes, high levels of expression of CYP3-like-1, CYP3-like-2 and CYP3-like-3 genes were observed in the digestive gland. However, the highest levels of expression were found in the labial palps and expression in the mantle margin was similar to the levels observed in the digestive gland. Lower CYP3-like expression levels were observed in gill, compared to other organs (Figure 4).

Response of *M. edulis* CYP1-like and CYP3-like genes to AHR agonists

The response of the mussel CYP genes to treatment with AHR agonists was evaluated with a PCB mixture (Aroclor 1254) containing dioxin-like AHR agonists, as well as non-dioxin-like congeners, and with three single compounds, a slowly metabolized dioxin-like PCB congener PCB126, a rapidly metabolized synthetic AHR ligand BNF, and the highly potent and rapidly metabolized AHR agonist FICZ. Short-term exposure experiments with bivalve species can be complicated by shell closure and the accompanying changes in metabolic function if the shell is closed for an extended period of time. We chose to inject the AHR agonists directly into the adductor muscle to insure a consistent initial exposure. Injection of mussels with Aroclor 1254 or FICZ did not cause any changes in the levels of mRNA expression of the CYP1-like or the CYP3-like genes in digestive glands of *M. edulis* after 24 h, compared to the control group (Figure 5). However, the injection with PCB 126 resulted in a modest increase in the levels of digestive gland CYP3-like-2 transcript expression, but there was not a statistically significant difference in transcript levels of the CYP1-like genes or the other two CYP3-like genes in either digestive gland or gill tissues. Treatment with BNF did not cause any changes in digestive gland CYP1-like and CYP3-like expression (Figure 6). However, BNF did elicit an increase in the expression of CYP3-like-1 and CYP3-like-2 genes in gill, compared to the controls (Figure 7).

Discussion

A preliminary study allowed us to identify many CYP sequences in the bivalve mollusk *M. californianus* (Zanette et al., 2010). To address the CYP complement and responses of selected CYPs in a definitive way we identified and cloned transcripts from *M. edulis* that appear to be homologues of two gene families important in xenobiotic metabolism in vertebrates, CYP families 1 and 3. Previous studies of CYP responses in bivalve mollusks have been hindered because of uncertainty regarding the identity of genes present, and a lack of specific substrate or antibody probes for molluscan CYPs, often leading to mistaken

conclusions. For example, putative CYP1A cross-reactive proteins detected by immunoblotting in mussels and clams were later identified as actin and tropomyosin, respectively (Grosvik et al., 2006). This is the first study to characterize and evaluate mRNA expression profiles of multiple *CYP1*-like genes, together with multiple *CYP3*-like genes in a bivalve mollusk. The results suggest distinct organ-specific patterns for the different genes. There was no hint of induction of *CYP1*-like mRNA in response to vertebrate AHR agonists, but two of the *CYP3*-like genes showed responses.

Phylogenetic analysis of *Mytilus edulis* CYP1-like and CYP3-like sequences

Molecular phylogenetic analyses of the five CYPs we analyzed from *M. edulis* consistently showed that these mussel sequences clustered with known CYP1s and CYP3s. The CYP1-like inferred proteins, shared 28% – 31% identity with the zebrafish CYP1A, while the CYP3-like sequences shared a higher % identity with vertebrate CYP3s, ranging from 36% to 39% identity to human CYP3A4. The usual system for CYP classification establishes cut-off identities of 40% and 55% between CYP protein sequences to be considered as belonging in the same family and subfamily, respectively (Nelson et al., 1996). However, with greater evolutionary distance, the percent identity for inclusion in a given CYP family can be lower than 40% (Nelson et al., 2004). Thus, both the phylogenetic analyses and the shared sequence identities suggest that these mussel sequences likely belong in CYP families 1 and 3, although here we employ the terms *CYP1-like* and *CYP3-like* for the *M. edulis* CYP sequences, following other examples for provisional nomenclature for invertebrate CYP genes (Goldstone et al., 2007; Verslycke et al., 2006). A definitive family assignment for CYPs in the Lophotrochozoa, which includes mollusks, may depend on other features. Shared synteny is one such feature, but the rate of synteny loss in different animal groups is variable, e.g., relatively slow in anemone, amphioxus and urchin, and very fast in some insects, which can render gene order largely uninformative. Synteny analysis for P450s in the genome of the mollusk *Lottia gigantea* is in progress (Nelson, 2011; Nelson et al., 2012).

The existence of *CYP1* or even *CYP1*-like genes in protostomes has not been established in the literature, and has been questioned by some authors (Goldstone, 2008; Goldstone et al., 2007; Goldstone et al., 2006; Rewitz et al., 2006). In addition to the genes we describe, four *CYP1*-like genes are present also in the genome of the oyster *Crassostrea gigas*, along with at least ten other slightly more distantly related CYPs (Zhang et al., 2012). As far as we are aware, ours is the first study to examine the expression of *CYP1*-like sequences in a bivalve mollusk. The importance of the CYP1-like proteins to physiology or xenobiotic metabolism in organisms where they do occur (i.e., in the basal lineage of deuterostomes, in echinoderms and tunicates) is not clear (Goldstone et al., 2007). This certainly is true as well of the bivalve *CYP1*-like genes.

The conserved nature of CYP Clan 3 genes in different animal groups has previously been observed. Comparison of *CYP3*-like genes from tunicate (Verslycke et al., 2006) and the *CYP30* gene from the bivalve mollusk *Mercenaria mercenaria* (Brown et al., 1998) with the vertebrate CYP3s indicate strongly that CYP Clan 3, and likely CYP family 3, is represented in mollusks. This would be consistent with a suggestion that a common CYP family 3 ancestral gene was present 800 to 1000 million years ago, before the split to deuterostomes and protostomes (Goldstone, 2008; Verslycke et al., 2006). We find it intriguing that the mussel CYP3-like sequences share a higher % identity with vertebrate CYP3s, than do the tunicate “CYP3s”, or the clam CYP30. The identity between the three *M. edulis* CYP3-like sequences, and the *M. mercenaria* CYP30 was not striking (34%-37%). Differences in the Clan 3 genes in different bivalve species will be explored in further studies.

Organ-distribution of *CYP1*-like and *CYP3*-like transcript levels in *Mytilus edulis*

Much higher levels of *CYP1*-like transcripts were observed in the *M. edulis* digestive gland, than in gonad/mantle, labial palp, mantle margin, adductor muscle, gill or foot. The digestive gland in bivalve mollusks is thought to have functions equivalent to the liver in vertebrates, with a role in elimination of xenobiotics and some endogenous substrates (Marigomez et al., 1999). The CYP1 family members in mammals are involved in carcinogenesis, oxidative stress, hepatotoxicity, diseases related to the metabolism of a variety of xenobiotics and endogenous substrates (Nebert and Russell, 2002). Assuming that organ-specific distribution in the expression of a gene could relate to function, some similarity in the function for the *Mytilus* CYP1-like and the vertebrate CYP1A could be predicted.

M. edulis CYP3-like genes also were similar in their patterns of organ-specific expression, and quite different from the *CYP1*-like genes. In mammals and fish, CYP3 proteins are very abundant in the liver and especially the gastrointestinal tract. In humans it is estimated that CYP3s metabolize between 40–60 % of the pharmacological compounds (Guengerich, 2008), as well as endogenous substrates like sterols, arachidonic acid, bile salts, and vitamin D (Nebert and Dalton, 2006). In the present study, the organ-specific transcript levels of the three *M. edulis* CYP3-like genes showed the highest levels in the labial palp, digestive gland, and mantle margin; intermediate levels in gonad, muscle and foot; and the lowest levels in gill. Little is known about the importance of the labial palps for xenobiotic metabolism in bivalves. However, their role in pre-ingestive food sorting (Milke and Ward, 2003) could result in high levels of exposure requiring the metabolism of xenobiotics or endogenous substrates.

Many of the tissues had relatively little variation in transcript expression between individual mussels, though variation was high in digestive gland and labial palp, which may be related to the functions of the organs. Variation in gene expression is commonly observed between individuals when sampling from wild populations of bivalves, due to factors including genetic diversity, seasonal variation and sex differences in physiology (Banni et al., 2011; Ciocan et al., 2011). We chose to collect mussels later in the summer after the spawning season, and did not look at sex-specific differences. Nevertheless, some variation in CYP gene expression could result from having both male and female mussels in the same treatment groups. Investigation of sex differences in CYP expression as linked to seasonal variation and gametogenesis are warranted.

Response of *M. edulis* CYP1-like and CYP3-like to vertebrate AHR agonists

The *M. edulis* CYP1-like genes did not show any response to three potent vertebrate AHR agonists or the PCB mixture Aroclor1254 (which can induce CYP1s, CYP2s and CYP3s in mammals). In fish, CYP1A induction has been widely employed as a biomarker of exposure to AHR agonists in the aquatic environment [e.g (Bucheli and Fent, 1995; Payne, 1976; Stegeman et al., 1986)]. The prominent use of bivalve mollusks in pollution monitoring programs has prompted numerous efforts to find some CYP gene, protein or catalytic activity that would provide a similar marker of exposure in these organisms (Cravo et al., 2009; Livingstone et al., 1989; Monari et al., 2007; Monari et al., 2009; Peters et al., 1998; Porte et al., 2001; Shaw et al., 2002; Snyder et al., 2001; Sole and Livingstone, 2005; Wootton et al., 1995). There has been little success, due to lack of knowledge of specific CYP sequences, probes, or substrates [e.g. (Grosvik et al., 2006; Livingstone and Farrar, 1984; Sole and Livingstone, 2005; Stegeman and Livingstone, 1998)]. AHR occurs in invertebrates (including bivalves) (Butler et al., 2004; Hahn, 2002; Liu et al., 2010; Qin and Powell-Coffman, 2004) but whether there is a link to any CYP regulation is unknown. While recent studies have shown increases [e.g.(Bebiano et al., 2007; de Toledo-Silva et

al., 2008)] as well as decreases (Cubero-Leon et al., 2012) in CYP expression, such changes still lack a mechanistic understanding that would support inference and prediction.

While it is possible that exposure longer than the 24 hr period we used could result in some response, our data are sufficient to indicate that there is not a direct transcriptional response at a time that in vertebrates is sufficient for strong induction of transcript levels via AHR activation. Thus, the lack of response seen here indicates that the *CYP1*-like genes may not satisfy the search for molluscan CYPs inducible by AHR agonists. An AHR homolog has been identified and cloned from *M. edulis* (M. E. Hahn, personal communication), and AHR has been identified in other bivalves (Butler et al., 2001; Liu et al., 2010). A recent report (Denison et al., 2011) indicates that halogenated and non-halogenated compounds can interact differently with vertebrate AHR possibly affecting its activation. However, the *M. arenaria* AHR was shown not to bind the halogenated AHR agonist TCDD or the non-halogenated agonist BNF (Butler et al., 2001). BNF typically is substantially less potent than TCDD. However, the *M. edulis* *CYP1*-like genes also did not respond to FICZ, the highly potent endogenous agonist for vertebrate AHR. This argues that the AHR does not regulate molluscan “*CYP1*”s. Thus, the *M. edulis* *CYP1*-like genes identified here may not be suitable biomarkers of exposure to AHR agonists, and may not be involved in (transcriptionally dependent) changes in monooxygenase activity occasionally seen in bivalves exposed to AHR agonists. While lack of induction could reflect lack of AHR activation by typical agonists, it is also possible that molluscan AHR simply is not involved in the regulation of *CYP1*-like genes. Thus, promoter regions for the *M. edulis* *CYP1*-like genes might not possess functional AHR response elements; such possibility can be examined once the recognition sites for mollusk AHR DNA binding have been determined.

There is a growing body of data showing that invertebrate AHRs do not bind known ligands for vertebrate AHRs, indicating important functional differences between invertebrate and vertebrate AHRs. There still could be some involvement of “vertebrate” ligands in invertebrate CYP regulation, perhaps through altering interactions with other ligands. This was suggested for benzo[a]pyrene modifying the effect of xanthotoxin in activation of the promoter of black swallowtail butterfly *CYP6B1* (Brown et al., 2005). *CYP6B1* is in CYP clan 3, where the mussel *CYP3*-like genes cluster. Expression of *CYP3*-like-1 and *CYP3*-like-2 in gill was induced slightly by BNF. It is tempting to invoke the AHR, although mammalian *CYP3*As can be induced by PAH also via the nuclear transcription factor NR1I2 (the pregnane X receptor, PXR; also known as the steroid xenobiotic receptor, SXR) (Kumagai et al., 2012). The PXR-related constitutive androstane receptor (NR1I3, CAR) evolved in the mammalian line and does not occur in invertebrates (Krasowski et al., 2005). Studies do suggest that a nuclear receptor NR1I in early deuterostomes (tunicates) is a co-orthologue of vertebrate vitamin D receptor and PXR, and that despite a more limited ligand specificity compared to vertebrate PXR (Fidler et al., 2012; Reschly et al., 2007), it appears to bind FICZ (Ekins et al., 2008). Homologues of VDR or PXR have not been found in mollusks, although the PXR/VDR dimer partner RXR (retinoid X receptor) does occur (Horiguchi et al., 2007; Nishikawa et al., 2004; Sternberg et al., 2008). RXR’s role in response to various xenobiotic chemicals in mollusks should be assessed.

In conclusion, we identified new *CYP1*-like and *CYP3*-like genes in *M. edulis* and described marked differences in their expression, including a lack of induction of the *CYP1*-like genes and slight induction of two *CYP3*-like genes by AHR agonists. Uncovering new CYP sequences and evaluating expression and responses of the full CYP complement to different contaminant classes in the mussel *M. edulis*, in relation to other gene responses (for example by RNAseq), could identify CYP genes that are regulated via contaminant exposure, and those possibly involved in endogenous substrate metabolism. Which receptors may be

involved in regulation of CYPs in bivalves is unclear; the possible involvement of novel receptors should not be ignored. Establishing receptor involvement could uncover new biomarkers of aquatic contamination, and provide a better understanding of physiologic mechanisms in those organisms that possess remarkable environmental, economic, and social importance. At present however, occasional CYP protein responses cannot be explained by a mechanism, and thus are not likely to be predictable. Finally, we emphasize that finding a molluscan CYP similar to a vertebrate CYP does not mean finding similar functions; that hypothesis must be addressed experimentally, by heterologous expression of the mussel CYPs and analysis of the catalytic functions of the individual CYPs directly.

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me_cyp3-like-1 MDILGFTVTPGVWLITIIILITLYLSLWHSWRRNGIFCEPKPTFLGLVPLFKKHSLAKADELVKKYGVSVGVNGL 80
me_cyp3-like-2 ..V..L.DI.S.IF..LLF.F....T..K.T....L.V....I.F.H..SIKDLAVK.VS.T.QD..P.....T 80
me_cyp3-like-3 -----E.....I.KEYF.K.SCNT.....R.....N.....QCH 41
dr_CYP3A65 --MFFS---AETWTLAF.VAL.VI.GS.P.RYFKKL.....A.L.F.TMLRY..D.PHVFMCFE...RIW.I.D.R 74
dr_CYP3C1 --MFDLSLSLVTWTLVVLV.TL.LI.GV.P.GFFKKL.....R.L.V.TALSYS-K.ICNF.I.CS...K.W.F.I.D.R 77
hs_CYP3A4 --MA.IPDAMETW.LLAVSLVL...GTHS.G.FKKL....T.L...NLSYH-K.FCMF.M.CH...K.W.F.G.D.Q 78

me_cyp3-like-1 LEVLVSIPAMIKEIFIKE-FSNETN-RPIPFKMDKETASSVGTAFDNHKKFLRSTLSPTESSGKOLMVPKIHRCCTDL 158
me_cyp3-like-2 I.....T.TDL.....-YQT...-VLIL.T.DLLD.MITA.E.D....I...MT...T...L.N.L.M.L...D.. 158
me_cyp3-like-3 I.S...A..E.L...V...-...P...F.V.PTERMLAG.AL.OGT.....I...TV..L...Q.EK..QA. 119
dr_CYP3A65 Q...CIM.QTI...TVL...CY.L...R.NFR--INGALYDA.SI.E.DD.RRI..V...S.T..RL.E.FGIMKKHSHT. 152
dr_CYP3C1 ..L...T.LE...T.LV.DCY.T...R.NMNPDLVGPF.DGITLVK.ER.RRI..S...Y.T..RL.EIF.IAMTHADRF 157
hs_CYP3A4 Q...AITT.D...TVLV...CY.V...R...FG--PVGFMK.AISI.E.EE...R...L...T...L.E...I.AQYGDV. 156

me_cyp3-like-1 VENIEQQR-EKGHIDMKVCGAYTMDVIASIAFGIEANSCKEENQFVKHAKAALGEIFKLKFLIMLFFFLKNLVSQ 237
me_cyp3-like-2 ..N..NGKLEQNDTVE.T..F.GF.L.....LQV.....D.E..TNT.KTLTDSVV.PY.VIV.I.....MGL 238
me_cyp3-like-3 ..HS..GDNEPV...L...F...S...V...R..YVG.K..I.QLASSFAVF..M.....ITGTI 199
dr_CYP3A65 .DSMGKTAKRGESA.I..FF...S...VT...SVDDID.LNN.KDP..TNI.KMLKFDLLNPL...AF..MAPVLEKM 232
dr_CYP3C1 IK.M.KKDP-NLPIKI.D.VAP.SL...V..SS.SVDFD.INN.DDP..TSI.SFFNINPLSPL...LAFCSAA..LAKM 236
hs_CYP3A4 .R.LRREAETGKPVTL.D.F...S...T..S...VNID.LNN.QDP..ENT.KLLRFDLPDF..S.TV...IPILE.L 236

me_cyp3-like-1 LS-----SKAVNDIFKTVVDSALERT-----KQO-EVYNDFLQMLNARHGDQTS-----DYFTKGDVQEFKNRGLNN 300
me_cyp3-like-2 FKAP-VFGESE...RNI.QQLVAE.K-----SNSNSHFR..I....SHK.K.KEKENGRI.ELGGL.FS.Y...M.E 312
me_cyp3-like-3 .LRGNRVG.D.IE...D.ALR...D.K-----E.KN.Q.Q.....M...KDEK-----...E 254
dr_CYP3A65 DFA--LFPTS.T...YAALQIKSD.DTKTVA.DNTKKRV...VDSQTGVKHKSD-----G.HTEK..SD 298
dr_CYP3C1 GIS--VF.RSTT..YKALRKIKDEHN-----ESNGRV...K..IQNQIPDD.VKDT-----AS.QPVK..TD 297
hs_CYP3A4 NIC--VFREF.TN.LRKS.KRMK.S.LED-----TQKHRV...IDSQNSKE-----TESHKA.SD 292

me_cyp3-like-1 IELKENAVTEPIAGYDTTANTLSFACYCLATNQDVQKCIDETENVLQGGTE-QMEDLVKLEYLDRFFNEVRLRYGAASR 379
me_cyp3-like-2 N.ILT.SMV..G..F...KT.P...A.V..LHP.M.ERLFT..D.E.GKSP..TYDS.PNMK..EM.LA...A.VP. 391
me_cyp3-like-3 M...S..M...L.....A...S.I.K.L.Q..DT..C.EK...Y..IS...Q...V.....T. 333
dr_CYP3A65 H.ILSQSMILIF...E.SSS...LF.N...PETMK.LQE..DETFFNQAVDY.T.MSMD...AALS.S...FPV.A. 378
dr_CYP3C1 H.ILSQSFILIG...E.ST...YLL.N...P.CLE.LVE...DKNFPLDI.ITYDA.MRMD..EMAIH.SM.VEP.GP. 377
hs_CYP3A4 L..VAQSII..IF...E..SSV...IM.E...HP...Q.LQE...DA..PNKA.PTYDTVLQM...MVV..T...EPI.M. 372

me_cyp3-like-1 FHRAAREDMIVCGVHLPKDVNVGVVYALHNEKYWPDEREDDEDRETDENKAKRPEYTFVFGVSPRICIGMRLALMEA 459
me_cyp3-like-2 INR.KS.I..N.MF...TD.TI.IS.....E..K...E.....T..M.SYL...L..V.....I.T 471
me_cyp3-like-3 .N.ECQS.IKIKDTF...GTDIS..SF.....EF...DK...E..ED.IS...S.I...I...L..L 413
dr_CYP3A65 LE.VCKKTVEIN.LL...LV.M..T...D.D..SE..S.K.E...K.G..ESIDP.MYM...L..N...F.QVTM 458
dr_CYP3C1 LE.VCKKTVEIN.IT...NTL..I.L.V.S.D.DL.E.S.NE.K.E...S.P.S.TEINQCA.M...L..N...F..MM 457
hs_CYP3A4 LE.VCKK.VEIN.MF...G.V.MI.S...D...TE..K.L.E..SKK..DNIDP.IYT...S...F...NM 452

me_cyp3-like-1 KMALVFMILQRFSPCSETEIPEVELEQGAILRAKNGIKININKR*----- 504
me_cyp3-like-2 ..Y..ISXV.N.K.VVG.K...MP..K.F.T.PL...H.K.E...*----- 516
me_cyp3-like-3 ....Y..N.S.TV.DT...TF.K.FVLK.Q..VV..A.P.KDTQ*----- 462
dr_CYP3A65 ..L.I.EI...DV.V.D..QV..L..GFNGLLSP.DP...KLQP.KLSQSPDVCCNNQKS* 518
dr_CYP3C1 LLV.KL..KY.VET.K..Q...Q.NF--FFQP.VP.T.KLIP..SHKEKQ*----- 506
hs_CYP3A4 ..L..IRV..N.S.K..K..Q..LK.SL.GLLQPEKPVV..RVES..DGTVSGA*----- 504

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Figure 2.

Alignment of *Mytilus edulis* (me) CYP3-like amino acid predicted sequences with fish (*Danio rerio*, dr) and human (hs) CYP3 sequences. Dark and light grey boxes denote identical or similar amino acids in the alignment, respectively. Marks denote structural CYP signatures of conserved motifs (C: helix C, I: helix I, K: helix K, PERF: PERF and *: heme binding domain).

Figure 3.

Phylogenetic trees of molluscan and vertebrate cytochrome P450 amino acid sequences. These maximum likelihood phylogenies include *Mytilus* full length sequences (“myted”; *M. edulis*), limpet (“lottia”; *L. gigantea*), sea urchin (“urchin”; *S. purpuratus*), zebrafish, and human sequences, along with selected other full-length bivalve sequences (*C. gigas* CYP356A1, *M. mercenaria* CYP30, *H. cumingii* CYP30). (A) CYP1-like and CYP17-like sequences (human CYP17A1 is the outgroup) (B) CYP3-like (CYP Clan 3) sequences.

Figure 4.

Levels of *CYP1*-like and *CYP3*-like transcripts in different organs of *Mytilus edulis*. Molecule numbers for *CYP* sequences were calculated based on standard curves and normalized to 28S transcript levels within each specific organ. *CYP* transcript levels are presented as the relative transcript molecule numbers per 100 ng of total RNA. Values shown are mean values with error bars representing one standard deviation. Equal letters indicate absence of difference among groups (ANOVA – Tukey HSD, $p < 0.05$, $n = 8$).

Digestive Gland

Figure 5.

Levels of CYP1-like and CYP3-like transcripts in *Mytilus edulis* injected with vegetable oil (control) or with the vertebrate CYP inducers Aroclor 1254 (mixture of PCB isomers) and 6-formylindolo[3,2-b]carbazole (FICZ). Molecule numbers for CYP sequences were calculated based on standard curves and normalized to 28S transcript levels within each specific treatment. CYP transcript levels are presented as the relative transcript molecule numbers per 100 ng of total RNA. The graph is plotted as Box and Whiskers (min., Q1, median, Q3, max.). Equal letters indicates absence of difference among groups (ANOVA – Tukey HSD, $p < 0.05$) ($n=8$).

Digestive Gland

Figure 6.

Levels of digestive gland *CYP1*-like and *CYP3*-like transcripts in *Mytilus edulis* injected with vegetable oil (control) or with the mammalian AHR agonists β-naphthoflavone (BNF) and 3,3',4,4',5-pentachlorobiphenyl (PCB 126). Molecule numbers for *CYP* sequences were calculated based on standard curves and normalized to 28S transcript levels within each specific treatment. *CYP* transcript levels are presented as the relative transcript molecule numbers per 100 ng of total RNA. The graph is plotted as Box and Whiskers (min., Q1, median, Q3, max.). Equal letters indicates absence of difference among groups (ANOVA – Tukey HSD, $p < 0.05$) ($n=8$).

Gill

Figure 7.

Levels of gill *CYP1*-like and *CYP3*-like transcripts in *Mytilus edulis* injected with vegetable oil (control) or with the mammalian AHR agonists β -naphthoflavone (BNF) and 3,3',4,4',5-pentachlorobiphenyl (PCB 126). Molecule numbers for *CYP* sequences were calculated based on standard curves and normalized to 28S transcript levels within each specific treatment. *CYP* transcript levels are presented as the relative transcript molecule numbers per 100 ng of total RNA. The graph is plotted as Box and Whiskers (min., Q1, median, Q3, max.). Equal letters indicates absence of difference among groups (ANOVA –Tukey HSD, $p < 0.05$) (n=8).

Table 1

Mytilus californianus primers used for the cloning of the new *CYP1*-like and *CYP3*-like genes in *Mytilus edulis*.

Primer name	Primer sequence 5'-3'
CYP1-1 forward	TGACGTGTTTAAAATTCGCATGG
CYP1-1 reverse	CTGCGTCCAATACCAAATGTAAG
CYP1-2 forward	TTGGGAAATACGAAAGCTACCTCCA
CYP1-2 reverse	TCCAGCAACAATGAAATCCCGTA
CYP3-3 forward	TTCCTGGTCCGGAACCAACTCC
CYP3-3 reverse	GCAGACCTTAGCGCCGTGTCG

Table 2

RACE specific-primers used for full-length cloning of *CYP1*-like-1, *CYP1*-like-2 and *CYP3*-like-3 sequences in *Mytilus edulis*.

Primer name	Primer sequence 5'-3'
CYP1-1 forward 1	GCAGAGAGGCAATACGTGAAGCGTTG
CYP1-1 forward 2	AACCCACTTCAGCCCCATGATGAC
CYP1-1 reverse 1	GTTCCGGCGAATTTCGTATTGTTCC
CYP1-1 reverse 2	GGCATAACAAATCGCAACCATGGCA
CYP1-2 forward 1	TGGGTAAGTGGCCGACCATAGTGC
CYP1-2 forward 2	AAATCACCAGCGTGGAATGGTTGATG
CYP1-2 reverse 1	TTCCCCAACACAAGGCCTTTCA
CYP1-2 reverse 2	TCATGGTGAACCTTCTTCTCGGCGTA
CYP3-3 forward 1	CCCAGGGAACACATTGGAAGTTTCT
CYP3-3 forward 2	GGAAAGAAAGCAGCCATTGGACAAC
CYP3-3 reverse 1	GTCTCCCCGCTGTGAGTGGATGTT
CYP3-3 reverse 2	CTTAGCGCCGTGTCGAAGAATTCAA

Table 3

Mytilus edulis primers employed in the *CYP1*-like-1, *CYP1*-like-2, *CYP3*-like-1, *CYP3*-like-2, *CYP3*-like-3 and 28S real time PCR reactions.

Primer name	Primer sequence 5'-3'
CYP1-1 forward	TGGTTGCGATTTGTTATGCCCTGGA
CYP1-1 reverse	GGCGGAAAGCAATCCATCCGTGA
CYP1-2 forward	TGGGTAAGTGGCCGACCATAGTGC
CYP1-2 reverse	TTCCCCAAACACAAGGCCTTTCA
CYP3-1 forward	TCAGTGCAATTGTCGTCGAAAGC
CYP3-1 reverse	TCATGGCGAGCGTTTAACATCAG
CYP3-2 forward	GGAGGATTGATGAGTTGGGAGGAC
CYP3-2 reverse	AGGCGGCAAAAGAAAGAGTGGTC
CYP3-3 forward	CCCAGGGAACACATTGGAAGTTTCT
CYP3-3 reverse	GTCTCCCCGCTGTGAGTGGATGTT
28S forward	CTGGCCTTCACTTTCATTGTGCC
28S reverse	GACCCGTCTTGAAACACGGACCA

Amino acid identities among CYP1-like predicted sequences from *Mytilus edulis* (me) and *Mytilus californianus* (mc), with CYP1 sequences from *Danio rerio* (dr) and human (hs). Unmasked identities are on the upper diagonal, sequence-masked identities are on the lower diagonal.

Table 4

	me CYP1-1	mc CYP1-1	me CYP1-2	hs CYP1A1	hs CYP1A2	dr CYP1A
me CYP1-1	ID	89	46	33	34	31
mc CYP1-1	90	ID	44	34	33	31
me CYP1-2	49	47	ID	28	29	28
hs CYP1A1	37	38	32	ID	71	55
hs CYP1A2	38	37	33	75	ID	53
dr CYP1A	35	35	31	59	57	ID

Amino acid identities among CYP3-like predicted sequences from *Mytilus edulis* (me) and CYP3 sequences from *Danio rerio* (dr) and human (hs). Unmasked identities are on the upper diagonal, sequence-masked identities are on the lower diagonal.

Table 5

	me_cyp3-1	me_cyp3-2	me_cyp3-3	dr_CYP3A65	dr_CYP3C1	hs_CYP3A4
me CYP3-1	ID	51	54	34	32	38
me CYP3-2	53	ID	43	33	33	39
me CYP3-3	60	48	ID	32	29	37
dr CYP3A65	38	38	35	ID	51	54
dr CYP3C1	35	37	33	56	ID	47
hs CYP3A4	42	44	41	59	51	ID