4-Nonylphenol disrupts osmoregulation in the European sea-bass *Dicentrarchus labrax*

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Introduction

4-Nonylphenol (4-NP) is a synthetic organic chemical able to mimick the action of the natural female hormone 17β-estradiol (E2) by binding to the E2 receptor.[1] 4-NP is a degradation product of nonylphenols polyethoxylates (NPEs), which are widely used as surfactants.[2] It can be found in seawater at maximal concentrations of around one to four micrograms per liter.[3] The most contaminated marine sites are estuaries, lagoons and coastal areas close to sewage treatment plants or industrial waste discharges. Reproductive disorders associated with 4-NP exposure have been well described in fish[4,5] but less attention has been paid to other hormone-regulated functions such as osmoregulation. In the past decade, several studies highlighted that 4-NP may disrupt endocrine control of osmoregulation in salmonids, notably during the smoltification process.[6-9] To date, the endocrine disruption of osmoregulatory pathways has been poorly studied in teleosts other than salmonids.

The European sea-bass *Dicentrarchus labrax* is an euryhaline marine teleost commonly found along the coasts of the north-east Atlantic Ocean and the Mediterranean Sea. *D. labrax* migrates in lagoons and estuaries where salinity is generally lower and more variable than in the open sea and where the concentrations of anthropogenic pollutants such as 4-NP are supposed to be higher. The endocrine regulation of osmoregulation in *D. labrax* involves several hormones including pituitary hormones such as somatolactine (SL), prolactin (PRL) and growth hormone (GH)[10] as well as cortisol, a glucocorticoid released by inter-renal cells[11] and the insulin-like growth factor I (IGF-I), predominantly released from liver cells.[12] The GH/IGF-I axis is known to be involved in the seawater acclimation process of teleosts whereas prolactin promotes ion uptake in fresh water.[12] Cortisol has been shown to interact with both of these hormones and is believed to have a dual osmoregulatory function.[13] The goal of the present study was to investigate whether 4-NP disrupts hypo-osmoregulation and its hormonal control in *D. labrax* following aqueous exposure.

Materials and Methods

Juvenile sea-bass (60.8±6.5 g and 19.0±1.2 cm, mean±SD) were obtained from the culture system at the IFREMER station at Palavas (Hérault, France). After their transfer to the laboratory in Montpellier, the fish were acclimatized in 3500 L tanks filled with aerated and filtered natural seawater. For the experiments, the fish were kept in 200 L glass aquaria (temperature: 19°C; dissolved oxygen: 9 mg/L; salinity: 37‰; pH 7.0-8.0; photoperiod: 12 h light/12 h dark). Fish were not fed during the experiments. Ten fish per aquarium were exposed during 6 days at a daily nominal concentration of 100 μg/L of 4-NP (CAS 84852-15-13, Sigma-Aldrich Chemicals, MO, USA). 4-NP was dissolved in methanol, while the control group received the carrier solvent alone (0.002% methanol). The exposure was performed under static conditions and half of the water was changed every 48 h. Water was sampled 5 min, 4 h, 8 h, 12 h and 24 h following 4-NP addition and 4-NP measurements were carried out by SPME/GC/MS. 4-NP was removed from contaminated water with granular activated carbon as recommended.[14] For sampling, animals were anesthetized with 200 ppm of phenoxy-2-ethanol (Galaxy Surfactants Ltd., Mumbai, India). Blood was collected from the dorsal aorta using a 1 mL syringe coated with heparin (Li-heparin, Sigma-Aldrich, France) and blood osmolality was measured rapidly after collection using an Advanced 3300 micro-osmometer (Advanced Instruments Inc., MA, USA). Pituitary glands and gills were dissected on ice and immediately stored in Trizol reagent (Invitrogen, France) at –80°C for molecular biology analyses. Animal manipulation and experiments were performed according to the recommendations of the European Union directive (2010/63/EU) and of the French law (decree 87/848) regulating animal experimentation.

Total RNA was extracted using Trizol reagent according to the manufacturer's instructions and 500 ng of the total RNA was treated with RNase-free DNase (Invitrogen, France). Quantification of total RNA was performed with a NanoDrop® ND-1000 V3300 Spectrophotometer (Nanodrop Technology Inc., USA) and the RNA integrity was checked by electrophoresis. Reverse transcription was performed using M-MLV reverse transcriptase and random primers (Invitrogen, France). Quantitative real-time PCR (qRT-PCR) analyses were performed with a Light-Cycler system (Roche, Mannheim, Germany), using 2.5 μL of the LightCycler-FastStart DNA Master SYBRGreen Mix (Roche), 0.75 μL of each primer (reverse and forward at 0.5 μM, Table 1) and 2 μL of cDNA. The results were normalized with the ribosomal protein L13a, a housekeeping gene already validated in the sea-bass.[15]

The data were expressed as the mean±standard deviation. Differences between control and groups of fish exposed to 4-NP were evaluated by t-test ($P \leq 0.05$). The calculations were performed using Statgraphics Centurion XV software (StatPoint, Inc., http://www.statgraphics.com/).

Results and Discussion

Alkylphenols such as 4-nonylphenol (4-NP) are one of the wide variety of environmental chemicals reported to have estrogenic effects, however little work has been done on their effect on osmoregulation in euryhaline teleosts, other than in salmonids. In the current study, a nominal concentration of 100 μg/L was used. Chemical analysis of water revealed that this nominal concentration corresponds to a mean 4-NP concentration of 40±20 μg/L. This concentration corresponds to 10 times the highest concentrations recorded in marine environments (4 μg/L along the northern Mediterranean coasts)[3]. At these concentrations, no mortality occurred during the treatment period. 4-NP exposure did not affect mean blood osmolalities compared to controls (367±11 mOsm/kg *vs* 358±9 mOsm/kg, n=10),

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but a difference in the distribution of osmolality values was observed (Figure 1). Blood osmolalities of controls range from 342 to 369 mOsm/kg with the majority of animals (50%) in the 360-370 mOsm/kg osmolality range. Blood osmolalities of the exposed animals range from 350-381 mOsm/kg with 40% of the animals having osmolalities over those recorded in controls (>370 mOsm/kg). This tendency of an increased blood osmolality after 6 days of aqueous 4-NP exposure suggests a reduced capacity to tightly regulate ion fluxes in seawater (SW). A hypo-osmoregulatory failure has also been noticed in the gilthead sea-bream (*Sparus aurata*) injected with 200 µg 4-NP/g body mass where increased blood osmolalities and a reduction in the renal Na^+/K^+-ATPase activity where measured after 10 days.[16] In the Atlantic salmon *Salmo salar*, 4-NP has been shown to affect smoltification through decreased branchial Na^+/K^+-ATPase activities and Na^+/K^+-ATPase α-subunit mRNA levels as well as a delayed downstream migration.[17] In this species, 4-NP seems also to alter hyper-osmoregulation.[18] In the present study, contrary to the Atlantic salmon mentioned above, branchial Na^+/K^+-ATPase α-subunit mRNA levels are significantly increased in 4-NP exposed fish (Figure 2). This is consistent with a hypo-osmoregulatory failure since the sea-bass, contrary to salmonids, has significantly higher branchial Na^+/K^+-ATPase α-subunit mRNA and Na^+/K^+-ATPase activities in fresh water (FW) compared to SW.[19,20]

Osmoregulation is an endocrine-driven function, mediated, in SW-acclimated teleosts, by the growth hormone/insulin-like growth factor I (GH/IGFI) axis and by cortisol that has a dual osmoregulatory function in several teleosts.[13] In the sea-bass, the pituitary somatolactin mRNA is significantly higher expressed in SW than in FW suggesting an involvement in hypo-osmoregulation however the exact underlining mechanisms are not known.[10] In order to determine the endocrine pathways by which 4-NP affects hypo-osmoregulation in the sea-bass, we measured the effect of 4-NP on the mRNA expression of two pituitary hormones thought to be involved in the sea-bass hypo-osmoregulation, GH and SL (Figure 2).

Pituitary GH is significantly decreased in the exposed animals which might contribute to their hypo-osmoregulatory failure (Figure 2). In the Atlantic salmon, plasma IGF-I levels were decreased in presence of 4-NP.[7] As GH has been shown to be the major secretagogue of hepatic IGF-I, the IGF-I levels in sea-bass might also be altered. Moreover, Hanson *et al*.[9] showed significantly decreased liver and gill growth hormone receptor (GHR2) and putative somatolactin receptor (GHR1) expressions in 4-NP- exposed (at 100 µg/L) rainbow trouts (*Oncorhynchus mykiss*). At the gill level and other osmoregulatory organs, 4-NP could also have a direct effect that needs to be investigated in future studies.

Although several physiological functions like reproduction, stress, Ca^{2+} regulation and acid-balance have been established for the pituitary somatolactin (SL) in teleosts, its involvement in hypo-osmoregulation is still not clear.[21] SL belongs to the growth hormone/prolactin family and some studies suggest that SL may exerts its effects by the way of the GH/IGF-1 axis and is involved in IGF-I regulation.[22] Its pituitary expression might also be affected by estrogenic compounds. In the sea-bass however, aqueous 4-NP exposure does not significantly affect SL mRNA expression even if there is a slight tendency of decrease (Figure 2). Further studies are required analyzing circulating plasma levels or investigating the SL putative receptor (GHR1) in order to determine if the SL effects are affected by 4-NP.

Even if the mechanisms through which 4-NP affects the GH/IGF-1 axis are still not clear, these data and those in the sea-bass suggest that the alteration of the GH/IGF-1 axis by this compound may be a common phenomenon among teleosts. The slightly higher blood osmolalities combined to decreased GH mRNA

Table 1. Primer sequences used in this study.

Gene	Accession #	Primer	Nucleotide sequences (from 5' to 3')
Somatolactin	AJ277390	SL-F SL-R	CATCACCAAAGCCTTACCC GGCACATCATACTGGAATAGGC
Growth hormone	GQ918491.1	GH-F GH-R	CACAACCTCCACCTGCTCG GCGTTGTGTCTCGTGCTTGTC
Na^+/K^+ ATPase-α1	AM419034	NKAα1-F NKAα1-R	AGAGGGATGTTGGCGATGAT CTGCTGGACGACAACTTTGC
L13a	DT044539	L13a-F L13a-R	TCTGGAGGACTGTCAGGGGCATGC AGACGCACAATCTTGAGAGCAG

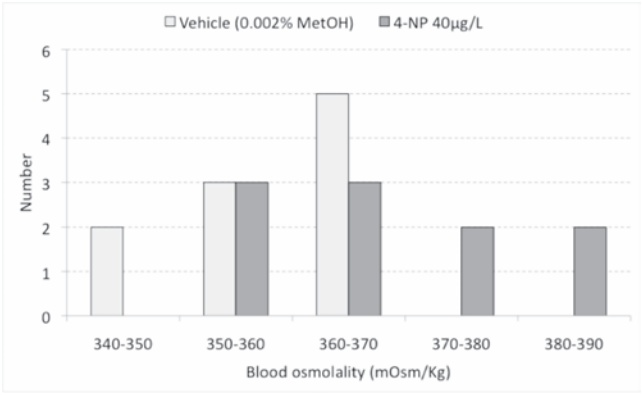

Figure 1. Blood osmolalities in *D. labrax* exposed to 40±20 µg/L of 4-NP or vehicle only (0.002% MetOH).

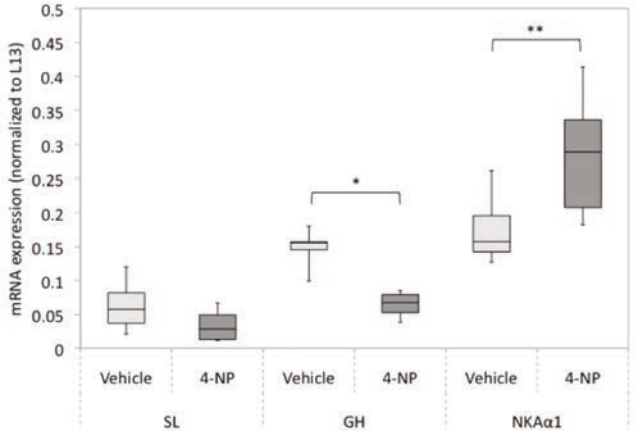

Figure 2. mRNA expression of somatolactin (SL) and growth hormone (GH) in the pituitary gland and Na^+/K^+ ATPase alpha subunit (NKAα1) in the gills of *D. labrax* exposed to 40±20 µg/L of 4-NP or vehicle only (0.002% MetOH). Data are normalized to the ribosomal RNA L13a and expressed as mean±SD (n=5 for pituitary gland and n=10 for gills). *P<0.05; **P<0.01 (t-test).

expression levels show a hypo-osmoregulatory failure in the sea-bass. McCormick *et al.*[7] suggested that estrogenic compounds may cause a general shift toward increased capacity for ion uptake and a shift away from ion secretory mechanisms which has to be further investigated in sea-bass. Further research with other estrogenic ligands (natural or synthetic) should help determining the contribution of estrogen signaling pathways towards ionic regulation disruption in fish.

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