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REVIEW ARTICLE

Piscine cytochromes P450 (CYP) and their response to antimicrobial drugs

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Abstract

Most research on the P450 (CYP) system in teleosts has been done on environmental factors that influence their induction. Less is known about CYP metabolism of aquaculture antibiotics. This article outlines the impact of aquaculture antimicrobials on the piscine CYP system, with particular regard to interactions with tetracyclines, fluoroquinolones, sulphonamides, berberine and chloramphenicol, as paradigms for current, potential and discontinued piscine antimicrobial drugs. It gives an overview of literature reports and advances in the field of biological significance of the CYP in fish. Emphasis has been placed on highlighting the most significant isoforms for biotransformation of drugs, and their drug response mechanisms. The challenge is to elucidate the differences in responses of CYP enzymes in different species to antimicrobial treatment as they may have relevance for the use of antimicrobials in aquaculture, especially as drug interactions with the fish CYP may alter their distribution, metabolism and elimination. They can impact the metabolism of other drugs metabolized by the same system with an effect on the physiology of fish administered these antimicrobials. Also, they can affect the persistence of residues and the length of the withdrawal period. For food animals such as farmed fish, this knowledge is a fundamental biomedical goal.

Keywords: cytochrome P450, CYP, fish, biotransformation, antimicrobial therapy

Introduction

In farming of aquatic organisms, medications are needed to maintain animal health and manage fish populations. However, the development of therapeutic agents for the treatment of fish diseases involves long and complex processes. When compared with mammals, fish have some differences in pharmacokinetics that need to be considered. For example, differences in bioavailability of tetracycline, quinolones and beta-lactam antimicrobials have been documented (Storey 2005). Oxytetracycline (OTC) and amoxicillin are not well absorbed from the intestines of fish. The doses of these antimicrobials are generally two to five times that needed to produce therapeutic systemic concentrations in mammalian species (Burka, Hammell, Horsberg, Johnson, Rainnie & Speare 1997). Also, for several antimicrobials, the cations present in seawater inhibit their absorption from the intestine, while their pharmacokinetics is affected by water temperature as well (Burka *et al.* 1997; Storey 2005). As in other animals, most pharmaceuticals are metabolized by CYP enzymes in fish, which makes them of critical importance both for detoxification or formation of toxic metabolites.

Drugs are usually lipophilic, which allows them to enter their site of action of target organs or tissues via cell membranes and exert their effect. As lipid soluble compounds, they are difficult to eliminate from the body. Metabolism, or biotransformation of these compounds into more polar, inactive metabolites, is generally an enzymatic process

(Hildebrand, Hümpel, Gieschen & Kraus 1994; Bernhardt 2006). The CYP mono-oxygenases are a large superfamily of proteins present in most tissues. They are of central importance in detoxification or activation of a number of foreign hydrophobic compounds, including many therapeutic agents, chemical carcinogens and environmental pollutants (Nebert & Gozales 1987). Many of these enzymes are inducible by the compounds they metabolize. This mono-oxygenase system perhaps has the widest-ranging spectrum of substrates, overlapping those of most of the other metabolizing enzymes (Schenkman 1999; Guengerich 2004). They accept as substrate molecules as small as ethanol and as large as the polycyclic aromatic hydrocarbon (PAH), benzo(a)pyrene (BP) or the antibiotic erythromycin, that is, ranging in molecular weight (MW) from just about 40 to over a 1000 kDa (Guengerich 1996).

CYP isoforms are found in almost every phylum in which they have been sought (Heffernan & Winston 1997). Although they were originally discovered in mammalian hepatic microsomal preparations, they have subsequently been found in many organs and tissues of numerous other animals and in some plants, fungi and bacteria. CYP gene superfamily comprises over 8000 genes and pseudogenes distributed across a wide range of biological domains (Nelson 2009; Parente, De-Oliveira, Beghini, Chapeaurouge, Perales & Paumgarten 2009). Already by 1998, there were more than 1000 CYP DNA sequences, and the problem of genetic nomenclature is becoming daunting (Nelson 1998, 2009).

A better understanding of the role of each CYP in drug metabolism and drug-induced toxicity is vital. At this time, however, most research on the CYP system in fish has been done on environmental factors that influence CYP induction. Much less is known about metabolism of aquaculture antibiotics by the CYP system (Snegaroff, Bach & Prevost 1989; Ishida 1992; Moutou, Burke & Houlihan 1998; Vaccaro, Giorgi, Longo, Mengozzi & Gervasi 2003; Topic Popovic, Babish & Bowser 2007; Yu & Yang 2009; Zhou, Li, Fang, Yang, Hu, Zhou & Zhou 2011; Hu, Li, Sun, Fang, Zhou, Hu & Zhou 2012; Topic Popovic, Howell, Babish & Bowser 2012).

Knowledge of the multiplicity, function and regulation of CYP forms in non-mammalian and non-traditional species continues to grow in importance. Research on aquatic species mono-oxygenase systems expanded rapidly in the mid-1970s so that already by the late 1970s and 1980s, several major reviews of microsomal CYP systems in aquatic species appeared (Bend & James 1978; Lech & Vodicnik 1984; Stegeman, Woodin & Binder 1984). Direct information regarding the identity of CYP forms in aquatic species is most abundant for fish. The first CYPs purified from fish were from the elasmobranch little skate (*Raja erinacea*) (Bend, Pohl, Arinc & Philpot 1977). So far multiple CYP forms have been purified, partially purified or cloned from the freshwater and marine fish, mainly from rainbow trout (*Oncorhynchus mykiss*), perch (*Perca fluviatilis*), scup (*Stenotomus chrysops*), cod (*Gadus morhua*), tilapia (*Oreochromis niloticus*), zebrafish (*Danio rerio*), killifish (*Fundulus heteroclitus*), goldfish (*Carassius auratus*), stickleback (*Gasterosteus aculeatus*) and medaka (*Oryzias latipes*) (Andersson & Förlin 1992; Stegeman 1995; Ueng & Ueng 1995; Gu, Xu, Wang, Gao & Zhao 2005; Luckenbach, Early, Rowe, Borski, Daniels & Godwin 2005; Tseng, Hseu, Buhler, Wang & Hu 2005; Goldstone & Stegeman 2008; Oh, Byung, Ha, Choi & Chung 2009; Zanette, Jenny, Goldstone, Woodin, Watka, Bainy & Stegeman 2009; Goldstone, McArthur, Kubota, Zanette, Parente, Jonsson, Nelson & Stegeman 2010; Hassanin, Kaminishi, Funahashi & Itakura 2012).

The aim of this article was to present a critical review of the impact of aquaculture antimicrobials on the piscine CYP system and to give an overview of recent literature reports and advances in the field of biological significance of the CYP enzymes in fish.

CYP system in fish

Although the research on mammalian CYPs continues to dominate the literature, there is a growing recognition of the biological significance of CYPs in fish. There are about 20 000 species of fish, representing nearly one-half of all known vertebrate species. These species comprise 200 families, 32 orders and 3 classes within the subphylum Vertebrata. They are extraordinary diverse, and inhabit virtually every niche within the world's fresh and marine waters (Stegeman 1989). They are also a direct food source for humans as well as conveyors of toxic chemicals to human beings (Sen & Arinc 1998).

Initially it was thought that fish lacked CYP-linked mono-oxygenases, but studies carried out in the late 1960s by Buhler and Rasmusson (1968)

and Dewaide and Hendersson (1968) showed that these enzymes were present in the livers of rainbow trout and other fish. Enzyme activities of fish hepatic microsomes were generally lower than in mammals and many fish CYPs had temperature optima of about 25°C, explaining why they were not detected in the earlier studies where incubations at higher temperatures were employed. Subsequently, as observed in mammals, multiple CYP forms were discovered in fish, predominately localized in the liver, but also found in lower concentrations in other tissues, like kidney, gut, gall bladder, gonads, nervous tissue, endocrine cells, gills, etc. (Andersson & Förlin 1992; Buhler 1995; Sarasquete & Segner 2000).

Fish hepatic microsomes exhibit typical reduced CO absorption spectra with a peak near 450 nm, and electron paramagnetic resonance characteristics with low-spin *g* values near 2.41, 2.25 and 1.91 typical for CYPs. Specific contents of CYPs in fish hepatic microsomes cluster between 0.2 and 0.5 nmol mg^{-1}. They range from less than 0.1 nmol mg^{-1} to nearly 2.0 nmol mg^{-1} microsomal protein. Such differences in content can occur within a single species, depending on strain, sex or chemical treatment (Andersson & Förlin 1992). Fish hepatic microsomes catalyse epoxidation, hydroxylation, dealkylation and oxidation reactions ascribed to CYP in other systems (Gillam 2007; Gillam & Hunter 2007). Substrates metabolized include many that are used to characterize CYPs in mammals, but activities found in teleosts might be catalysed by CYP forms largely unrelated to the catalyst for the same activity in mammalian systems. Conversely, CYP forms that are structural homologues in teleosts and mammals could have different activities (Stegeman 1993). One of the major differences between mammalian and fish CYPs is that some fish species have much higher constitutive levels of aryl hydrocarbon hydroxylase (AHH) than mammalian counterparts (Lee, Yoon, Haasch & Lech 1992). CYP1B1 was found to be the major isoform involved in AHH activity in human lymphocytes (Toide, Yamazaki, Nagashima, Itoh, Iwano, Takahashi, Watanabe & Kamataki 2003). Goksøyr, Andersson, Hansson, Klungsoyr, Zhang and Förlin (1987) described certain characteristic features of the fish xenobiotic metabolizing systems: generally lower specific activities compared with mammals, but high capability to activate procarcinogens to reactive intermediates; high responsiveness of mono-oxygenase activities to the PAH class of inducers (3-MC, β-naphthoflavone BNF, BP, etc.), but an apparent refractiveness to induction by the phenobarbital. In fish, CYPs have mostly been studied as catalysts for bioactivation of carcinogens and biomarkers of environmental contamination (Råbergh, Vrolijk, Lipsky & Chen 2000).

As in the mammalian system, multiple forms of CYPs belonging to the families of CYP1A, CYP1C, CYP2B, CYP2E, CYP2M, CYP2K, CYP2R, CYP2U, CYP3A, CYP11A, CYP17, CYP19 and CYP26 are found in fish (Godard, Goldstone, Said, Dickerson, Woodin & Stegeman 2005; Parente *et al.* 2009; Goldstone *et al.* 2010). Intensive research on fish CYPs rapidly reveals novel cytochromes, like cloning and characterization of CYP26 in zebrafish *Lythrypnus zebra* (Haque, Andreola & Deluca 1998), CYP2N1 and CYP2N2 in killifish (Oleksiak, Wu, Parker, Karchner, Stegeman & Zeldin 2000), cloning of CYP1B1 in scup and plaice *Pleuronectes quadrituberculatus* (Godard, Leaver, Said, Dickerson, George & Stegeman 2000; Leaver & George 2000), cloning, sequencing and tissue expression of CYP3A27 in rainbow trout (Lee, Wang-Buhler, Cok, Yu, Yang, Miranda, Lech & Buhler 1998), and many others. There are 54 CYP isoforms reported to be purified, partially purified, or cloned from aquatic species (Arinc & Sen 1999; Uno, Ishizuka & Itakura 2012). The number of fish species from which full-length coding regions of CYP1A genes have been sequenced has increased from four (rainbow trout, plaice, toadfish *Opsanus beta* and scup) to over a dozen, which include CYP1A sequences from tomcod *Microgadus tomcod*, butterflyfish *Chaetodon ocellatus*, sea bream *Sparus aurata*, sea bass *Dicentrarchus labrax*, Atlantic salmon *Salmo salar*, medaka *O. latipes*, mummichog *F. heteroclitus*, yellow catfish *Pelteobagrus fulvidraco*, crucian carp *hybridized Prussian carp* and killifish (Morrison, Weil, Karchner, Sogin & Stegeman 1998; Uno *et al.* 2012). Zebrafish was found to have a total of 94 CYP genes, distributed among 18 gene families found also in mammals (Goldstone *et al.* 2010). In mammals, sex, diet and age are among the factors known to influence mono-oxygenase systems. Marked sex differences also occur in CYP activities in fish (Gray, Woodin & Stegeman 1991; Topic Popovic *et al.* 2007). Moreover, there are changes in activity associated with species, strain, season, water temperature and gonadal status of fish (Goksøyr & Förlin 1992; Husøy, Myers, Willis, Collier, Celander & Goksøyr

1994). Although no detailed study is available on the influence of immunomodulating factors on the fish biotransformation system (Marionnet, Chambras, Taysse, Bosgireaud & Deschaux 1998; Reynaud & Deschaux 2006), it can generally be said that disease may influence CYP activity through interference with endocrine homoeostasis, impairment of intermediary metabolism, release of toxins, changes in tissue morphology and prolonged changes in energy intake (Guengerich 1996). Also, drug interactions on fish health have received considerably less attention compared to that on human health. Chemical interactions can affect biomarker responses in situations of mixed exposure to complex mixtures of environmental pollutants and drugs, which can result in misinterpretation of biomarker data. In that sense, inhibition of catabolic CYP enzyme activities (i.e. CYP1A and CYP3A) are linked to adverse outcomes in fish such as bioaccumulation of procarcinogens and estrogens, which can lead to carcinogenesis or cause endocrine disruption (Celander 2011).

CYP1A1 and CYP1A2 in fish

Although several isoforms have been identified in fish, CYP1A has received the most attention as the major hydrocarbon-inducible CYP. Subfamily CYP1A proteins in fish have reduced CO maxima at 447 nm, are the primary catalysts for 7-ethoxyresorufin *O*-deethylase (EROD), ethoxycoumarin *O*-deethylase, and AHH, metabolize BP on the benzo-ring, are strongly inhibited by α-naphthoflavone, are inducible by PAHs, and show reciprocal cross-reactivity with antibodies and to mammalian 1A1 (Stegeman 1993). Members of CYP1 family, including those belonging to CYP1A and 1B subfamilies, are known to play a prominent role in the activation of a number of environmentally occurring procarcinogens and many drugs (Goldstone & Stegeman 2006; Goldstone, Goldstone, Morrison, Tarrant, Kern, Woodin & Stegeman 2007; Parente *et al.* 2009). For many years, it had been assumed that fish express only one *CYP1A* gene product that has been characterized as a CYP1A1 protein (Råbergh *et al.* 2000). However, two genomic *CYP1A* clones were isolated by Berndtson and Chen (1994) from 3-MC-treated rainbow trout and were characterized as *CYP1A1* and *CYP1A2* based on sequence homology, presence or absence of xenobiotic regulatory elements in 5′ flanking regions, and significant differences in intron sequence. Also, there are distinctions in temporal patterns of AHH and EROD induction, and disparate results regarding multiple protein or mRNA products induced in fish by common 1A1 and/or 1A2 inducers (Goksøyr & Förlin 1992; Stegeman 1993). Differences have been reported in the catalytic and immunological properties of hepatic microsomes from BNF (inducer of CYP1A1 in mammals) and isosafrole (inducer of CYP1A2 in mammals)-treated trout (Buhler 1995; Stegeman 1995; Buhler & Wang-Buhler 1998). It is well established that the distinct difference between cytochromes CYP1A1 and 1A2 resides in their spin state. While CYP1A1 is a low spin haeme protein, CYP1A2 exists as a high spin haeme protein (Arinc & Sen 1999). Although *CYP1A* gene subfamily evolved early during vertebrate evolution and seems to be highly conserved across vertebrate taxa (Goldstone *et al.* 2007), constitutive levels and inducibility of CYP1A protein and catalytic activity exhibit a rather large variability between fish species and populations (Parente *et al.* 2009). Also, the position of the ancestral *CYP1A* locus remains a question: opposing orientations of the pufferfish and frog *CYP1A* genes pose a conundrum with regard to determining whether *CYP1A1* or *CYP1A2* resides in the ancestral *CYP1A* gene locus (Goldstone & Stegeman 2006).

CYP1A1 is localized in endothelial cells of heart, pillar cells of gills, kidney, hepatocytes, sinusoidal endothelium and billiary epithelial cells of fish (Lester, Braunbeck, The, Stegeman, Miller & Hinton 1993). Contrary to mammals, where CYP1A shows a heterogeneous distribution throughout the hepatic parenchyma, no zonation can be observed in fish liver (Sarasquete & Segner 2000). However, the levels of the CYP1A1 protein found in livers of rainbow trout are low in fish not exposed to PAHs or halogenated aromatic compounds (Buhler & Wang-Buhler 1998). Upon immunohistochemical examination of liver sections unexposed to such inducers, the CYP1A1 was barely detectable (Buhler 1995). Relative molecular mass of CYP1A1 cross-reacting protein bands in Western blots of hepatic microsomes, probed with an anti-fish antibody, varies among the teleost species, and ranges between 54 and 59 kDa. Also, a *CYP1A3* gene has been isolated and sequenced from the rainbow trout (Carvan, Ponomareva, Solis, Matlib, Puga & Nerbert 1999). Four CYP type 1 family enzymes (CYP1B, CYP1C1,

CYP1C2 and CYP1D) have been isolated from diverse fish species (Uno *et al.* 2012).

CYP3A4 in fish

CYP3A is a gene subfamily composed of multiple forms of CYP3A enzymes as characterized by immunochemistry, catalytic activities, and cDNA cloning and expression. CYP3A is inducible by steroidal chemicals and by a variety of compounds, including naturally occurring and synthetic glucocorticoids and macrolide antibiotics (Quattrochi & Guzelian 2001). Husøy *et al.* (1994) demonstrated organ distribution and cellular localization of cod CYP3A-like isozymes in control and BNF-treated cod, analysed by imunohistochemistry and showed its occurrence in liver hepatocytes, respiratory epithelial cells of gills, intestinal and caecal mucosal epithelium, epithelium of renal tubules and pancreatic acinar cells.

A strong similarity in the structure and catalytic function was found between trout and human CYP3A4 (Miranda, Wang, Henderson, Zhao, Guengerich & Buhler 1991), where polyclonal antibodies (IgG) generated against trout CYP LMC5 reacted strongly with CYP3A1 in dexamethasone-induced rat liver microsomes and with CYP3A4 in human hepatic microsomes in immunoblots. Buhler (1995) reports CYP3A4 isolation from livers of untreated trout, having a MW of 59 kDa, reduced CO maxima of 448 nm, and significant activity for the 6β-hydroxylation of testosterone and progesterone and for the *N*-demethylation of benzphetamine. Upon Western blot analysis, concentrations of CYP3A4 from the livers of trout were found to be 32% higher in liver microsomes from sexually mature males than in females (Buhler & Wang-Buhler 1998).

Control of CYP3A expression is thought to be mediated by the Ah (aryl-hydrocarbon) receptor/ARNT pathway in zebrafish, whereas, the pregnane X receptor (PXR) is suggested to activate CYP3A expression in Atlantic salmon (Finn 2007; Uno *et al.* 2012). Pregnane X receptor regulates the expression of CYP3A isozymes, as well as other CYP isoforms, by binding as a heterodimer with the 9-*cis* retinoic acid receptor (Bainy 2007). Pregnane X receptor genes have been cloned from a variety of vertebrate species, including zebrafish (Bainy & Stegeman 2004). However, there is a lack of knowledge of how pharmaceuticals and other xenobiotics interact with CYP3A and PXR in fish. Wassmur, Grans, Kling and Celander (2010) established that the rainbow trout PXR is less responsive to prototypical PXR agonists than its mammalian counterparts and that it also responds differently to glucocorticoid receptor agonists. As there are also species differences, to understand how pharmaceuticals affect biotransformation in fish, sites of interaction on biotransformation pathways need to be characterized.

Therapeutic agents and CYPs

In recent years, a huge progress has been achieved in CYP research and better understanding of the role of each CYP in drug metabolism and drug-induced toxicity (Lu 1995; Cravedi 2002; Reynaud & Deschaux 2006; Li, Yang, Zhang, Lin, Yu & Hu 2008; Zanette *et al.* 2009; Goldstone *et al.* 2010; Uno *et al.* 2012). Drug response varies greatly across groups and individuals. This variability is due to many pharmacological factors. In phase I and phase II metabolism, this variability may be a reflection of enzyme inhibition, enzyme induction and/or genetic differences and disease (Meyer 1994; Cozza & Armstrong 2001).

Impact of aquaculture antimicrobials on the fish CYP system

Many countries throughout the world, including Europe and the United States of America, have strictly regulated controls on use of veterinary medicines, particularly for use in food animal species, including fish. In the recent decades, the European legislation regarding veterinary pharmaceuticals has introduced restrictions of the use of medicines for the treatment of farm animals, and as a result, available veterinary medicinal products for fish are now authorized. The basic Directives of European legislation on veterinary medicinal products have been frequently and substantially amended over time. The main document regarding the availability of aquaculture medicines is the Regulation for the establishment of maximum residue limits (CEC 1990; CR 2010). Currently approved in the EU are amoxicillin, florfenicol, flumequine, oxolinic acid, OTC, sarafloxacin and sulfadiazine trimethoprim, while certain compounds, including chloramphenicol (CP) and the nitrofurans are specifically prohibited for use in food animals in Europe and in the United States of America.

In the United States of America, all drugs legally used in aquaculture must be approved by the FDA's (Food and Drug Administration) Center for Veterinary Medicine (FDA/CVM). The Center defines drug ingredients, manufacturers, species, routes of delivery, dose forms, withdrawal periods, tolerances, and uses by species, including dose rates and limitations. The most common route of delivery of these legal antibiotics to fish occurs through mixing with specially formulated feed. In the United States of America, there are currently only three FDA-approved and available antibiotic drugs for use in fish. They are OTC, ormetoprim and sulfadimethoxine, and florfenicol. FDA will sometimes allow veterinarians to prescribe the use of medicated feed for fish species other than those listed on the label. For example, OTC medicated feed approved for use in catfish, may be prescribed off label for hybrid striped bass by a licensed veterinarian (Durborow & Francis-Floyd 1996).

Despite strict regulations, except via medicated feed, fish may get exposed to antimicrobial drugs via aquatic environment. Discharge from sewage treatment plants has been identified as their primary source (Lindberg, Wennberg, Johansson, Tysklind & Andersson 2005; Smith, Iftikar, Higgins, Irshad, Jandoc, Lee & Wilson 2012). Numerous pharmaceuticals and other environmental contaminants are metabolized by CYP enzymes in fish, and therefore CYP enzymes are of critical importance both for detoxification and for the formation of toxic metabolites (Dorne, Skinner, Frampton, Spurgeon & Ragas 2007; Beijer, Abrahamson, Brunstrom & Brandt 2010). Although generally effect levels for pharmaceuticals are higher than those found in the environment (Corcoran, Winter & Tyler 2010), the risks to wild fish populations have not been thoroughly characterized, and there has been a lack of consideration given to the likely chronic nature of exposures, or the potential mixture effects.

Interactions with tetracyclines

Environmental exposure to OTC and sulfathiazole was proven to indirectly increase the catalytic activity of aromatase, potentially through transcriptional level modulation (Ji, Choi, Lee, Park, Khim, Jo, Choi, Zhang & Giesly 2010). It also resulted in greater expression of *CYP17*, *CYP19* or *3βHSD2*, which play crucial role in steroidogenic pathways. Topic Popovic *et al.* (2012) determined responses to OTC treatment and investigated whether antibodies to known mammalian CYP forms (CYP1A1 and CYP3A4) would react with their respective CYP isoforms in fish [hybrid striped bass (*Morone saxatilis* male × *Morone chrysops* female), channel catfish (*Ictalurus punctatus*), and Nile tilapia]. In addition, expression of these CYPs as well as the activity of CYP1A2 and CYP3A4 was also assessed. Both goat anti-rat CP1A1 and rabbit anti-human CYP3A4 showed good cross-reactivity with the three species tested in this study. Although some antibodies only recognize proteins from species closely related to the sources of the immunogen (Al-Arabi & Goksøyr 2002), the antibodies used in this study recognized epitopes from the divergent groups examined. Considerable non-specific binding was displayed, which was expected regarding the source of the primary antibodies (Topic Popovic *et al.* 2007). Immunological cross-reactivity has previously been demonstrated between fish CYP3A and both rat CYP3A1 and human CYP3A4 (Miranda *et al.* 1991). Also, clear, putative, OTC-related responses were found in liver and the hepatic phase I xenobiotic metabolizing enzyme system of the three different fish species in this observational study. This observational approach demonstrated species differences both in control activities and in the timing and extent of hepatic responses to OTC. Further, resorufin benzyl ether (BzRes) was proven to be a better substrate than benzyloxy-4-trifluoromethylcoumarin (BFC) for monitoring CYP3A activities in fish. The data of the study indicated that antibiotics should also be evaluated in the target species under conditions of use. The variable effects in the response among different fish species to antibiotics in this study are a valid reason to critically use assays developed for mammals for evaluating drug response in fish. Moreover, the unique responses of CYP enzymes in different fish species to OTC treatment may have relevance for the use of other antibiotics in aquaculture.

Interactions with fluoroquinolones

The first indications that CYP1A were involved in the metabolism of oxolinic acid were described by Ishida (1992), who treated carp (*Cyprinus carpio*) with polychlorinated biphenyl, a CYP1A inducer, before the treatment with oxolinic acid. CYP1A was also responsible for difloxacin (DIF) metabolism in crucian carp liver (Fu, Yang, Zhang, Yu &

Hu 2011) and its *N*-demethylation in Chinese idle (*Ctenopharyngodon idellus*) kidney (Yu & Yang 2009; Yu, Yang, Wang, Yu & Hu 2010). After DIF treatment, CYP1A expression gradually decreased and was significantly lower than the control. CYP1A1 transcript level was down-regulated by DIF, suggesting that DIF is both the substrate and inhibitor of CYP1A.

Enrofloxacin (EF) was assayed for the effect on induction of CYP1A mRNA in Atlantic tomcod (Williams, Lech & Buhler 1997). Pretranslational suppression of CYP1A induction was found in spawning females. Enrofloxacin exhibited potent inhibition on the CYP1A-related EROD activity as well as CYP1A expressions at both protein an mRNA levels in crucian carp (Hu *et al.* 2012). In sea bass, EF was proven able to provoke the inactivation of the CYP3A enzyme (Vaccaro *et al.* 2003). A single dose of i.p. injection of 3 mg kg^{-1} of EF or multi-doses of 1 mg kg^{-1} were sufficient to cause a generalized depression of CYP activities in sea bass. Enrofloxacin was an effective inactivator of the erythromycin *N*-demethylase activity (Vaccaro *et al.* 2003; Hu *et al.* 2012), while immunoblot analysis showed a strong decrease in staining intensity of a protein band immunoreactive with anti-CYP3A27. Therefore, it can be postulated that fluoroquinolone-antibiotics such as EF in sea bass have an adverse reaction with the CYP enzymes, and hence a strong potential to cause long-lasting interactions. Compared with OTC, oxolinic acid was better absorbed and faster excreted from the treated fish (Björklund, Råbergh & Bylund 1991).

However, significant differences were noted in *O*-dealkylation and Western blotting responses between compounds of fluoroquinolonic drug class when rainbow trout were fed medicated diets with oxolinic acid and flumequine (Moutou *et al.* 1998). Both oxolinic acid and flumequine effects were related to the CYP1A subfamily. The induction of day 10 post-treatment with oxolinic acid was confirmed by immunoblotting, while the lack of evidence for CYP1A induction after flumequine administration suggests that flumequine caused an activation of CYP1A, probably by protein stabilization. Also, the effects of oxolinic acid on *O*-dealkylation were delayed and longer lasting compared with those of flumequine (Moutou *et al.* 1998). Such differences in CYP response post-treatment can be explained with differences in the pharmacokinetics of oxolinic acid and flumequine in rainbow trout.

Interactions with sulphonamides

The antibacterial drug sulfamethoxazole did not markedly inhibit gill EROD activity of three-spined sticklebacks (*G. aculeatus*) in the work of Beijer *et al.* (2010), although inhibition of EROD activity by sulfamethoxazole has been reported in fish hepatocytes *in vitro* (Laville, Ait-Aissa, Gomez, Casellas & Porcher 2004). Combination ormetoprim–sulfadimethoxine incorporated in diet was administered to summer flounder (*Paralichtys dentatus*) in study by Topic Popovic *et al.* (2007). Immunoblots of pre-treatment and treated summer flounder hepatic microsomes probed with goat anti-rat CYP1A1 antibodies exhibited specific bands in the region of 65–70 kDa. Dealkylation of the hepatic CYP1A2 marker 3-cyano-ethoxycoumarin was significantly increased relative to pre-treatment fish through 10 days and returned to pre-treatment levels by post-treatment day 21. When identical samples were tested for BFC and BzRes activities, males and females yielded different curves. Benzyloxy-4-trifluoromethylcoumarin activity was markedly higher in males, but females had higher BzRes activity, with both substrates specific for CYP3A4 enzymes. In this study (Topic Popovic *et al.* 2007), the authors demonstrated the usefulness of mammalian antibodies for summer flounder CYP Western blotting and quantified CYP1A2 and 3A4 metabolism by modifying kits developed for mammalian microsomes, which could facilitate future work on piscine hepatic microsomes. As an observational study with no concurrent, untreated fish at the post-treatment sampling times, inferences were limited to comparisons with pre-treatment fish and over sampling times. While CYP1A1 and CYP3A4 enzyme activities exhibited sharp increases and decreases through post-treatment day 10, activities for both isozymes returned to pre-treatment levels by post-treatment day 21. Considering the age of the summer flounder used and the relatively short observational period, it is unlikely that the extent of fluctuations seen in enzyme activity, 50–100%, was due to normal, possibly confounding physiological factors. Further, these temporal perturbations in CYP enzyme activity correlate strongly with the presence of potentiated sulphonamide residues found in summer flounder (Kosoff, Chen, Wooster, Getchell, Clifford, Craigmill & Bowser 2007). Thus, as it has been demonstrated repeatedly in mammalian systems, therapeutic

compounds that are not substrates of phase I oxidative enzymes have the potential for interfering with the biotransformation of other therapeutic compounds or environmental chemicals. For food animals such as the summer flounder, the implications of this prospect impact the withdrawal time of additional therapeutics or the presence of environmental contaminants in edible tissues.

Interactions with berberine and CP

Berberine, a quaternary isoquinoline alkaloid, could enhance the serum bactericidal activity in fish by activating the complement system, giving it a potential for prevention or treatment of fish diseases (Ji, Zhang, Li & Gong 2012), and has long been considered as an antibiotic candidate for controlling systemic bacterial infections in fish, especially in synergistic action with EF (Zhang, Aihua, Xie & Cheng 2009). In the work of Zhou *et al.* (2011), berberine acted as a potent inhibitor of crucian carp CYP1A, reducing not only CYP1A mRNA expression in a dose-dependent manner, but also directly inhibiting this enzyme competitively. Also, high berberine doses inhibited CYP3A through the downregulation of its expression at the both mRNA and protein level. The authors compared the identity of this crucian carp CYP1A isoform with those of human at the amino acid level and found that it had a stronger identity with human CYP1A2 than CYP1A1. Hence, the mechanisms underlying the effects of berberine on crucian carp CYP1A isoform and human CYP1A expressions may be quite different.

Chloramphenicol, a broad spectrum antibiotic, has previously been used for treatment of fish bacterial diseases, particularly furunculosis and diseases caused by pseudomonads (Snieszko 1954). In trout liver cells, the biotransformation of labelled CP, CP-glucuronide, was found to be the major metabolite, along with the CP-base, CP-alcohol and CP-oxamic acid, while the metabolic rate of the 3H-CP reached 0.2 nmol per hour per 106 trout hepatocytes (Cravedi & Baradat 1991). Snegaroff *et al.* (1989) measured interactions between CP and mono-oxygenases by measuring aldrin epoxidase (AE) and found that the AE activities in trout liver were severely inhibited under higher concentrations, but not inhibited by CP under concentrations below a certain threshold. Chloramphenicol was also shown to have an inhibitory effect on the hepatic EROD activities in rainbow trout, both *in vivo* and *in vitro* (Snegaroff *et al.* 1989), however, it had very little or no inhibitory effect on trout lauric acid (ω-1)-hydroxylase and dimethylbenz[*a*]anthracene hydroxylase activities (Miranda, Henderson & Buhler 1998). Understanding the inhibition mechanism would minimize the selection of drugs that could either inhibit or destroy CYPs. Decreased CYP levels could result in unacceptably high plasma drug level and long drug half-life.

Conclusion

There is an array of antimicrobial drugs evaluated for the effects on CYPs in fish (Table 1). While the majority of these compounds are regularly detected in the aquatic environment from human-use and agriculture, some are utilized in aquaculture for treatment of fish diseases. However, drug interaction studies are lacking outside of mammalian species and not enough is known about the effects of aquaculture antibiotics on the piscine CYP system. Although fish CYPs are likely inducible by mammalian inducers, the specific isoforms responsive to such inductions are still unclear, and information on fish CYP induction potential and catalytic activity is often inferred from mammalian systems, assuming similar patterns. As both CYP1A1 and CYP3A4 have become important markers of chemical exposure in many species including fish, perturbations of the activities of these enzymes may have consequences in the metabolism of antimicrobial drugs, especially CYP3A4, arguably the most important enzyme involved in the metabolism of xenobiotics.

Antimicrobial drugs must be evaluated extensively in the target organism. Drug interactions with the fish CYPs may alter their distribution and metabolism, as well as elimination. They can impact the metabolism of other drugs and xenobiotics metabolized by the same system with an effect on the physiology of fish administered these antimicrobials. The differences in responses of CYP enzymes in different fish species to antimicrobial treatment may have relevance for the use of antimicrobials in aquaculture. The variable effects in the response among different fish species to antimicrobial drugs are a valid reason to critically use assays developed for mammals for evaluating drug response in various fish. Also, the use of CYP antibodies across phyla can be of value if caution is exercised in the

Table 1 Antimicrobial drugs used in aquaculture, evaluated for the effects of CYPs in fish. Most classes and compounds listed are broad-spectrum antibiotics, active against a wide range of Gram-negative and Gram-positive pathogenic microorganisms. Some agents (chloramphenicol (CP), sulfamethoxazole and berberine) are bacteriostatic, but may act as bactericidals in high concentrations or when used against highly susceptible organisms

Class and compound	Mechanism of action	CYP interaction	Fish species	Reference
Class: Tetracycline Compound: Oxytetracycline	Inhibit cell growth by inhibiting translation. They bind to the 30S ribosomal subunit and prevent the amino-acyl tRNA from binding to the A site of the ribosome	Increase in the catalytic activity of aromatase and greater expression of *CYP17*, *CYP19*, *3βHSD2* (medaka); Induction of CYP1A and CYP3A; decrease in 3-cyano-ethoxycoumarin (CEC) and resorufin benzyl ether (BzRes) activities (tilapia);Increase in CEC, 7-benzyloxy-4-trifluoromethylcoumarin (BFC), BzRes activities (channel catfish).Inhibition of 7-ethoxyresorufin *O*-deethylase (EROD) and 7-pentoxyresorufin *O*-deethylase (PROD) activities (Am. alligator)	*Oryzias latipes, Oreochromis niloticus, Morone saxatilis* male × *Morone chrysops* female, *Ictalurus punctatus*	Mayeaux and Winston (1998), Topic Popovic *et al.* (2007), Ji *et al.* (2010), Topic Popovic *et al.* (2012)
Class: Fluoroquinolone Compounds: Oxolinic acid Difloxacin Enrofloxacin (EF) Flumequine	Interfere with the activity of the bacterial DNA gyrase and topoisomerase IV, needed for the transcription and replication of bacterial DNA. DNA is the primary quinolone target for Gram-negative bacteria; topoisomerase IV is the preferential target in Gram-positive organisms. DNA replication and transcription is inhibited	Activation of CYP1A by protein stabilization (EF, sea bass); depression of the microsomal *N*-demethylation of aminopyrine (APD) and erythromycin (ERND) *N*-demethylase, EROD, 7-ethoxycoumarin *O*-deethylase (ECOD) and 6β-testosterone hydroxylase (sea bass);Inhibition of EROD activity, expression at protein and mRNA levels; ERND inhibition (crucian carp,EF); Inhibition of CYP activity (flumequine, oxolinic acid); induction of CYP1A levels (oxolinic acid), increase in EROD and benzyloxyresorufin *O*-dealkylase (BROD) activity (rainbow trout);*N*-demethylation (CYP1A) of difloxacin (Ch. idle);Strong inhibition of CYP2E (grass carp);Inhibition and downregulation of CYP1A mRNA by difloxacin (crucian carp)	*Oncorhynchus mykiss, Cyprinus carpio, Ctenopharyngodon idellus, Microgadus tomcod, Carassius auratus gibelio, Dicentrarchus labrax*	Williams *et al.* (1997), Moutou *et al.* (1998), Vaccaro *et al.* (2003), Wang, Yang, Zhang, Yu and Hu (2008), Fu *et al.* (2011), Hu *et al.* (2012)

Table 1 (continued)

Class and compound	Mechanism of action	CYP interaction	Fish species	Reference
Class: Sulphonamide Compound: Sulfamethoxazole Ormetoprim–sulfadimethoxine Sulfathiazole	Inhibit the enzymatic conversion of pteridine and p-aminobenzoic acid (PABA) to dihydropteroic acid by competing with PABA for binding to dihydrofolate synthetase, an intermediate of tetrahydrofolic acid (THF) synthesis. THF is required for the synthesis of purines and dTMP and inhibition of its synthesis inhibits bacterial growth. Pyrimethamine and trimethoprim inhibit dihydrofolate reductase, another step in THF synthesis, and act synergistically with the sulphonamides	CYP1A immunoreactivity with sulfamethoxazole (zebrafish);Increase in the catalytic activity of aromatase and greater expression of *CYP17*, *CYP19*, *3βHSD2* (medaka, sulfathiazole);Slight inhibition of gill EROD activity (three-spined stickleback, sulfamethoxazole); Inhibition of EROD activity (fish hepatocytes *in vitro*);Induction of CYP1A and CYP3A; increased dealkylation of CEC, increased activities of BzRes and BFC (relative to sex) (summer flounder, ormetoprim-sulfadimethoxine)	*Danio rerio, Oryzias latipes, Gasterosteus aculeatus, Paralichtys dentatus*	Laville *et al.* (2004), Topic Popovic *et al.* (2007), Beijer *et al.* (2010), Ji *et al.* (2010), Madureira, Rocha, Cruzeiro, Rodrigues, Monteiro and Rocha (2012)
Class: Fenicole Compound: CP	Reversibly bind to the L16 protein of the 50S subunit of bacterial ribosomes, where transfer of amino acids to growing peptide chains is prevented (by suppression of peptidyl transferase activity), inhibiting peptide bond formation and subsequent protein synthesis	Weak inhibition of EROD and PROD activities (common carp) Inhibition of EROD; inhibition of aldrin epoxidase under higher concentrations (rainbow trout)	*Oncorhynchus mykiss, Cyprinus carpio*	Snegaroff *et al.* (1989), Machala, Nezveda, Petrivalsky, Jarosova, Piacka and Svobodova (1997), Miranda *et al.* (1998)
Class: Quaternary isoquinoline alkaloid Compound: Berberine	Inhibitor of canalicular multispecific organic anion transporter 1 (cMOAT); complement consumption	Inhibition of CYP1A and CYP3A activities (EROD and ERND activity); decrease in CYP3A protein; inhibition of *CYP1A* and *CYP3A* expression. High doses inhibit CYP3A through downregulation of its expression at both mRNA and protein level (crucian carp)	*Carassius auratus gibelio, Ctenopharyngodon idella*	Zhang *et al.* (2009), Zhou *et al.* (2011), Ji *et al.* (2012)

interpretation. Substrates developed for use in mammalian systems have been assayed in fish, and are useful for CYP-mediated functional comparison between mammalian and fish species. Standardized protocols for drug impact on fish CYPs will facilitate those studies and standards will be necessary for the future application on teleosts.

As the metabolism of aquaculture antimicrobials by the CYP enzymes could also determine the metabolism of other xenobiotics and endogenous compounds, along with persistence of residues and the length of the withdrawal period before fish being apt for human consumption, and taking into consideration other challenges and concerns regarding drug approval/administration/dosing/metabolism, impact of antimicrobial drugs on piscine CYPs requires further in-depth studies, equal to their mammalian counterparts.

Conflict of interest

The authors declare no conflict of interest.

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