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Cytochrome P4501A mRNA and protein induction in the striped bass
(*Morone saxatilis*)

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Abstract

The striped bass (*Morone saxatilis*) support a valuable recreational fishery, and are among the most important piscivorous fish of the San Francisco Estuary. This species has suffered a significant decline in numbers over the past decades and there is indication that contaminants are important contributors. Polycyclic Aromatic Hydrocarbons (PAHs) and Polyhalogenated aromatic hydrocarbons (PHAHs) including PCBs and dioxins are widespread in the Estuary, they typically bioaccumulate through trophic levels, reaching highest levels in top predators, and are known to affect fish health and development. The aim of this study was to investigate the dynamics of cytochrome P4501A (*Cyp1a*) induction simultaneously at different levels of biological organization (RNA transcription and protein synthesis) as a biomarker of exposure to PAHs and PHAHs. We utilized β -naphthoflavone (BNF) as a model PAH to induce *Cyp1a* responses in juvenile striped bass in both dose-response, and time-response assessments and determined *Cyp1a* mRNA and protein levels. Significant responses were measured in both systems at 10 mg BNF.Kg⁻¹, a concentration used for time-response studies. Messenger RNA levels peaked at 6 h post-injection, while protein levels increased progressively with time, significantly peaking at 96 h post-injection; both remaining elevated throughout the duration of the test (8 days). Our data suggest that rapid induction of gene transcription following exposure and subsequent cumulative protein synthesis could provide a useful means of identifying temporal variants in exposure to *Cyp1a* inducers in *Morone saxatilis*. The potential application of this combined *Cyp1a* gene and protein biomarker in this species for field studies is discussed.

Keywords: *Cyp1a*, biomarker, fish, *Morone saxatilis*, “beta-naphthoflavone”, “Polycyclic Aromatic Hydrocarbon”

1. Introduction

The San Francisco Estuary is subject to diverse anthropogenic pressures, including the release of numerous contaminants that have greatly altered the functioning of this ecosystem (Bennett and Moyle 1996; Sommer et al. 2007; Strange 2008). Among contaminants, polycyclic aromatic hydrocarbons (PAHs) and polyhalogenated aromatic hydrocarbons (PHAHs) including polychlorinated biphenyls (PCBs) and dioxins are widespread in the Estuary (Davis et al. 2007; Oros et al. 2007). These chemicals generally occur as complex mixtures originating from a variety of sources: storm water runoff, wastewater treatment plant effluent, atmospheric deposition and dredged material disposal. PAHs and especially PHAHs typically bioaccumulate through the food web, reaching highest levels in top predators (Hylland 2006; Beyer and Biziuk 2009). In fish, water or food exposure to PAHs and PHAHs has been reported to result in numerous biological effects, such as weight loss, immunotoxicity, hepatotoxicity, cardiotoxicity, edema formation, genotoxicity and tumor promotion (e.g. Tanguay et al. 2005; Reynaud and Deschaux 2006, Pinkney et al. 2010; Nahrgang et al. 2010; Wills et al. 2010; Hiken et al. 2011). Such compounds are known to affect early life stages development, i.e. malformations of embryo and larvae (Barron et al. 2004; Sundberg et al. 2006, Billiard et al. 2008) and cause alteration of endocrine system function and reproduction (Monosson 1999/2000). These deleterious effects may reduce survival leading to population level effects.

The striped bass (*Morone saxatilis*) were introduced from the East Coast of USA in the late 1880s, now support a valuable recreational fishery, and are among the most important piscivorous fish in the San Francisco Estuary. Along with a number of other pelagic fish species in the estuary, this species has suffered a significant decline in numbers (Bennett and Moyle 1996; Feyrer et al. 2007).

Extensive research on this and other pelagic fish species have also indicated that invasive species, food limitation, intra- and inter-specific competition and predation, increased entrainment, habitat losses, disease and impaired offspring (Ostrach et al. 2008; Sommer et al. 2007) likely contribute to population declines, and there is indication that contaminants are important contributors (Stevens et al. 1985; Bailey et al. 1994; Bennett et al. 1995; Ostrach et al. 2008). Monitoring and assessment of the striped bass population in the San Francisco Estuary has been extensive over the past 50 years for various reasons. Striped bass exploit the entire estuary ecosystem including the rivers, Delta region, outer bays and nearshore Pacific Ocean (Stevens et al. 1985, Sommer et al. 2007). Striped bass are an apex predator in the system and as such are important sentinels for this ecosystem (Ostrach et al. 2009). The majority of fish species declining in the San Francisco Estuary are listed as either threatened or endangered and cannot be studied in the laboratory due to their status as well as the difficulty of handling and lack of appropriate methods for experimentation. Unlike these other species, striped bass are not a listed species and are amenable to breeding and experimentation in captivity (Ostrach et al. 2008).

To understand the effect of contaminants on fish we must use systems that measure the extent of sublethal biological effects resulting from chemical exposure. Molecular and biochemical biomarkers, in particular, are valuable tools for monitoring contaminant exposure (Tom and Austlander 2005; Sarkar et al. 2006; Fedorenkova et al. 2010), however, most require validation for better interpretation of field data (e.g. Cannon et al. 2010). Toxic responses are directly linked to a contaminant's mode of action, and are generally preceded by modifications in gene transcription. RNA is not the final product of a gene, as the gene function is carried out by the resulting protein. The level of protein may be regulated through transcriptional, post-

transcriptional, and post-translational mechanisms. Thus measurements of mRNA transcription may or may not be indicative of subsequent protein levels and activity.

Induction of cytochrome P4501A (*Cyp1a*) is a powerful biomarker of exposure to PAHs and PHAHs (i.e. PCBs and dioxins) and has been used for biomonitoring assessments in a number of aquatic organisms (Anderson et al. 1994; Stegeman and Hahn 1994; van der Oost et al. 2003; Hylland 2006). Cytochrome P4501A is a monooxygenase involved in xenobiotic biotransformation and detoxification of many PAHs, PCBs and dioxins, and is induced by the binding of these compounds with Aryl hydrocarbon receptor (AhR), i.e. a cytosolic ligand-activated transcription (Stegeman and Hahn 1994; Hahn 2001). The induction of *Cyp1a* may then be regulated at various post-transcriptional steps (Abdelhamid et al. 2010; Amara et al. 2010). Cytochrome P4501A can also be activated by other pathways and plays a strong role in steroid hormone biosynthesis, tryptophan and retinol metabolism (review in Monostory et al. 2009). Induction of *Cyp1a* varies between fish species (Wirgin et al. 1996, Willet et al. 1997, Oris and Roberts 2007), so that a specific evaluation needs to be performed before using this biomarker on a sentinel species in environmental monitoring programs. In this study we thus focus on both *Cyp1a* gene expression and protein content in order to provide a combined biomarker specific for the striped bass *Morone saxatilis*.

The aims of this study are two-fold, investigating the dynamics of *Cyp1a* induction at different biological levels of organization (RNA transcription and protein synthesis), and to explore the relationship between the measured parameters. By conducting sublethal exposure response studies that integrate molecular and biochemical assessments, we are better able to understand the relationship between gene expression and effects at higher levels of biological organization. In the

present study, we utilize the PAH model, β -naphthoflavone (BNF), to induce *Cyp1a* responses in juvenile striped bass in both i) dose response and ii) time response experiments, in order to identify and utilize transcriptional and translational differences as measures of temporal variants of xenobiotic exposure.

2. Methods

2.1. Fish handling: One-year old striped bass juveniles (fork length: 19.9 ± 2.5 cm; body mass: 114 ± 40 g) used in this study were produced from a domestic brood stock maintained at the Center for Aquatic Biology and Aquaculture at UC Davis. Fish were moved to the experimental facility two days prior to the start of the experiment. They were maintained in aerated circular (diameter: 60 cm; height: 35 cm) flow-through freshwater tanks (10 fish per tank) at 19 – 20 °C water temperature and under natural photoperiod. Fish were fed daily at 2.5% body weight with #3 Crumbled Salmon/Trout chow (Silver Cup, Murray, UT 84157), but were fasted for 24 h prior to test initiation to avoid any post-anesthesia risk (e.g. regurgitation of food and/or fecal contamination of recuperation water)

2.2. BNF exposures and sample preparation: Fish were exposed to BNF through intra-peritoneal injections in two experiments: (i) a 24h dose response to BNF concentrations (Sigma-Aldrich Corporation) of 0.1, 1, 10, and 25 mg.kg⁻¹, and (ii) a time response study, commencing at time 0 h with either a 10.0 mg β -naphthoflavone.kg⁻¹ or solvent control (SC), followed by a metabolic detoxification time of 6 h, 12 h, 24 h, 2 d, 4 d and 8 d. Each group of fish (N=10) for both dose and time response was placed in an individual circular tank (see above). Corn oil (Sunny Select, Super store industries, Lathrop, CA 95330) was used for BNF dilution and as solvent control. Fish

sampled at time zero received no injection. Prior to injection, fish were anesthetized with MS-222 (tricaine methanesulfonate, Sigma, St. Louis, MO, USA) at 100 mg.L⁻¹. Upon termination of the experiment, fish were euthanized using an overdose of MS-222, their livers dissected and immediately snap frozen in liquid nitrogen. Liver tissue was then ground in liquid nitrogen to a fine powder using mortar and pestle, aliquots were placed into two separate microcentrifuge tubes, to ensure a homogeneous sample, and stored at -80°C for subsequent molecular and biochemical analyzes.

2.3. *Cyp1a* Transcription assessment:

RNA extraction and cDNA synthesis: Total RNA was extracted from powdered liver tissue using RNeasy Mini Kit (Qiagen Inc., Valencia, CA), with on-column DNase digestion, as per manufactures protocol. Samples were quantified using a NanoDrop spectrophotometer and their quality verified by electrophoresis on 1% agarose gel. Complementary DNA (cDNA) was synthesized using 100 units of SuperScript III (Invitrogen, Carlsbad, CA, USA), 600 ng random hexadeoxyribonucleotide (pd(N)6) primers (random hexamer primer), 10U RNaseOut (RNase inhibitor), and 1mM dNTPs (all Invitrogen, Carlsbad, CA, USA).

Quantitative PCR: We measured *Cyp1a* gene (precisely CYP1A1) expression in striped bass using specific probes and primers designed for quantitative PCR (qPCR), as detailed in Table 1. TaqMan Universal PCR Mastermix (Applied Biosystems) was used in cDNA amplifications using an automated fluorometer (ABI PRISM 7900 Sequence Detection System, Applied Biosystems). Quantitative PCR data was analyzed using the relative quantification 2^(-Delta Delta CT) method (Livak and Schmittgen, 2001), using *β-actin* expression as a reference to which *Cyp1a* expression was normalized. Statistical assessments were conducted on Log₂ normalized data (see below).

162

163 **2.4. *Cyp1a* Protein quantification:** A *Cyp1a* competitive-ELISA for striped bass was developed
164 following Tom et al. (2002). A brief method description is provided below.

165 2.4.1. *Cyp1a* protein production: A strain of *Escherichia coli* that contained an antigenic fragment
166 of *Lithognathus mormyrus Cyp1a* gene was obtained from Dr. Moshe Tom, Israel Oceanographic
167 and Limnological Research, Haifa, Israel. Colonies were grown over night in Lysogeny Broth
168 medium with 100 µg/mL ampicillin at 37 °C to produce a starter culture. This was used to
169 inoculate one liter of Terrific Broth medium with 50 µg/mL ampicillin and 1.0 mM thiamine at 37
170 °C. Four hours later heme precursor δ-aminolevulinic acid and isopropyl β-D-1-
171 thiogalactopyranoside (IPTG) inducer were added to a final concentration of 0.5 mM, and the
172 culture was incubated at 28 °C for 24 hours. After centrifugation at 5,000 x g for 15 minutes in
173 250ml flasks, bacteria were transferred into 50 mL centrifuge tubes and spun again. Cells were
174 resuspended into 30 mL of 0.1 M potassium phosphate buffer, 20% glycerol, 0.1 mM
175 Dithiothreitol (DTT) and 0.1 mM Ethylene Diamine Tetraacetic Acid (EDTA) (pH 7.4). Egg white
176 lysozyme (0.5 mg/ml) was added to the buffer and incubated for one hour at 4 °C. A 1/10,000
177 dilution of nonselective protease inhibitor (Sigma P8340) and 1 µg/ml DNAase I were added. The
178 resulting spheroplast suspension was then sonicated with a high powered probe sonicator at 25
179 Watts, 8 times for 30 seconds on ice and then centrifuged at 30,000 x g for 2 hours. The *Cyp1a*
180 containing cytosol was saved and the pellet was discarded.

181 2.4.2. *Cyp1a* protein purification: The *Cyp1a* containing cytosol was purified on a Ni⁺
182 Nitrilotriacetic acid Agarose (Qiagen) column equilibrated with running buffer 50 mM potassium
183 phosphate buffer containing 0.2 M NaCl, 20 mM glycine, 20% glycerol, 0.1 mM EDTA and 0.1

mM DTT. The poly-His tail that was added on the recombinant protein fragment has a high affinity for the Ni⁺ ion and allows for the crude extract to pass through and enrich the solution with the protein of interest. The protein was eluted from the column with running buffer containing 50 mM histidine. Fractions were analyzed for total protein with the BioRad DC protein assay, and further analyzed with Sodium Dodecyl Sulfate (SDS) polyacrylamide gel (Invitrogen NuPAGE) to determine the fractions containing high concentrations of *Cyp1a*. These fractions were pooled, and analyzed with the BioRad DC protein assay to determine total protein content and by SDS gel electrophoresis to determine the *Cyp1a* protein concentration. SDS gels showed a prominent band at 55kDa matching the molecular weight of the *Cyp1a* protein fragment with a concentration of 0.18 µg/µL in the resulting stock.

2.4.3. *Cyp1a* competitive-ELISA optimization and measurement: For the competitive-ELISA, we used the monoclonal mouse anti-fish *Cyp1a* antibody (C10-7, Biosense Laboratories) that has been shown to cross-react with a variety of fish species including rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), carp (*Cyprinus carpio*), turbot (*Scophthalmus maximus*) and Atlantic cod (*Gadus morhua*). The produced *Cyp1a* protein fraction was coated on high binding 96 well plates (Costar 3925, Black) at 100 or 200 ng/mL *Cyp1a* protein fragment in 0.05 M Sodium Bicarbonate, pH 9.6. Plates were then blocked with PBS buffer pH 7.5 containing 1% Hammerstein Casein and 1.0 mM EDTA. Plates were then co-incubated with increasing concentrations of *Cyp1a* protein fragment with 1/5,000 or 1/10,000 diluted Mouse anti-fish *Cyp1a* antibody in PBS buffer pH 7.5 containing 1% Hammerstein Casein, 1.0 mM EDTA, and 0.05% Tween 20, and then rinsed with PBS and 0.05% Tween 20. Following incubation with 1/10,000 diluted Goat Anti Mouse Horse Radish Peroxidase (HRP) conjugate (Jackson Immuno Research) and rinsing with PBS and 0.05% Tween 20, HRP substrate (35.4 µM ADHP (Anaspec, Inc.), 1.76

mM Urea Hydrogen Peroxide in 0.05 M Sodium Phosphate buffer, pH 7.4) was added. After approximately 30 minutes, fluorescence was measured at 544 nm excitation with 590 nm emission on a Wallac Victor 2 Fluorescence Plate Reader. The results confirmed that the antigenic *Cyp1a* protein fragment was present. The competitive *Cyp1a* ELISA described above was shown to be optimized by coating plates overnight with 200 ng/ml *Cyp1a* at 4°C, co-incubating S9 fraction sample with 1/10,000 dilution of Mouse anti-fish *Cyp1a* in the competitive binding step, and incubating with 1/10,000 dilution of Goat anti Mouse-HRP (Ostrach et al., 2009; Spearow et al., 2011).

S9 fraction sample was prepared: powdered liver tissues of each sample was homogenized in 10 volumes by weight of ice-cold extraction buffer consisting 0.02M 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) 0.15M KCl, (pH 7.5) 0.22mM 4-(2-aminoethyl) benzenesulfonyl fluoride and 1/200 protease inhibitor cocktail (Sigma). After centrifugation at 10,000 g for 15 minutes at 4C, the S9 supernatant fractions of *Morone saxatilis* liver samples were obtained for *Cyp1a* protein measurement through competitive-ELISA as mentioned above.

2.5. Statistical Analyses: Data-sets were not normally distributed; therefore assessments were tested for significance using non-parametric tests. Time responses were assessed using Kruskal-Wallis analysis of variance, with Siegel-Castellan post-hoc multiple correction tests. Differences between data-points and respective solvent controls were assessed using Mann Whitney U tests. Dose response data were compared to controls using Mann Whitney U tests. Analyses were performed using the R software (R Development Core Team, 2008).

3. Results

3.1. Dose Response to BNF exposure:

Striped bass hepatic *Cyp1a* mRNA and protein responses to BNF exposure were dose dependent at 24 h post injection (Fig. 1), with both q-PCR and competitive ELISA results correlating positively with increased BNF exposure ($p<0.001$). *Cyp1a* mRNA abundance was significantly increased 3.83-fold by 1 mg β -naphthoflavone.Kg⁻¹ relative to solvent controls ($p<0.05$), and almost 13-fold by 10.0 mg β -naphthoflavone.Kg⁻¹ ($p<0.05$). *Cyp1a* Protein content was significantly upregulated by 10 mg β -naphthoflavone.Kg⁻¹; ($p<0.01$).

3.2. Timed Response to BNF exposure:

There was a significant time-dependent response in hepatic *Cyp1a* mRNA abundance and protein content in juvenile striped bass exposed to 10.0 mg β -naphthoflavone.Kg⁻¹ (Fig. 2). *Cyp1a* mRNA abundance peaked at 6 h with a 40.80 fold-increase relative to time zero ($p<0.001$), then decreased with metabolic detoxification time to 8 d, though remaining significantly up-regulated at 12 h, 24 h and 48 h relative to time zero ($p<0.01$). The quantity of *Cyp1a* protein, however, increased with time, being significantly higher relative to time zero from 48 h onwards ($p<0.001$), peaking at 96 h post-injection (9.3 $\mu\text{g.mg}^{-1}$ total protein) and remained elevated at 8 d post-injection (6.33 $\mu\text{g.mg}^{-1}$ total protein). We note that control fish (SC) also show some increase in mRNA and especially protein levels during the first 24 hours after the beginning of the experiment, which may be due to the effects of handling stress. However, *Cyp1a* mRNA and protein levels were significantly higher with respect to solvent controls throughout the 6 h to 8d post-injection period (Fig. 2).

4. Discussion

The cytochrome P4501A is considered an excellent indicator of PAHs and PHAHs exposure, with a number of reviews highlighting its use in monitoring (Anderson et al. 1994; Stegeman and Hahn 1994; van der Oost et al. 2003; Hylland 2006). Liver microsomal 7 ethoxyresorufin-O-deethylase (EROD) activity is commonly used as a proxy of *Cyp1a* induction. Although *Cyp1a* is the main enzyme catalyzing the de-ethylation of ethoxyresorufin, other CYP enzymes also can contribute to EROD activity (Spearow et al. 2011). It has also repeatedly been reported that EROD can substantially be inhibited by elevated levels of PCBs, fluoranthene (a prevalent PAH), dioxins and heavy metals (e.g., Viarengo et al. 1997; Gooch et al. 1989; Willett et al. 2001; Ortiz-Delgado et al. 2008). EROD activity is technically easy to measure and it is still considered as a robust biomarker (Oris and Roberts 2007) however, this enzymatic activity biomarker shows some limitations that can make interpretation sometimes difficult in field studies (Spearow et al. 2011). Although, several studies have focused separately on *Cyp1a* induction at different organizational levels: gene expression (e.g. Rees 2003; Chung-Davidson 2004; Aluru 2008) and protein content or EROD activity (e.g. Gravato and Santos 2002; Miller et al. 2004; Ortiz-Delgado et al. 2008), relatively few have compared gene expression and protein level (see: Kloepper-Sams and Stegeman 1989; Hahn and Stegeman 1994). In this study we focus on both *Cyp1a* induction through gene expression and protein content with state of the art techniques in order to provide a combined biomarker applicable for *Morone saxatilis*. Molecular and biochemical biomarkers are promising tools to assess the effects of chemicals on exposed organisms; however, data interpretation is sometimes the limiting factor hindering their use. Nevertheless, through assessing the combined responses at different levels of biological organization, it is possible to gather more extensive

information towards a more reliable interpretation of data, thus substantiating the use of biomarkers in monitoring efforts. Although, ongoing validation is required in order to apply these biomarkers in field situations, the current study strongly supports the use of *Cyp1a* in striped bass as a monitoring tool of sublethal exposure, particularly when two or more assessment methods can be applied synchronously.

This study has been successful in refining and validating a *Cyp1a* protein assay technique for striped bass developed by Ostrach et al. (2009), based on methods by Tom et al. (2002). Results were comparable to responses reported by Kloepper-Sams and Stegeman in *Fundulus heteroclitus* (1989; 1994), though the newly developed approach, and current techniques permit the presentation of these responses in a more specific manner for *Morone saxatilis*. The present study applied a competitive-ELISA technique that uses a recombinant *Cyp1a* and that allows for a higher-throughput quantitative evaluation of fish *Cyp1a* (Tom et al. 2002), than the semi-quantitative western blot method used in the reference study by Kloepper-Sams and Stegeman (1989). In the present study, hepatic *Cyp1a* mRNA abundance in juvenile striped bass responded rapidly to BNF exposure peaking within six hours post-exposure. The corresponding biotransformation and detoxification protein was induced progressively peaking at four days post-exposure and remaining elevated throughout the duration of the experiment. Thus the rapid increase in mRNA abundance and subsequent cumulative protein synthesis can provide a useful mean of identifying temporal variants in exposure, where mRNA levels are used to detect recent exposures and protein levels are indicative of past exposures. The time course of *Cyp1a* induction may also vary following single or continuous exposure (e.g. Levine and Oris 1999) and to different kind of contaminants; for instance poorly metabolizable AhR inducers such as PCBs or dioxins show prolonged induction even at gene expression level (e.g. Hahn and Stegeman 1994). In field

conditions, chronic exposure is the common environmental situation and fluctuations in pollution levels occur. Time course of biomarker are important in evaluating changes in exposure due to pollution sources or prevention actions. Therefore further studies are evidently required with different modes of exposure and using different *Cyp1a* substrates, inhibitors and mixtures before application to field conditions. The difference between these conditions would therefore need to be established based on statistical differences in expression from negative control samples, or previously determined basal expression levels (Tom and Auslander 2005). Nevertheless models that make use of the shorter half life of *Cyp1a* mRNA, relative to that of *Cyp1a* protein, could potentially detect temporal variants in exposure. This is likely to hold true, not only for *Cyp1a*, but for a number of other biomarkers of toxicant exposure and effect.

Increasing the informative power of biomarkers, by measuring multiple levels of organization along with establishing suites of biomarkers, indicative of exposure and effect of multiple groups of chemicals, will allow a more comprehensive evaluation of contaminant impacts in exposed organisms. This is especially needed for field monitoring. Current monitoring efforts in the San Francisco Estuary have highlighted the presence of a number of contaminants, including PAHs and PHAHs (SFEI 2008). The newly developed *Cyp1a* ELISA described here, along with knowledge based applications of numerous other molecular and biochemical biomarkers, and numerous other molecular and biochemical biomarkers (Burge et al. 2004; Geist et al. 2007; Picha et al. 2008; Washburn et al. 1996; Durieux et al. 2010; Spearow et al. 2011) will permit the assessment and understanding of the sublethal effects of complex mixture of chemical contaminants on striped bass. In addition, as an apex predator, striped bass is a sentinel species in this threatened ecosystem such that the approach developed and described here can be an extremely useful and effective mean of assessing, understanding and potentially mitigating sublethal contamination in estuarine

habitats where striped bass reside. These promising results show the need for continuing research towards establishing links between cellular responses to toxic contaminants with effects on growth, development, behavior, reproduction and survival.

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Table 1. Primer and Probes for *Cyp1a* (precisely CYP1A1) and β -actin used for quantitative PCR assessments of gene expression in striped bass.

Gene	Species for starting sequence	NCBI GenBank accession number	Primer	Roche Probe
<i>Cyp1a</i>	<i>Dicentrarchus labrax</i>	AJ251913	Right:	
			GCGGCACAACCCCAGAGTA	No. 65;
			Left:	CTGGAGGA
			CAGCTTTCATGACGGTGTGAG	
β -actin ^a	<i>Dicentrarchus labrax</i>	AJ493428	Right:	
			GCAATGAGAGGTTCCGTTGC	No. 11;
			Left:	CTTCCAGC
			GCAGGACTCCATACCGAGGAA	

^a reference gene

Figures captions

Figure 1. Hepatic *Cyp1a* mRNA levels (A) and *Cyp1a* protein levels (B) in juvenile *Morone saxatilis*, at 24 h following the injection of 0.1, 1, 10 and 25 mg β -naphthoflavone.Kg⁻¹. Values represent means $\pm$ SE. With sample size for the respective concentrations: N = 9, 10, 10, 8 and 9 (A); 10, 10, 10 and 10 (B). Significant differences from solvent control are indicated: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Figure 2. Timed responses in hepatic *Cyp1a* mRNA (A) and protein levels (B) in juvenile *Morone saxatilis*, following the injection with 0 (solvent control: SC) and 10 mg β -naphthoflavone.Kg⁻¹ (BNF 10) at 0, 6, 12, 24, 48, 96 and 192 h. Values represent means $\pm$ SE. With sample size for the respective timed responses: N = 9, 8, 8, 8, 10, 4 and 10 (A: BNF 10); 10, 8, 9, 10, 4 and 10 (A: SC); 9, 8, 10, 10, 10, 4 and 10 10 (B: BNF 10); 10, 10, 9, 10, 4 and 10 (B: SC). Significant differences from solvent control are indicated: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

532

533 Fig. 1

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535 Fig. 2