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Induction of vitellogenin synthesis in immature male yellowfin seabream (*Acanthopagrus latus*) exposed to 4-nonylphenol and 17β-estradiol

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Abstract

In the present study, the effects of xenoestrogen 4-nonylphenol (4-NP) and 17β-estradiol (E2) on the synthesis of vitellogenin (VTG) in immature male yellowfin seabream (*Acanthoparus latus*) were assessed to determine the potential use of this species as a biological model for studying endocrine disrupters in the Musa estuary in southern seawaters of Iran. A total number of 104 fish were injected by 10, 50, 100, 150 and 200 μg g^{-1} $week^{-1}$ of 4-NP and 2 μg g^{-1} $week^{-1}$ of E2, and their blood plasma was obtained for biochemical analysis on days 0, 7 and 14. The hepatosomatic index (HSI) of each fish was also recorded at each sampling time. The results showed that 4-NP significantly induced VTG synthesis in *A. latus*, which was detected by gel electrophoresis as two major protein bands (~210 and 191 KDa). The induction of VTG was also determined indirectly by measuring the alkali-labile phosphate, total plasma calcium and protein. All of these VTG indicators significantly increased in the plasma of 4-NP- and E2-treated fish. Meanwhile, a significant dose-dependent increase was observed in HSI, indicating the activation of hepatic VTG production in 4-NP- and E2-injected fish. Furthermore, the plasma alanine aminotransferase and aspartate aminotransferase concentrations significantly increased in 4-NP-treated fish, presumably reflecting a situation of hepatic tissue damage due to 4-NP toxicity. Based on the results, it is suggested that *A. latus* could be used successfully as a model species for future studies of endocrine disrupting contaminants in the Musa estuary.

Keywords

Acanthopagrus latus, vitellogenin, 4-nonylphenol, 17β-estradiol, xenoestrogen

Introduction

Over the past two decades, public concern has been increasing about xenobiotic chemicals that may interact with and disrupt the endocrine systems of humans and wildlife populations (Damstra et al., 2002; Matthiessen, 2003a). These compounds are commonly referred to as endocrine disrupting chemicals (EDCs), which include a diverse range of synthetic and natural compounds (Goksøyr, 2006; Tapiero et al., 2002). Among EDCs, those that elicit an estrogenic response have received great attention because of their capability to mimic or antagonize the actions of endogenous hormones (Folmar et al., 2000; WHO/IPCS, 2002). Now, there is clear

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evidence of EDCs (particularly xenoestrogens) that may influence health, development and reproduction of many animal species, including humans (Colborn et al., 1993; Gross-Sorokin et al., 2006; Segner, 2005; Segner et al., 2003). However, the great bulk of our knowledge concerning adverse effects of EDCs derives from fish studies, particularly from freshwater species. In recent years, considerable attentions have been focused on the applicability and predictability of laboratory animals for assessing the effects of EDCs on aquatic ecosystems. In this regard, a number of potential biomarkers have been validated and used in the study of endocrine disruption in fish and other aquatic organisms (Arukwe et al., 2000; Jones et al., 2000; Kleinkauf et al., 2004; Mitchelmore and Rice, 2006; Matozzo et al., 2008; Zaccaroni et al., 2009). Induction of vitellogenin (VTG) synthesis in immature and male fish has been the most commonly used biomarker of exposure to estrogenic contaminants in the aquatic environments (Marin and Motazzo, 2004; Wheeler et al., 2005).

The Musa estuary is the largest estuarine water body located in southern Iran. This is an ecosystem with unique biological and ecological characteristics, which is an important habitat for various species of aquatic animals. Nevertheless, this region is surrounded by several petrochemical companies and commercial ports that continually discharge their wastewaters with various toxic chemicals into the estuary (Malmasi et al., 2010). Previously, it has also been demonstrated that a huge volume of materials (approximately 3,000,000 tons) either used or produced in only one of these petrochemical factories, Bandar Imam Petrochemical Complex, could act as xenoestrogen. This accounts for about 51% of its raw materials and products (Ebrahimi and Shamabadi, 2007). On the other hand, a great volume of effluent discharges from sailing activities, oil refineries and urban areas provide another source of xenoestrogens input into the Musa estuary. In recent years, the presence of sterile fish have been claimed frequently by aquaculturists of the region who are looking for the fertile mature fish for breeding in local catch. It is, therefore, suggested that some contaminants act as EDCs and adversely affect the reproduction and development of fish that are living in this water body.

Among marine fish, protoandrous and protogynous species that have a critical period of sexual differentiation may be more endangered by EDCs than other species (Matthiessen, 2003b). In the present study, sexually immature male yellowfin seabream (*Acanthopagrus latus*), a protandrous hermaphrodite fish belonging to the family Sparidae was chosen as a model species. This is an important marine species in Iran in terms of fisheries and aquaculture. Several useful features such as being highly resistant to stressful conditions, easy adaptation to captivity, availability throughout the year and providing sufficient amount of blood and tissue for multiple analysis made *A. latus* eligible for the current study.

The objectives of this study were (1) to investigate the effects of xenoestrogen 4-nonylphenol (4-NP) on VTG synthesis in sexually immature male *A. latus* in laboratory and (2) to evaluate the potential use of this species as a biological model in subsequent field biomonitoring studies of EDCs in the Musa estuary.

Materials and methods

Chemicals

Branched 4-NP and 17β-estradiol (E2) were purchased from Tokyo Chemical Industry (TCI Europe, Zwijndrecht, Belgium) and Sigma (St Louis, Missouri, USA), respectively. 2-Phenoxyethanol was obtained from Merck Schuchardt (Hohenbrunn, Germany). The stock solutions of 4-NP and E2 were prepared by dissolving appropriate amount of chemicals in ethanol–coconut oil (1:9) and were stored in the dark glass bottles at 4°C.

Fish collection and maintenance

A total of 150 sexually immature male fish were captured from the Musa estuary (Zangi and Jafari creeks) and immediately transferred to the Mariculture Research Station of the South Iranian Aquaculture Research Center in Mahshahr. Of them, 104 fish (mean body mass 133.7 ± 2.4 g) were randomly placed into eight fiberglass tanks containing 300 L of filtered, ultraviolet-treated and aerated seawater (pH 8.1, salinity 45% and temperature 26°C) and maintained under 12 h:12 h light:dark photoperiod. The fish were acclimated to this condition for 10 days before the experiment was started. The fish were not fed during acclimation and experimental period. About 80% of experimental seawater was changed every day, and the feces were siphoned out during water exchange.

Fish treatment and sampling

The fish were anesthetized with 0.1% 2-phenoxyethanol and injected intraperitoneally with 10, 50, 100, 150 or 200 μg g^{-1} $week^{-1}$ of 4-NP in half doses twice a week.

Additionally, to compare the estrogenic response of 4-NP-treated fish, one group was injected with E2 (2 μg g^{-1} $week^{-1}$). The concentrations used were chosen based on the previous work (Christensen et al., 1999). Moreover, the solvent controls received ethanol–coconut oil only (100 μl), whereas the controls were not injected at all. On days 0, 7 and 14, the fish were taken from the tanks, anesthetized and their total length and weight were measured. The blood was drawn from the caudal artery using heparinized syringes and immediately transferred into ice-chilled vials. The plasma was collected after centrifugation (1000 × *g*, 10 min) and stored at −80°C until further use. After each bleeding, an individual fish was placed into separate plastic bags, labeled and stored in ice until dissection. The livers were quickly dissected, weighed and fixed in 10% natural buffered formalin and stored at room temperature for later histological evaluation. The hepatosomatic index (HSI) was calculated as follows:

$$\text{HSI}\ (\%) = (\text{total liver weight}/\text{total body weight}) \times 100$$

Vitellogenin analysis

The VTG in the plasma samples was identified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) performed according to the method of Laemmli (1970) using an 8% separating gel and 4% stacking gel. Before electrophoresis, the plasma was diluted at 1:2 ratio with sample buffer (63 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.0025% bromophenol blue and 2.5% 2-mercaptoethanol) and boiled for 5 min. The gels were run in an Omni Page Maxi-Vertical Electrophoresis Unit (Cleaver, Warwickshire, England) with a constant current of 165 V up to 4 h. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 for 50 min. A protein molecular weight marker (RECOM™ SPECTRUM, No. REC 008, Banqiao City, Taiwan) was used to calibrate the gel. VTG was also determined indirectly by measuring the alkali-labile phosphate (ALP) according to Hallgren et al. (2009). In brief, 100 μl of plasma were mixed with 54 μl acetone to precipitate plasma proteins. Then, two consecutive washing steps using Tris and ethanol, respectively, were performed to remove free phosphates of plasma. The remained protein pellets were mixed with 100 μl of 1 M NaOH and incubated for 90 min at 70°C to allow hydrolysis of bound phosphates. The levels of free phosphates were determined in the aqueous phase by a colorimetric assay of acidified phosphomolybdate complexes. Each of the individual samples was measured in triplicate by ultraviolet/visible UNICO spectrophotometer (model 2100, New Jersey, USA) and the results were expressed in microgram of PO_4^{-3} per milliliter plasma.

The total plasma calcium levels were determined by colorimetric measurement with methylthymol blue using a commercially available kit (Man Co., Tehran, Iran). Plasma levels of total protein were determined by standard procedures used in clinical biochemistry laboratories according to Biuret method with bovine serum albumin as a protein standard (Pars Azmoon Co., Tehran, Iran).

Enzymatic analysis

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured according to the method proposed by International Federation for Clinical Chemistry modified by a commercially available kit (Pars Azmoon Co., Tehran, Iran). In ALT assay, the enzyme catalyzes transaminase reaction between L-alanine and 2-oxoglutarate to form glutamate and pyruvate. Pyruvate is converted to lactate and NAD^+ by lactate dehydrogenase. AST was assayed in a coupled reaction with malate dehydrogenase in the presence of NADH. The end point was recorded using Auto-analyzer (Technicon RA1000, New York, USA) at 340 nm and 37°C.

Statistical analysis

All values are expressed as means ± standard error. Two-way analysis of variance was used to examine the significance of treatment, time and their interaction ($p < 0.05$). Once a significant difference was observed, Duncan's *post hoc* test was used to determine the significant differences between groups.

Results

There were six mortalities during the exposure period. All the mortalities occurred on the second week of exposure and belonged to 100 ($n = 1$), 150 ($n = 2$) and 200 μg g^{-1} ($n = 2$) of 4-NP- and 2 μg g^{-1} ($n = 1$) of E2-treated groups. Moreover, after dissection, the gender of two fish from 4-NP (50 μg g^{-1}) and E2 groups was determined as female. Therefore, their plasma samples were excluded from the experiments analysis. The presence of VTG in the plasma samples of 4-NP treated fish was verified by SDS-PAGE as

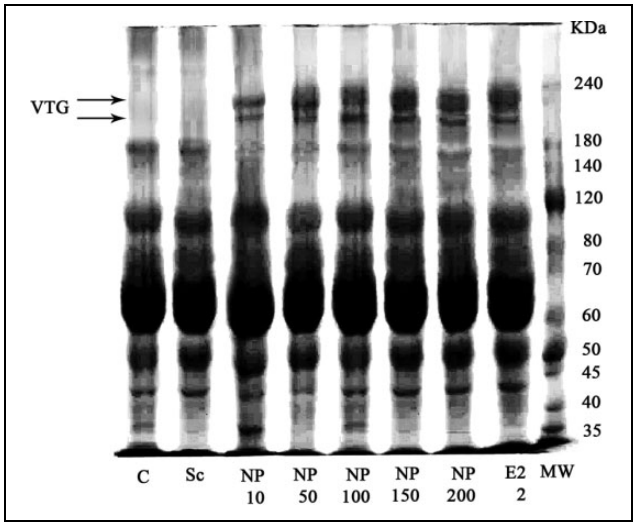

Figure 1. SDS-PAGE pattern of the plasma proteins from immature male *A. latus* treated with 4-NP or E2 after 14 days of treatment. C: control; Sc: solvent control; 4-NP: 4-nonylphenol; E2: 17β-estradiol (at the indicated nominal concentrations in microgram per gram body weight); MW: molecular weight protein standards; SDS-PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

two protein bands at ~210 KDa (wide) and 191 KDa (thin), but the mentioned bands were not observed in the plasma of untreated fish. These bands were also observed in E2-treated fish (Figure 1).

The induction of VTG in plasma was also investigated by the measurement of ALP. The plasma ALP levels exhibited a significant increase in the samples collected on day 7. The increase in ALP continued until the end of experiment in the second week. At this point it was distinguishable from the first week in the majority of 4-NP-treated groups at the same doses ($p < 0.05$; Figure 2(a)). The plasma ALP level did not significantly change during the first week of experiment with 10 μg g^{-1} 4-NP, while this level exhibited a moderate but significant increase with respect to the controls and solvent controls over the second week of experiment ($p < 0.05$). In all the treated groups, the total plasma calcium levels (another indirect indicator for VTG synthesis) significantly increased after 7 and 14 days (Figure 2(b)). A significant increase was also found in the groups treated with 4-NP after 14 days compared with those observed after 7 days of exposure to the same treatment ($p < 0.05$). The total plasma protein levels clearly elevated in response to 4-NP (except of 10 μg g^{-1}) after 7 and 14 days of treatment, compared with the controls and solvent controls (Figure 2(c)). The HSI showed a strong dose-dependent increase in response to 4-NP after 7 and 14 days of treatment (Table 1). The mentioned increase was more marked after 14 days of treatment. A significant increase in all above-mentioned parameters was also observed for single dose of E2, as seen for the highest dose of 4-NP.

As shown in Figure 3, a significant increase was observed in plasma levels of both ALT and AST after 7 and 14 days in the groups treated with 50, 100, 150 and 200 μg g^{-1} $week^{-1}$ doses ($p < 0.05$). However, the injection of 10 μg g^{-1} 4-NP had no significant effect on both plasma levels of ALT and AST, compared with the controls and solvent controls.

Discussion

The results showed that the injection of different doses of 4-NP caused a marked increase in plasma VTG concentration in immature male *A. latus*.

VTG is a female specific protein with a high molecular weight (300–600 kDa native and/or 160–200 kDa subunits), whose levels in male and immature fish are very low or nonmeasurable (Denslow et al., 1999). 4-NP treatment of immature male *A. latus* resulted in the induction of two major protein bands with molecular weight of approximately 210 and 191 KDa. A similar band induction was also observed in the plasma of E2-treated fish, while no band was detected in the plasma of controls and solvent controls. Hence, the protein bands isolated by SDS-PAGE in the present study were identified as VTG. The appearance of two different bands of VTG has already been reported in several fish species such as guppy (*Poecilia reticulata*, 191 and 156 KDa), zebrafish (*Danio rerio*, 193 and 134 KDa), white sturgeon (*Acipenser transmontanus*, 210 and 190 KDa) and paiche (*Arapaima gigas*, 184 and 112 KDa) (Chu-Koo et al., 2009; Li and Wang, 2005; Linares-Casenave et al., 2003; Van den Belt et al., 2003).

Typically, VTG is considered as a dimeric molecule, which consists of homologous monomers (Mosconi et al., 1998). However, it is possible that the two monomers are not identical by their molecular weights (Specker and Sullivan, 1994). This difference may have a genetic origin or be due to the post-tran slational modification of VTG (Watts et al., 2003). On the other hand, Nilsen et al. (2004) stated that the formation of different molecular weight bands could be the result of degradation of VTG during SDS-PAGE process. Since the published data concerning VTG structure in the *A. latus* are not available, we are not certain whether this is a normal structure of

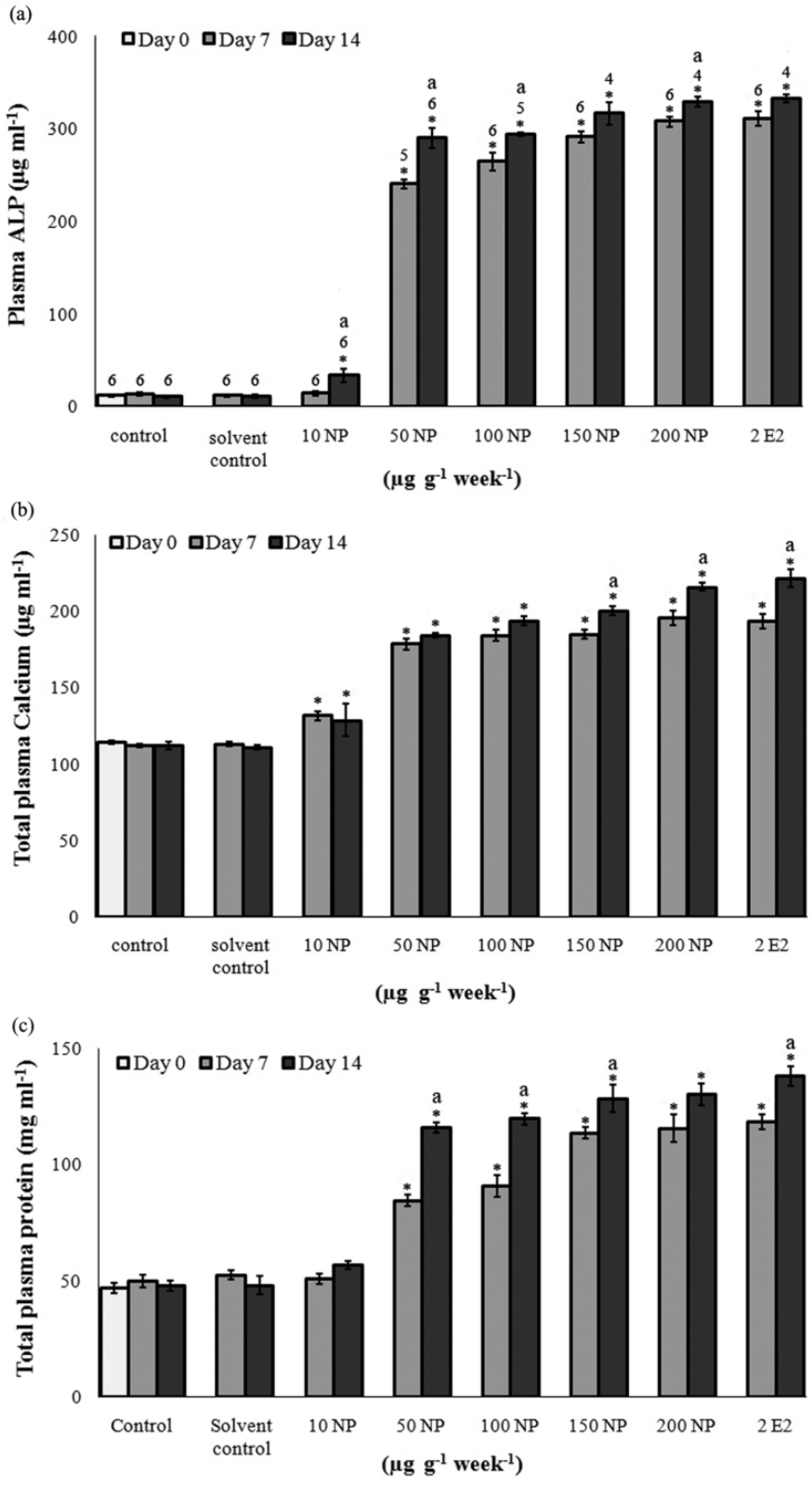

Figure 2. Effects of 4-NP and E2 on the concentrations of ALP (VTG) (a), total plasma calcium (b) and total plasma protein (c) in immature male *A. latus*. The fish were sampled on days 0, 7 and 14; number of sampled fish is indicated at the top of the columns (Figure 2(a)). *Significant difference from control ($p < 0.05$). [a]Significant difference from day 7 in the same concentration. E2: 17β-estradiol; ALP: alkali-labile phosphate; VTG: vitellogenin; 4-NP; 4-nonylphenol.

Table 1. Mean hepatosomatic index (mean ± standard error) of yellowfin seabream after 7 and 14 days of exposure to 4-NP and E2 via injection.

Concentration ($\mu g\ g^{-1}\ week^{-1}$)	Day 0	Day sampled	
		Day 7	Day 14
Control	0.58 ± 0.02	0.57 ± 0.02	0.58 ± 0.03
Solvent control		0.58 ± 0.02	0.57 ± 0.02
10 NP		0.74 ± 0.03	0.71 ± 0.04
50 NP		1.33 ± 0.06[a]	1.43 ± 0.11[a]
100 NP		1.32 ± 0.04[a]	1.97 ± 0.11[a,b]
150 NP		1.41 ± 0.09[a]	2.35 ± 0.12[a,b]
200 NP		1.68 ± 0.13[a]	2.94 ± 0.23[a,b]
2 E2		2.38 ± 0.08[a]	3.36 ± 0.25[a,b]

HSI: hepatosomatic index; 4-NP: 4-nonylphenol; E2: 17β-estradiol.
[a]Significant difference from control ($p < 0.05$).
[b]Significant difference from HSI at day 7 in the same concentration ($p < 0.05$).

VTG or its degradation products during sampling or sample preparation. Regardless of VTG degradation, two types of VTG have been reported in some teleosts (Fukada et al., 2003; Tyler et al., 1999; Watts et al., 2003). In addition, one band form and lower size of VTG subunit that have been reported in other sparid species, such as gilthead seabream (*Sparus aurata*, 180 kDa; Mosconi et al., 1998), common dentex (*Dentex dentex*, 170 kDa; Pavlidis et al., 2000), common seabream (*Pagrus pagrus*, 189 kDa; Kokokiris et al., 2001) and black bream (*Acanthopagrus butcheri*, 100–200 KDa; Codi King et al., 2008), might be due to structural differences of this protein even between closely related species.

VTG is a highly phosphorylated protein. Hence, its indirect quantification is achieved by the measurement of ALP, which has been widely used in different aquatic organisms like fish and bivalve mollusks (Gagnaire et al., 2009; Kramer et al., 1998; Ricciardi et al., 2008; Verslycke et al., 2002). ALP level in fish has shown a strong correlation with actual VTG levels measured using specific immunotechnique assays (Verslycke et al., 2002). In the present study, the plasma ALP levels were increased by 4-NP and E2 treatments, whereas no significant change was observed in untreated fish. This elevated levels of plasma ALP clearly indicates the VTG induction in 4-NP-treated fish. Similarly, Christensen et al. (1999) observed a significant increase in plasma levels of ALP in male flounders (*Platichthys flesus*) treated with different doses of 4-NP. Induction in plasma ALP concentration was also found in adult fathead minnows (*Pimephales promelas*) exposed to E2 through waterborne exposure (Kramer et al., 1998). Furthermore, E2 treatment of juvenile Eurasian perch (*Perca fluviatilis*) through diet resulted in a significant increase in plasma ALP levels with respect to controls during 85 days (Mandiki et al., 2005). A notable increase in plasma ALP levels has also been reported in the sand goby (*Pomatoschistus minutus*) exposed to 4-*tert*-octylphenol, another xenoestrogen compound (Robinson et al., 2004).

Previously, it was shown that the phosphorus content of VTG may vary (range from 1% to 1.7%) between different species (Babin and Vernier, 1989; Specker and Sullivan, 1994). Our results showed that the background level of ALP in the plasma of *A. latus* is naturally low (10.82 $\mu g\ ml^{-1}$). While induction of VTG synthesis was coinciding with a sharp increase in ALP levels in the plasma. This means that VTG in *A. latus* contains considerable amounts of phosphorus. Rapid increase in ALP in the plasma of exposed fish together with low background level in control groups makes it easy to distinguish between 4-NP exposed and nonexposed fish suggesting that ALP is an appropriate biomarker for endocrine disruption in *A. latus*.

The vitellogenic response to 4-NP was also verified by the change in total plasma calcium. VTG is a calcium-rich glycolipoprotein and the concentration of total plasma calcium is directly correlated to plasma VTG levels (Goksøyr, 2006). When VTG is synthesized, a great amount of calcium is attached to the molecule. By this way, the molecule becomes more soluble in blood (Follett and Redshaw, 1974). Previous investigations indicated that the treatment of fish by E2 results in a concomitant increase in plasma calcium and VTG concentrations (Gillespie

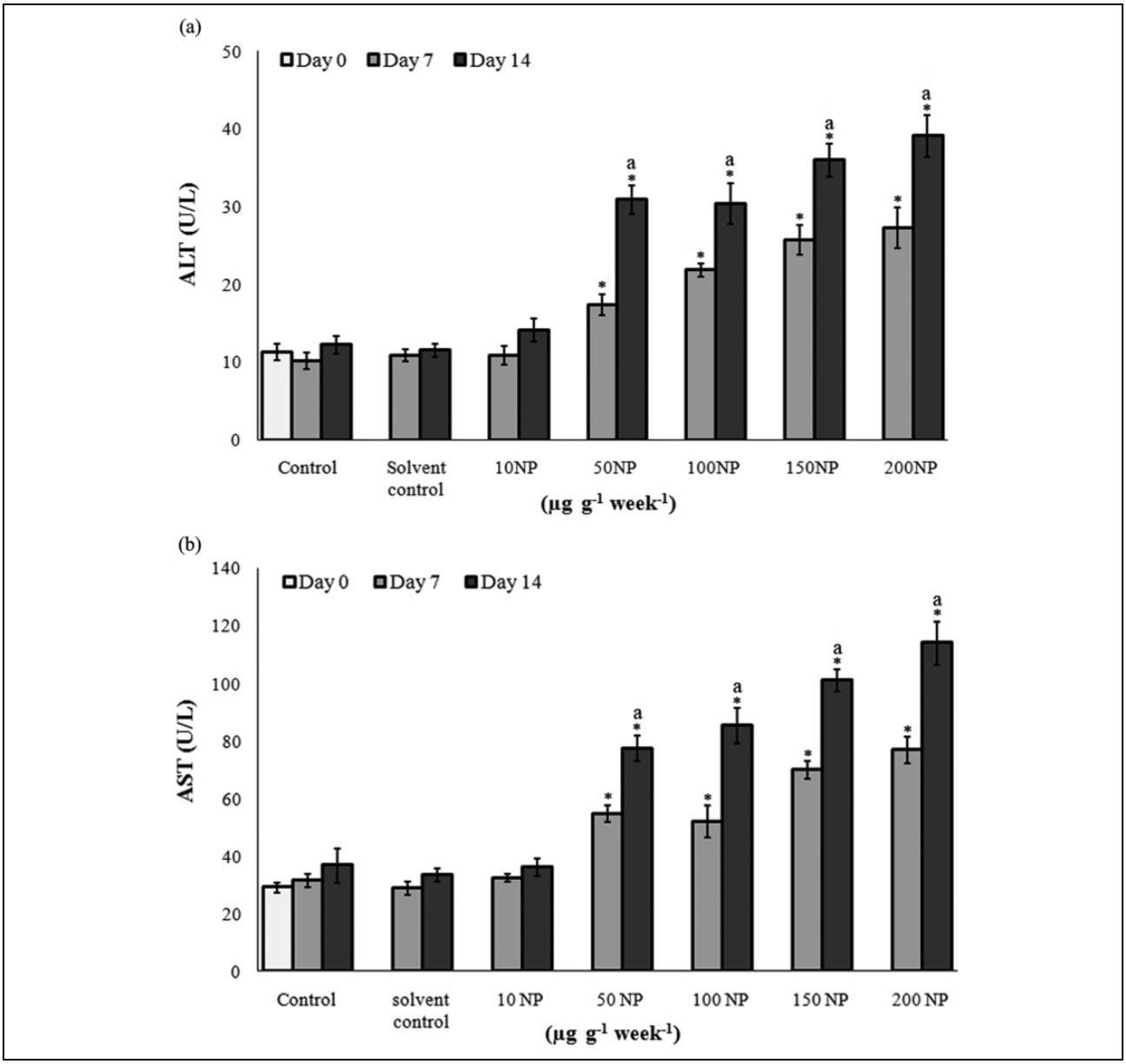

Figure 3. Effects of different doses of 4-NP on ALT (a) and AST (b) concentrations in the plasma of immature male *A. latus* after 7 and 14 days of treatment. Number of fish as in Figure 2(a). *Significant difference from control ($p < 0.05$). [a]Significant difference from day 7 in the same concentration. 4-NP: 4-nonylphenol; ALT: alanine aminotransferase; AST: aspartate aminotransferase.

and Peyster, 2004; Linares-Casenave et al., 2003; Madsen et al., 1997). Similarly, McCormick et al. (2005) found that the intraperitoneal administration of juvenile Atlantic salmon (*Salmo salar*) by 4-NP and E2 led to the induction of VTG and total calcium in the plasma. This was consistent with the results of Christiansen et al. (1998), who reported on sexually immature salmonids exposed to 4-NP. In the present study, the exposure of *A. latus* to different doses of 4-NP exhibited a stimulatory effect on calcium concentrations in the plasma of all the treated groups, while no alteration was observed in the controls and solvent controls. However, Gillespie and Peyster (2004) suggested that calcium level could be used as an indirect measure of VTG only if there is an obvious difference in plasma calcium (at least 55–70 mg l^{-1} in *P. promelas*) between the estrogen exposed and control fish. Such difference was also observed in the plasma of all the groups treated with 4-NP or E2 except for the group exposed to 10 µg g^{-1} of 4-NP.

The total plasma protein concentrations of 4-NP- and E2-treated fish significantly increased compared with the controls and solvent controls. This increased protein level can also be accounted for the increase in plasma VTG. This is in agreement with previous study in which injection of male flounders with different doses of 4-NP resulted in elevated total plasma protein concentrations (Christensen et al., 1999). In another work, Verslycke et al. (2002) observed the significant induction of plasma protein in rainbow trout (*Oncorhynchus mykiss*) following both waterborne exposure and intraperitoneal injection of 17α-ethinylestradiol (EE2). The induction of plasma protein was also reported in European eel (*Anguilla anguilla*) exposed to waterborne EE2 (Versonnen et al., 2004). Nevertheless, the protein induction (induced by 200 $\mu g\ g^{-1}$ of 4-NP) in *A. latus* was much stronger than EE2-treated *O. mykiss* and *A. anguilla* ($\sim$45 and 75 $mg\ ml^{-1}$, respectively), even though the estrogenic compound employed in those studies were more potential than NP (Folmar et al., 2002; Van den Belt et al., 2004). This could be related to higher potency of hepatic estrogen receptors (ERs) to bind the estrogenic ligands and induce the ER-mediated reporter gene which finally leads to VTG expression in *A. latus*.

A strong dose-dependent increase was also observed in HSI during the experimental period, which indicates the activation of hepatic VTG production in response to 4-NP or E2. This is consistent with previous studies, which have shown how exposure to NP resulted in an increase in HSI (Cardinali et al., 2004; Christensen et al., 1999; Ishibashi et al., 2006). On the other hand, in some species like killifish (*Fundulus heteroclitus*) and Chinese rare minnows (*Gobiocypris rarus*), no significant changes in the HSI have been observed following NP exposure (Pait and Nelson, 2003; Zha et al., 2008). Different results may be due to species differences, route of administration or employed doses of NP. However, our findings support the role of HSI as a simple and crude indicator of estrogen exposure, as previously suggested by Verslycke et al. (2002).

The normal structure and function of liver in fish make it the main target organ for various pollutants (Di Giulio and Hinton, 2008). ALT and AST are the two major aminotransferase enzymes, which have frequently been studied as potential biomarkers that reflect hepatocellular injuries (Bhattacharya et al., 2008; Kim et al., 2008; Vedel et al., 1998). ALT and AST levels in plasma are normally low. The increased levels of ALT and AST in plasma are always associated with liver damage, destruction of tissues or changes in cell membrane permeability, which would finally lead to leakage of the enzymes from cells into the plasma (Haschek et al., 2009; Schiff et al., 2007). AST enzyme naturally exists in a variety of tissues including liver, heart and skeletal muscles, kidney, brain, pancreas, lungs, leukocytes and erythrocytes. ALT is found in large amount in liver (Schiff et al., 2007). Hence, ALT is the more specific enzyme for hepatocellular injuries. Despite frequent studies about endocrine disrupting effects of NP, only few studies have focused on aquatic toxicity of this xenoestrogen (Bhattacharya et al., 2008). The results of present study clearly showed that the concentration of both ALT and AST in the plasma of 4-NP-treated fish has increased in a dose-dependent manner. However, the levels of ALT and AST in the plasma of controls and solvent controls as well as treated fish with the lowest dose of 4-NP did not change during the exposure period. Similarly, exposure of male flounder to different doses of 4-NP increased the plasma levels of ALT after 14 days of treatment (Christensen et al., 1999). Recently, the dual changes in the concentration of ALT and AST in the plasma of African catfish (*Clarias gariepinus*) have been reported by Senthil Kumaran et al. (2010). The enzyme levels were induced by the lower doses of NP while increasing concentration of NP caused a significant decline in plasma ALT and AST levels. It has been shown that NP exposure could lead to histopathological changes in liver by destruction of cell membranes and cell necrosis, which resulted in cytosolic leakage of aminotransferases into the blood stream (Bhattacharya et al., 2008). Therefore, it is suggested that the increased concentration of ALT and AST in the present study could be due to the changes in the membrane permeability of hepatocytes, liver tissue damage or injury to other tissues. Accordingly, it seems that the extent of damage is directly correlated with 4-NP concentration. ALT and AST are known to be involved in the catabolism of amino acids for energy production in animals in response to stress conditions (de Smet and Blust, 2001). Thereby, another possible reason for an increase in these enzymes in the plasma of 4-NP-treated *A. latus* could be to provide energy in order to cope with the stress condition.

As described above, a rapid and marked increase in different plasma indicators of VTG was observed only 7 days after treatment. These results revealed that *A. latus* is a highly estrogen sensitive species.

Although increased levels of protein, calcium and HSI are considered as the general response of the increased plasma VTG, their measurements can be useful to estimate the estrogenic response of a species to EDCs, along with the more specific methods. Furthermore, among the methods used, ALP was found to be the most relevant method for VTG assay in *A. latus*. This suggestion is clearly supported by emerged bands in the gel electrophoresis (Figure 1). In considering the fact that the histopathological studies on liver are time consuming and require expertise, our results clearly indicated that the measurement of ALT and AST in plasma could be an alternative indicator for the assessment of NP toxicity in *A. latus*. Interestingly, all the measured parameters of *A. latus* in this study showed little variation among the individual fish within the each group. This may be an advantage for the laboratory and field studies in order to avoid any excessive sampling replicates to detect changes and to increase their realism.

Conclusion

The result of present study showed that the exposure of immature male yellowfin seabream to 4-NP induced VTG synthesis in this species. Induction of VTG in the plasma of fish was directly detected by SDS-PAGE as well as determined indirectly by measuring ALP, total plasma calcium and total plasma protein. Among indirect methods, ALP showed a higher sensitivity in detecting changes in VTG levels in the 4-NP-treated fish, which was consistent with the obtained results from SDS-PAGE. The HSI values in this species had a rapid and strong increase in response to 4-NP or E2, which also indicated hepatic production of VTG. Moreover, the 4-NP treatment increased both plasma levels of ALT and AST that reflected the toxic effect of NP in *A. latus*. Measurement of plasma level of these enzymes associated with VTG biomarker provides a more comprehensive approach to evaluate biochemical, physiological and pathological alterations in the fish exposed to environmentally relevant xenoestrogens. Our overall results provide a number of hints that yellowfin seabream could serve as a useful biomonitor agent for estrogenic compounds in the Musa estuary.

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