Characterization of drug metabolizing enzymes and assessment of aging in the gilthead seabream (*Sparus aurata*) liver

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ABSTRACT: Organic anthropogenic compounds such as polycyclic aromatic hydrocarbons, polychlorinated biphenyls, nitroaromatics, dioxins, various pesticides and natural compounds can enter the aquatic environment. These lipophilic compounds are readily taken up into the tissues of aquatic organisms where biotransformation via Phase I and Phase II metabolism can in part, determine the fate and toxicity of the xenobiotics. In this study on gilthead seabream (*Sparus aurata*), liver microsomal ethoxyresorufin O-deethylase (EROD), methoxyresorufin O-demethylase (MROD), penthyloxyresorufin O-depenthylase (PROD), benzyloxyresorufin O-debenzylase (BROD), aniline 4-hydroxylase (A4H), N-nitrosodimethylamine N-demethylase (NDMA-DE), aminopyrine N-demethylase (APND), caffeine N-demethylase (CN3D) and erythromycin N-demethylase (ERND) were determined. A4H, ERND, NDMA-ND, EROD and PROD activities increased in gilthead seabream (*Sparus aurata*) liver microsomes of different ages, while CN3D and APND activities decreased. MROD activities were barely detectable in mature fish whereas BROD activities were not detectable at all ages studied. Results were also recorded at the protein level by Western blotting using anti-CYP1A, CYP2E and CYP3A antibodies. In conclusion, elevated levels of gilthead seabream liver microsomal CYP450 enzyme activities might reveal possible exposure to various exogenous compounds, which might affect the desired responses to drugs, hormones and dietary supplements used during breeding.

Keywords: *Sparus aurata*; gilthead seabream; drug metabolizing enzymes; fish

List of abbreviations

EROD = ethoxyresorufin O-deethylase; MROD = methoxyresorufin O-demethylase; PROD = penthyloxyresorufin-depenthylase; BROD = benzyloxyresorufin O-debenzylase; A4H = aniline 4-hydroxylase; NDMA-DE = N-nitrosodimethylamine N-demethylase; APND = aminopyrine N-demethylase; CN3D = caffeine N-demethylase; ERND = erythromycin N-demethylase; GST = glutathione S-transferase; PCB = polychlorinated biphenyls; PCDDs = polychlorinated dibenzodioxins; PCDFs = polychlorinated dibenzofurans; PAHs = polycyclic aromatic hydrocarbons

Organisms are often exposed to complex mixtures of pollutants, including polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polycyclic aromatic hydrocarbons (PAHs), alkyltin compounds, and metals. Pollutants that bioaccumulate in the organism first cause effects at the molecular and cellular levels. This may lead to adverse effects in the organism, which in turn may cause changes at the population and the community level in subsequent years. Fish populations living in highly polluted areas often have high incidences of gross pathological lesions and neoplasms that may be associated with elevated levels of toxic chemicals in sediments (Payne et al., 1987). High levels of neoplasms in fish collected from a creosote (mixture of petroleum products) polluted site in Puget Sound, WA, USA have been reported (Malins et al., 1985). Even though

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chemical analyses are able to measure a wide range of pollutants quantitatively and accurately, a complex mixture of chemical pollutants cannot be fully assessed. Furthermore, it does not reveal the impact of chemical pollution on the aquatic environment. The use of biochemical markers, however, fulfils this purpose. Biomarker responses are, broadly speaking, of two kinds: those that measure only exposure to a pollutant and those which measure both exposure to and the toxic effects of environmental chemicals (Peakall, 1992; Walker, 1998). The conjugation of xenobiotics and oxidative metabolites is also affected by aging. Various factors that may have a significant impact upon drug metabolism are affected by aging. For example, absorption may be altered by increased gastric pH or decreased splanchnic flow (Tsang and Gerson, 1990; Bowman et al., 1991). In addition, distribution may be altered by age-related decreases in lean body mass relative to total body mass (Brozek, 1952; Forbes and Reina, 1970; Bruce et al., 1980) or decreases in intracellular water content from 42% in young adults to 33% in the elderly (Schoeller, 1989). Hepatic clearance of drugs can decrease with age (Bach et al., 1981; Greenblatt et al., 1982), and it has been estimated that age-related decreases in xenobiotic metabolism can account for over 80% of toxic reactions to drugs observed in elderly populations (Woodhouse and Wynne, 1992).

Since the P450 enzymes are a super-family of numerous (more than 150) isoforms with diverse substrate specificity it is not possible to analyze the effect on all the enzymes. However we checked the effect on the isoforms of the genes CYP1A1, CYP1A2, CYP2B1/2, CYP2E1, CYP 1A, 2A, 2B, 2D and 3A. It is known that CYP1A1 family metabolize the polyaromatic hydrocarbons, while CYP2E1 metabolizes ethanol and CYP1A2 metabolizes aromatic amines. In this study, gilthead seabream (*Sparus aurata*) fish liver microsomes and the cytosol of fish of different ages (ranging from 1.5 to 24 months) were used as sample material. The fish used in this study, gilthead seabream (*Sparus aurata*), were bought from Pinar Fish in Izmir, on the Aegean coast of Turkey.

MATERIAL AND METHODS

Sampling and detection of protein

Liver microsomes and cytosolic samples were prepared according to the method of Arinc and Sen (1999). Protein concentrations were evaluated by the method of Lowry et al. (1951). Liver microsomal EROD, MROD, PROD, BROD, A4H, NDMA-DE, APND and ERND were determined from these gilthead seabream fish liver microsomes.

Detection of enzymatic activity

EROD assay. A 0.5mM stock substrate solution was first prepared by dissolving an appropriate amount of 7-ethoxyresorufin in DMSO. Then a daily 10μM solution was prepared by diluting 1 : 50 with 0.4M potassium phosphate buffer with pH 7.8 containing 1M NaCl. A standard 1mM stock of resorufin was prepared by dissolving an appropriate amount of resorufin in DMSO. The 5μM daily solution was prepared by diluting 1 : 200 with 0.4M potassium phosphate buffer with pH 7.8. A typical reaction mixture contained 0.1M potassium phosphate buffer pH 7.8, 1M NaCl, 1.2 mg BSA, 1.5μM 7-ethoxyresorufin, 50 or 100 μg microsomal protein, 0.5mM NADPH generating system in a final reaction volume of 1.0 ml in a fluorometer cuvette. The reaction was initiated by the addition of substrate and followed for three minutes in a spectrofluorometer at 535 nm (excitation) and 585 nm (emission) wavelengths. Finally a known amount of resorufin was added as an internal standard to the reaction mixture and the increase in fluorescence was recorded.

MROD, PROD, BROD assays. A 1.5mM stock substrate solution was first prepared by dissolving an appropriate amount of 7-methoxyresorufin and 7-pentoxyresorufin, benzyloxyresorufin in DMSO. MROD, PROD and BROD activities were measured as with the EROD assay.

NDMA-ND assay. The microsomal N-demethylase activity of control liver microsomes was determined calorimetrically by measuring the quantity of formaldehyde formed during the reaction according to the method of Nash (1953) as modified by Cochin and Axelrod (1959) with minor modifications. A typical assay mixture contained 100mM HEPES buffer pH 7.8, 2.5mM N-nitrosodimethylamine 1 mg microsomal protein and 0.5mM NADPH generating system to a final volume of 0.5 ml. The NADPH generating solution was composed of 2.5mM glucose-6-phosphate 2.5mM $MgCl_2$, 14.6mM HEPES pH 7.8, 0.5mM $NADP^+$ and 0.5 units of glucose-6-phosphate dehydrogenase. All the constituents were incubated at

37 °C for 5 min (Nash, 1953; Cochin and Axelrod, 1959). The N-nitrosodimethylamine demethylation reaction was initiated by the addition of 0.075 ml of the NADPH generating system to the incubation mixture and to zero time blanks to which 0.5 ml of 0.75 N perchloric acid was added before the addition of the cofactor, and incubated at 25 °C for 10 min aerobically with moderate shaking in a water bath. At the end of the incubation period, the enzymatic reaction was stopped by the addition of 0.5 ml 0.75 N perchloric acid. The contents of the tubes were transferred into eppendorf tubes and centrifuged at 12 000 × g for 15 min for the removal of denatured microsomal proteins using a centrifuge in a cold room. Finally, 0.5 ml aliquots of supernatant solution were transferred to test tubes and were mixed with freshly prepared 0.375 ml Nash reagent (prepared by the addition of 0.1 ml of acetylacetone, just before use, to a 25 ml solution containing 7.7 g ammonium acetate and 0.15 ml of glacial acetic acid). The mixture was incubated at 50 °C for 10 min in a water bath and the intensity of yellow colour development was measured at 412 nm using a spectrophotometer. Freshly prepared 0.5mM formaldehyde solution was used as a standard. The tubes contained standards at four concentrations (12.5, 25, 50 and 100 nm) as well as containing other incubation constituents mixed by Nash reagent and incubated at 50 °C for 10 min to give the same colour reaction. A Standard formaldehyde calibration curve was constructed and used for calculation of enzyme activities.

C3ND, APND and ERND assays. Typical assay mixture to determine C3ND and APND activities contained 100mM HEPES buffer pH 7.8, 0.1mM caffeine/0.1mM aminopyrene/5mM erythromycin substrate, 1 mg microsomal protein and 0.5mM NADPH generating system in a final volume of 0.5 ml. All the other experiments were carried out as the NDMA-ND assay and enzyme activities were calculated.

A4H assay. A4H activity of liver microsomes was determined by measuring the quantity of p-aminophenol (pAP) formed according to the method of Imai et al. (1966). A standard calibration curve was constructed and used for calculation of enzyme activities which was expressed as nmol of pAP/min/mg.

GST assay. Glutathione S-transferase (GST) activity was determined from the gilthead seabream (*Sparus aurata*) fish liver cytosols using the method of Habig et al. (1974).

RESULTS

Enzyme studies

NDMA-ND and A4H activity is associated with the cytochrome P4502E isozyme. A4H activity increased with aging. While at 1.5 months the activity was 0.002 ± 0.001 nmol/dakika/mg protein, at 24 months the value was 0.058 ± 0.002 nmol/min/mg protein. Figure 1 shows that NDMA-ND activity increased in gilthead seabream liver microsomes of different ages (ranging from 1.5 to 24 months). At 1.5 months the activity was 0.019 ± 0.007 nmol/min/mg protein, while at 24 months the value had increased to 0.323 ± 0.014 nmol/min/mg protein.

The E3-ND reaction is associated with cytochrome P4503A isozymes which are located mostly in the liver and intestine. ERND activity increased in gilthead seabream liver microsomes of different ages. At 1.5 months the activity was 0.09 ± 0.006 nmol/dakika/mg protein; at 24 months this had increased to 0.189 ± 0.008 nmol/min/mg protein (Figure 1).

The C3ND and MROD reaction is associated with cytochrome P4501A2 isozymes which are induced by PAH, PCB and dioxin-type chemicals. MROD, the activity of P4501A2 from liver microsomes was determined using the methoxyresorufin O-demethylase reaction which is measured spectrofluorometrically at 585 nm (Burke et al., 1974). MROD activities were not determined. CN3D activity decreased in gilthead seabream liver microsomes of different ages. At 1.5 months of age activity is 0.438 ± 0.014 nmol/min/mg protein, while at 24 months the value has decreased to 0.069 ± 0.012 nmol/min/mg protein (Figure 1).

Aminopyrene N-demethylase activity is associated with the cytochrome P4502C isozyme. APND activity decreased in gilthead seabream liver microsomes of different ages. Figure 1 shows that at 1.5 months of age the activity is 0.944 ± 0.024 nmol/min/mg protein, while at 24 months this activity had decreased to 0.056 ± 0.011 nmol/min/mg protein.

EROD activities of P4501A1 from liver microsomes were determined using the 7-ethoxyresorufin O-deethylase reaction. This reaction is based on the conversion of 7-ethoxyresorufin to resorufin which is measured spectrofluorometrically at 585 nm. The reaction rate of EROD (pmol resorufin/min) was obtained from the linear portion of the resorufin formation versus incubation time plots. Figure 2 shows that EROD activity

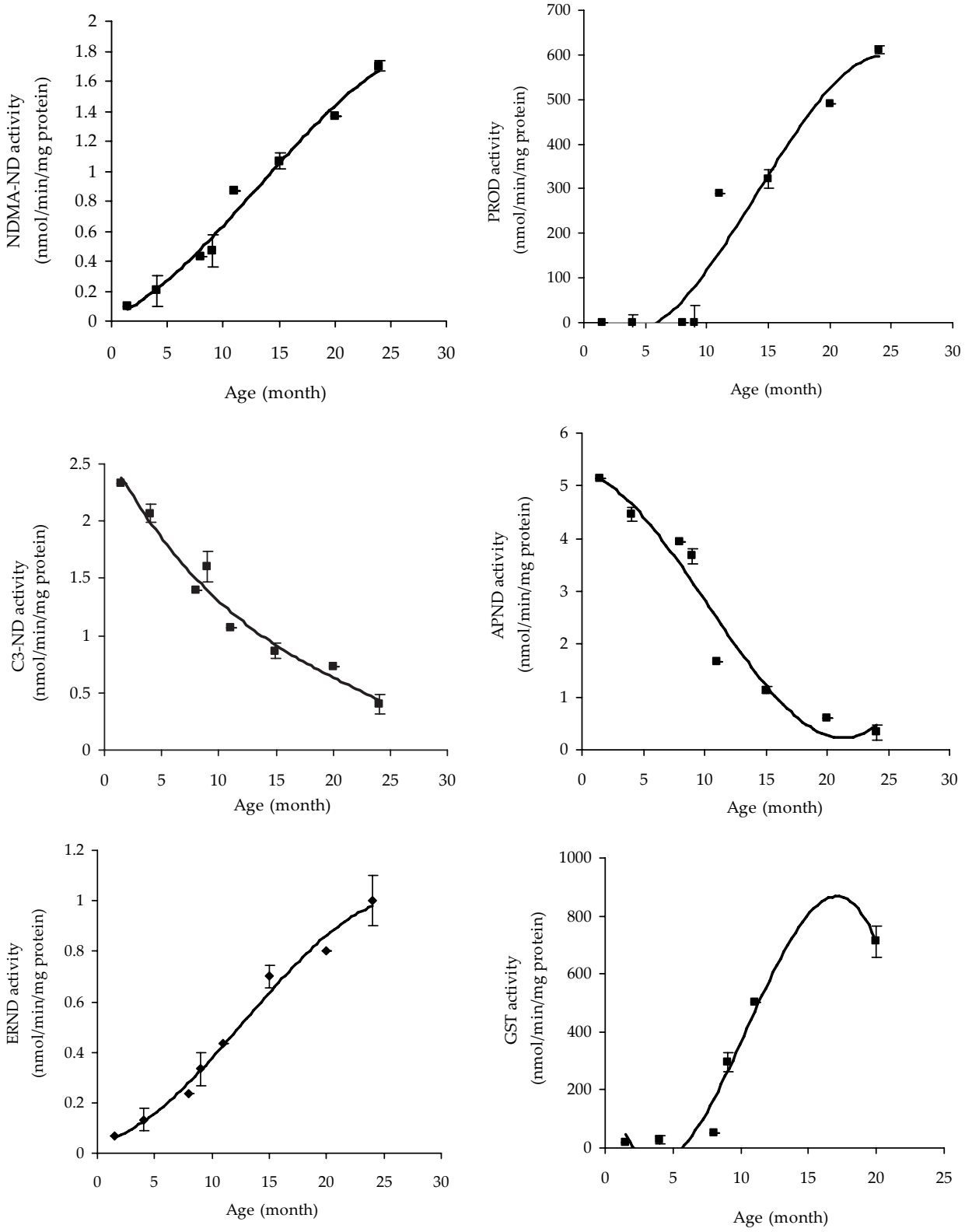

Figure 1. Alteration of NDMA-ND, A4H, C3ND, APND, ERND and GST enzyme activities in fish of different ages.

increased in gilthead seabream liver microsomes of different ages. At nine months the activity was 88.6 ± 1.11 pmol/min/mg protein; at 24 months this value had increased to 998.4 ± 13.7 pmol/min/mg protein. From 1.5 to 9 months of age activities were not detectable.

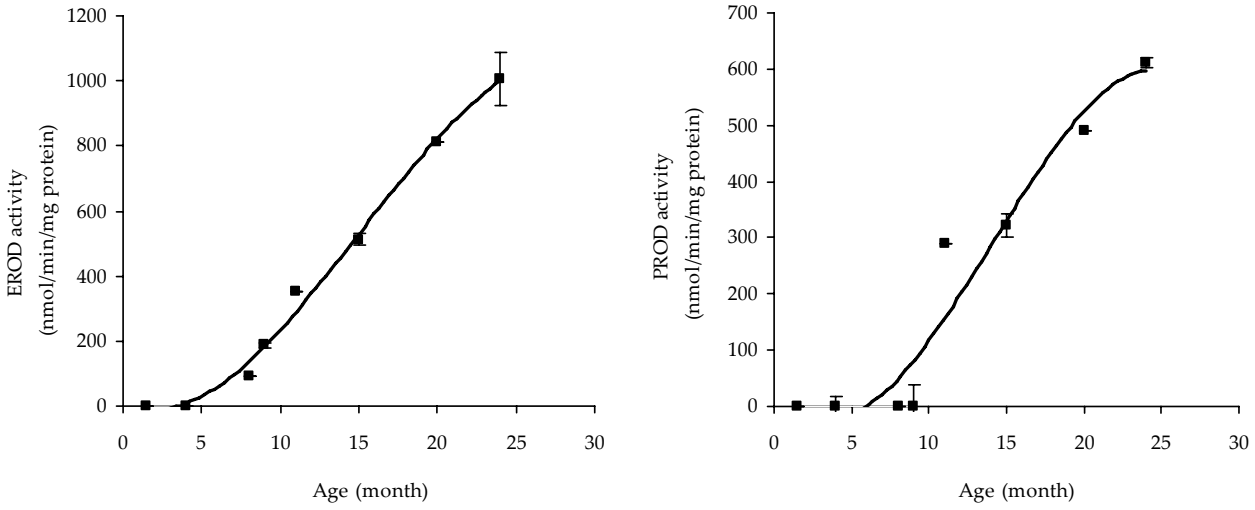

Figure 2. Alteration of EROD and PROD enzyme activities in fish of different ages.

PROD, the activity of P4502B1/2 from liver microsomes was determined using the benzyloxyresorufin O-debenzylase reaction which is measured spectrofluorometrically at 585 nm. The reaction rate of PROD (pmol resorufin/min) was obtained from the linear portion of the resorufin formation versus incubation time plots. PROD activity increased in gilthead seabream liver microsomes of different ages. At 15 months activity was 285.6 ± 20.9 pmol/dakika/mg protein, while at 24 months the activity had risen to 613.7 ± 16.4 pmol/min/mg protein. From 1.5 to 15 months of age activity was not detectable (Figure 2).

It is clear that both P450s and GST play a pivotal role in determining cellular sensitivity to environmental chemicals, and that modulation of the expression of these enzymes by various agents is an important part of their mechanism of action. Because of that we determined total GST activities. GST activity was determined from the gilthead seabream fish livers cytosols using the method of Habig et al. (1974). GST-CDNB activity increased in gilthead seabream liver cytosols of different ages (ranging from 1.5 to 20 months). At 1.5 months the activity was 17.7 ± 0.5 pmol/min/mg protein, while at 20 months this activity had increased to 690.9 ± 32.0 pmol/min/mg protein (Figure 1).

Thus, our results clearly demonstrate elevated A4H, ERND and NDMA-ND activities and decreasing EROD and GST activities. No significant changes were observed with MROD and PROD activities (Table 1). These alterations in XME can contribute to the varying susceptibility and response of these fish to different drugs and/or therapeutics used for treatments.

DISCUSSION

Most lipophilic chemicals including drugs, pesticides, carcinogens, environmental pollutants and naturally occurring compounds undergo enzyme-mediated oxidative, hydrolytic or conjugative biotransformations in liver and in extrahepatic tissues yielding more polar metabolites that can be easily excreted. The great majority of them are almost devoid of any activity but in certain instances metabolites are produced that may retain or enhance the effects of the parent compounds or even acquire different pharmacological or toxicological properties. Thus, metabolism plays a critical role in determining both the efficacy and the residence time of drugs in the body as well as in modulating the response to toxic chemicals. The expression and the activity of drug-metabolizing enzymes (DME) may be influenced by both external (e.g. diet, exposure to different chemicals) and internal factors (Gibson and Skett, 1994) which include species, breed, genetic polymorphisms, age, gender, and physiopathological conditions such as pregnancy or the so called "acute phase response" to physical, microbiological or parasitological agents (Monshouwer and Witkamp, 2000). Focusing on age, it is generally accepted that the sensitivity to the effects of drugs and other foreign chemicals is often greater in young and elderly individuals

Table 1. CYP-Dependent Enzyme and GST activities in male *Sparus aurata* of various age (average ± SD)

Enzymes (nmoles/min/mg protein)	Gilthead seabream age groups (month)							
	1.5 (35)*	4 (75)*	8 (52)*	9 (48)*	11 (45)*	15 (36)*	20 (35)*	24 (25)*
Aniline 4-hydroxylase (A4H)	0.002 ± 0.001	0.007 ± 0.001	0.010 ± 0.002	0.014 ± 0.002	0.030 ± 0.002	0.039 ± 0.002	0.044 ± 0.006	0.058 ± 0.002
Aminopyrine N-demethylase (APND)	0.944 ± 0.024	0.852 ± 0.027	0.733 ± 0.013	0.668 ± 0.026	0.311 ± 0.015	0.198 ± 0.025	0.115 ± 0.013	0.056 ± 0.011
Benzyloxyresorufin O-debenzylase (BROD)	ND	ND	ND	ND	ND	ND	ND	ND
Caffeine N-demethylase (CN3D)	0.438 ± 0.014	0.383 ± 0.025	0.258 ± 0.013	0.292 ± 0.017	0.193 ± 0.027	0.152 ± 0.020	0.145 ± 0.019	0.069 ± 0.012
Erythromycin N-demethylase (ERND)	0.09 ± 0.006	0.030 ± 0.010	0.048 ± 0.010	0.056 ± 0.006	0.099 ± 0.018	0.128 ± 0.008	0.144 ± 0.012	0.189 ± 0.008
Ethoxyresorufin O-deethylase (EROD)	ND	ND	ND	0.088± 0.001	0.280± 0.007	0.424 ± 0.018	0.667 ± 0.081	0.998 ± 0.014
Methoxyresorufin O-demethylase (MROD)	ND	ND	ND	ND	ND	ND	0.015 ± 0.006	0.059 ± 0.005
N-nitrosodimethyl-amine N-demethylase (NDMA-ND)	0.019 ± 0.007	0.043 ± 0.008	0.079 ± 0.006	0.091 ± 0.008	0.091 ± 0.008	0.196 ± 0.01	0.241 ± 0.02	0.323 ± 0.014
Pentoxyresorufin O-depenthylase (PROD)	ND	ND	ND	ND	ND	0.286 ± 0.021	0.350 ± 0.038	0.614 ± 0.016
Glutathione S-transferase (GST-CDNB)	0.018 ± 0.005	0.028 ± 0.002	0.040 ± 0.004	0.295 ± 0.025	0512 ± 0.054	NM	0.691 ± 0.032	NM

ND = not detectable, NM = not measured
*number in paranthesis indicates fish numbers

than in adult ones as the result of several factors including differences in the rate of absorption, binding to plasma proteins, distribution, biotransformation, and excretion (Baggot et al., 2001). Data generated from extensive studies performed in humans (Wilkinson, 1997; Tanaka, 1998) indicate that many enzyme-mediated metabolic pathways show marked age-related changes and a considerable body of information is becoming available also for species of veterinary interest, especially for dogs (Kawalek and El Said, 1990), sheep (Kawalek and El Said, 1994), pigs (Kaddouri et al., 1992), cattle (Peggins et al., 1984), and chickens (Coulet et al., 1996). However, because of the short duration of the commercial life of many species, most of the above studies are centred on the changes in enzyme expression and activity occurring in newborn individuals when they become mature and only few reports have investigated DME activity in aged animals (Kawalek and El Said, 1990). Unlike the majority of domestic species, the horse is characterized by a relatively long lifespan: animals slaughtered for commercial purposes may be aged 10–15 years or even more (20–25) in the case of race or saddle horses at the end of their career. Despite this, the only published report dealing with the effects of age on DME was conducted on 22 horses of both sexes but tested only five substrates (Lakritz et al., 2000). In a previous report (Nebbia et al., 2003) a comparison was made between the expression of equine liver microsomal monooxygenases and that of food-producing and laboratory species. The main aim of the present study was to investigate the development of a wide array of oxidative (cytochrome P450-dependent), hydrolytic and conjugative hepatic DME in 50 female horses aging from up to one year to over 12 years. Moreover, in consideration of both the propensity to accumulate cadmium displayed by the equine liver (Baldini et al., 2000) and the well documented effects of this metal on hepatic DME (Kadiiska et al., 1985), liver cadmium levels were measured and correlated with all examined parameters.

Liver drug metabolism decreases with age in animals. The effect of aging on microsomal cytochrome P450 (P450)-dependent drug metabolism in humans has not yet been fully elucidated. Various factors that may have a significant impact upon drug metabolism are affected by aging, e.g., aging is known to decrease hepatic cytochrome P450 content (Jeffrey and William, 1997). In the study, adult (three months of age) and aged (26 months) male mice were used and EROD (CYP1A1) activities were found to be high in aged mice. Similarly, MROD (CYP1A2) activities were observed to be elevated in aged mice. On the other hand, GSTM1 did not change significantly (Michailova et al., 2005).

Gilthead seabream fish liver microsomes and cytosols of different ages were used. A4H, ERND, NDMA ND, EROD and PROD activities increased in gilthead seabream (*Sparus aurata*) liver microsomes of different ages. APND, CN3D activities decreased in gilthead seabream liver microsomes of different ages whereas MROD and BROD activities were not detectable.

CONCLUSIONS

The following conclusions can be drawn:

- Age-related and statistically significant changes between groups were observed in all examined activities except BROD.
- A general increasing trend was detected in A4H, ERND, NDMA-ND, EROD, PROD and GST-CDNB activities of maturing fishes.
- A decreasing trend was observed with APND and CN3D and their trend was similar.
- No BROD activity was detected in all age groups studied.
- The most pronounced increase was observed for EROD activity.
- The increasing trend observed in A4H and NDMA-ND activities was similar suggesting that they were catalyzed by the same CYP isoforms (CYP2E).
- EROD and MROD activities of different age groups, which are known to be catalyzed by CYP1A in fish, were quite different. Thus, *Sparus aurata* CYP1A shows quite different substrate specificity from that of other fishes.
- CN3D activity, known to be catalyzed by CYP1A2 or orthologues in rats and mammals showed an opposite trend to EROD activity. Therefore, it seems to be catalyzed by a CYP isoform not belonging to the CYP1A subfamily in *Sparus aurata*.
- CYP3A mediated activity, ERND, exhibited an age-related significant rise during maturation in seabreams.
- Conjugative phase II activity (cytosolic GST-CDNB activity) showed an increase in liver in *Sparus aurata* over the examined age range. In addition, it exhibited a correlation with EROD activity.

In conclusion, our results suggest that some important age-related differences in xenobiotic metabolism do occur in the *Sparus aurata* liver and are substrate specific, which might affect obtaining desired responses to drugs, hormones and dietary supplements used during breeding. Furthermore, marked species differences were suggested in age-related xenobiotic metabolism in *Sparus aurata*.

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