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Dietary intake of 17α-ethinylestradiol promotes leukocytes infiltration in the gonad of the hermaphrodite gilthead seabream

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ABSTRACT

A wide variety of chemicals discharged from industrial and municipal sources have been reported to disrupt the endocrine system of animals, which may be exposed via the food chain and contaminated water. 17α-Ethinylestradiol (EE_2), a drug used in oral contraceptives and hormone replacement therapy, has a widespread presence in the aquatic environment. Current knowledge on the sensitivity of marine fish to estrogenic environmental chemicals is limited. We report here the effects of dietary intake of EE_2 on gilthead seabream, a marine hermaphrodite teleost, focusing on the immune events that take place in the gonad. When seabream males were fed with 5, 50, 125 and 200 μg EE_2/g food for 7, 14, 21 and 28 days an infiltration of acidophilic granulocytes and B lymphocytes occurred in the testis as the same time that spermatogenesis is disrupted. Moreover, the dietary intake of EE_2 promoted a dose-dependent up-regulation of the expression of genes coding for cytokines, chemokines and adhesion molecules correlated with a leukocyte infiltration.

1. Introduction

A wide variety of chemicals discharged from industrial and municipal sources have been reported to disrupt the endocrine system of animals, which may be exposed via the food chain and contaminated water (Jones et al., 2004). Endocrine disrupting chemicals (EDCs) mimicking or antagonizing the action of hormones are a particular cause for concern. Recent evidence indicates that endocrine disruption as a consequence of estrogen exposure may result, on occasions, in the near collapse of wild fish populations (Kidd et al., 2007). As global consumption of pharmaceuticals rises, an inevitable consequence is an increased level of contamination of surface and ground waters with these biologically active drugs, and thus in turn a greater potential for adverse effects in aquatic wildlife (Corcoran et al., 2010). 17α-Ethinylestradiol (EE_2), a pharmaceutical compound used for oral contraceptives and hormone replacement therapy, has a widespread presence in the aquatic environment (Ternes et al., 1999), where it reaches concentrations of 0.5–62 ng/l in European sewage and superficial waters (Hinteman et al., 2006; Johnson et al., 2005; Kuch and Ballschmiter, 2000). Several fish species have been bath-exposed to environmental concentrations of EE_2 to assess any effects on reproduction (Hashimoto et al., 2009; Hogan et al., 2010; Kaptaner and Unal, 2010; Lai et al., 2002a; Lange et al., 2008; Marlatt et al., 2010; Peters et al., 2007; Xu et al., 2008). However, little attention has been paid to other physiological processes, such as immune responses that are known to be affected by estrogens (Lai et al., 2002a). Moreover, the resistance of EE_2 to degradation, which could support its bioaccumulation throughout the food chain as it has been predicted by some food-web models (Lai et al., 2002b), should not be underestimated and, for instance, the determination of the impact of the intake of low levels of EE_2 with the food on fish reproduction but also on other physiological processes such as immune responses is mandatory.

The gilthead seabream (*Sparus aurata* L.) is a seasonally breeding, marine, protandrous hermaphrodite teleost with a bisexual

Abbreviations: EDCs, endocrine disrupting chemicals; EE_2, 17α-ethinylestradiol; AG, acidophilic granulocytes; E_2, 17β-estradiol; Mc, macrophages; Ly, lymphocytes; ERs, estrogen receptors; GSI, gonadosomatic index; MG, gonad mass; MB, body mass; T, testosterone; 11KT, 11-ketotestosterone; mAb, monoclonal antibody; TNFα, tumor necrosis factor α; CCL4, CC chemokine ligand 4; IL8, CXC chemokine interleukin 8; SELE, leukocyte adhesion molecule E-selectin; IgM, heavy chain of immunoglobulin M; IgT, heavy chain of immunoglobulin T; *rsp18*, ribosomal protein S18 gene; IgM, immunoglobulin M; PCNA, proliferating cell nuclear antigen; MCSFR, macrophage colony stimulating factor receptor; IgM+, IgM-positive cells; IgT+, IgT-positive cells.

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gonad, which, during the male phase, has a functional testicular area and a non-functional ovarian area. The testis undergoes abrupt morphological changes especially after spawning, including a massive infiltration of acidophilic granulocytes (AG) (Chaves-Pozo et al., 2003, 2005a; Liarte et al., 2007). These immune cells are produced in the head–kidney, the main haematopoietic organ in fish, but, when they infiltrate the testis, they show heavily impaired functions (Chaves-Pozo et al., 2005b). Interestingly, it is the gonad itself which actively regulates the presence of these immune cells in the testis by stimulating their extravasations from the blood (Chaves-Pozo et al., 2005b, 2008a). Endogenous increases of 17β-estradiol (E_2) in serum are correlated with AG migration into the gonad after spawning (Chaves-Pozo et al., 2008b, 2010), while exogenous E_2 accelerates the final events of spermatogenesis, inhibits the proliferation of spermatogonia in early stages, and induces AG infiltration (Chaves-Pozo et al., 2007). However, AG does not express any of the three estrogen receptors (ERs) (ERa, ERb1, ERb2) known in gilthead seabream (Liarte et al., 2011; Pinto et al., 2006). Interestingly, macrophages (Mc) and lymphocytes (Ly) are always present in the interstitial tissue of the gilthead seabream gonad (Liarte et al., 2007) and express ERa (Liarte et al., 2011). Moreover, Mc are known to be a key cell type in the immune-modulatory role played by E_2 in the gilthead seabream gonad (Chaves-Pozo et al., 2010; Liarte et al., 2011).

In the present study, gilthead seabream were fed for 7, 14, 21 and 28 days with pellet diet containing EE_2 ranging from an environmental concentration (5 μg/g EE_2/g food) to non-environmental ones (50, 125 and 200 μg/g EE_2/g food) in order to asses the potential risks to wild gilthead seabream populations focusing on the local immune regulation of the spermatogenesis.

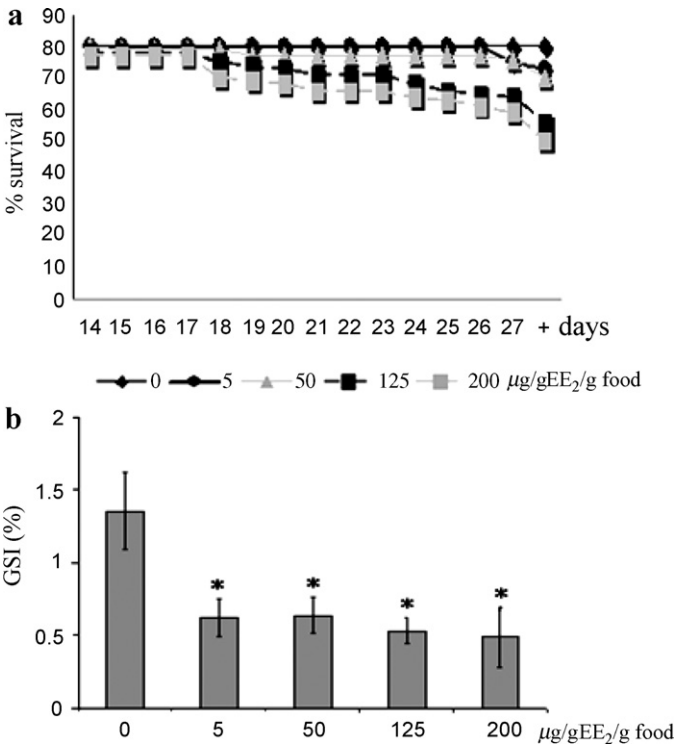

Fig. 1. Effects of EE_2 dietary intake on fish survival (a) and gonadosomatic index (b) after 28 days of 0, 5, 50, 125 and 200 μg EE_2/g food. Asterisks denote statistically significant differences compared with control, determined by ANOVA and Tukey post hoc test ($P < 0.05$).

2. Materials and methods

2.1. Animals and experimental design

Healthy specimens of gilthead seabream (*Sparus aurata* L., Actinopterygii, Perciformes, Sparidae) were bred and kept at the Centro Oceanográfico de Murcia (IEO, Mazarrón, Murcia). The experiment was conducted to test the effects of the dietary intake of 5, 50, 125 and 200 μg EE_2 (purity 98%; Sigma)/g food for 0, 7, 14, 21 and 28 days.

The experiment was performed using mature gilthead seabream males ($n = 400$) at the spermatogenesis stage with a body weight of 320 g kept in 2 m^3 tanks with the water temperature ranging from 14.6 to 17.8 °C. Specimens were kept with a flow-through circuit, a suitable aeration and filtration system and natural photoperiod. The environmental parameters, mortality and food intake was recorded daily. The EE_2 was incorporated in the commercial food (44% protein, 22% lipids, Skretting, Spain) at doses of 0, 5, 50, 125 and 200 μg/g food, using the ethanol evaporation method (0.3l ethanol/kg of food) as described elsewhere (Shved et al., 2007). The specimens were fed three times a day *ad libitum* and fasted for 24 h before sampling. Specimens ($n = 6$ fish/group and time) were anesthetized with 40 ppm of clove oil. Afterwards decapitated, weighed, and the gonads were removed and weighed, while serum samples from trunk blood were obtained by centrifugation and immediately frozen and stored at −80 °C until use. The gonads were processed for light microscopy and gene analysis as described below. The experiments described comply with the Guidelines of the European Union Council (86/609/EU), the Bioethical Committee of the University of Murcia (Spain) and the Instituto Español de Oceanografía (Spain) for the use of laboratory animals.

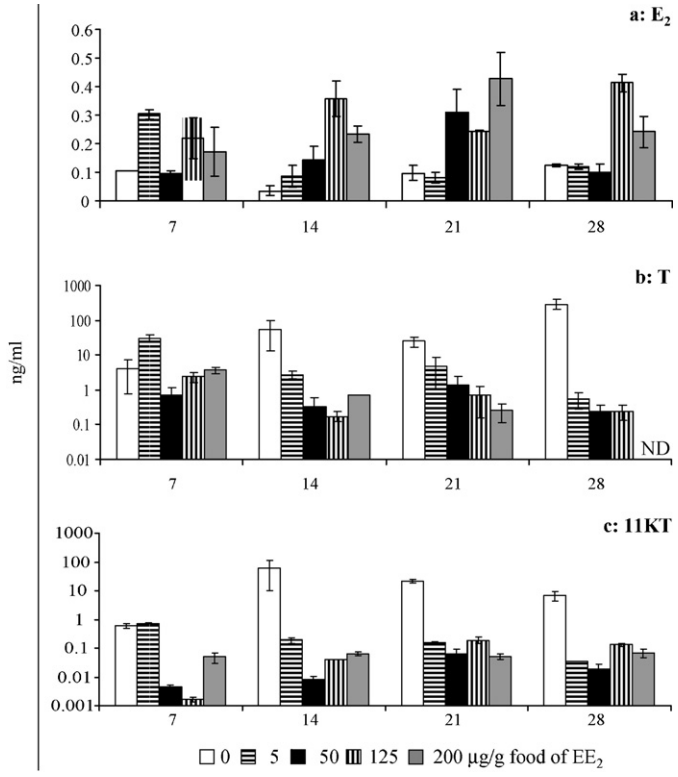

Fig. 2. Effects of EE_2 dietary intake on 17β-estradiol (E_2) (a), testosterone (T) (b), and 11-ketotestosterone (11KT) (c) serum levels. Data were obtained from a pool of serum obtained after mixing the same amount of serum from 6 fish/group and represent the means ± S.E.M. of duplicate samples. ND: non-detected.