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Low-dose exposure to alkylphenols adversely affects the sexual development of Atlantic cod (*Gadus morhua*): Acceleration of the onset of puberty and delayed seasonal gonad development in mature female cod

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ABSTRACT

Produced water (PW), a by-product of the oil-production process, contains large amount of alkylphenols (APs) and other harmful oil compounds. In the last 20 years, there have been increasing concerns regarding the environmental impact of large increases in the amounts of PW released into the North Sea. We have previously shown that low levels of APs can induce disruption of the endocrine and reproductive systems of Atlantic cod (*Gadus morhua*). The aims of this follow-up study were to: (i) identify the lowest observable effect concentration of APs; (ii) study the effects of exposure to real PW, obtained from a North Sea oil-production platform; and (iii) study the biological mechanism of endocrine disruption in female cod. Fish were fed with feed paste containing several concentrations of four different APs (4-*tert*-butylphenol, 4-*n*-pentylphenol, 4-*n*-hexylphenol and 4-*n*-heptylphenol) or real PW for 20 weeks throughout the normal period of vitellogenesis in Atlantic cod from October to January. Male and female cod, exposed to AP and PW, were compared to unexposed fish and to fish fed paste containing 17β-oestradiol (E_2). Approximately 60% of the females and 96% of the males in the unexposed groups were mature at the end of the experiment. Our results show that exposure to APs and E_2 have different effects depending on the developmental stage of the fish. We observed that juvenile females are advanced into puberty and maturation, while gonad development was delayed in both maturing females and males. The AP-exposed groups contained increased numbers of mature females, and significant differences between the untreated group and the AP-treated groups were seen down to a dose of 4 μg AP/kg body weight. In the high-dose AP and the E_2 exposed groups, all females matured and no juveniles were seen. These results suggest that AP-exposure can affect the timing of the onset of puberty in fish even at extremely low concentrations. Importantly, similar effects were not seen in the fish that were exposed to real PW.
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1. Introduction

Offshore oil production platforms discharge vast quantities of produced water (PW) into the sea. PW is a by-product of current oil-production technology, and consists of seawater containing an extremely complex mixture of dispersed oil, polycyclic aromatic hydrocarbons (PAHs), alkylphenols (APs), organic acids, metals, and traces of production chemicals (Neff, 2002; Durell et al., 2006). The release of increasing amounts of APs and other potential endocrine disrupting chemicals (EDCs) into the sea have led to speculation that these compounds may induce endocrine disruption in marine fish (Thomas et al., 2004a, 2009; Scott et al., 2006b; Boitsov et al., 2007; Tollefsen et al., 2007).

APs have been shown to bind to the oestrogen receptor, mimicking the effects of the natural female sex hormone oestrogen and disrupting the endocrine and reproductive systems (Nimrod and Benson, 1996; Servos, 1999; Tollefsen and Nilsen, 2008). APs affect a number of reproductive parameters in fish, including gonadal development (Meier et al., 2007), induction of plasma vitellogenin (Vtg) in male and juvenile fish (Jobling and Sumpter, 1993; White et al., 1994), inhibition of spermatogenesis (Jobling and Sumpter, 1993; Gimeno et al., 1998; Miles-Richardson et al., 1999; Weber et al., 2002), and oogenesis (Tanaka and Grizzle, 2002; Weber et al., 2003).

Normal development of the reproductive system is controlled by the hypothalamic–pituitary–gonadal (HPG) axis. At puberty, activation of the HPG system, by neurotransmitters secreted from the

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hypothalamus, trigger development of secondary sexual characteristics which ultimately lead to the capacity to sexually reproduce (Zohar et al., 2010; Taranger et al., 2010). The recently discovered neuropeptide, kisspeptin, has been shown to be crucially important for the triggering of puberty and for future fertility in mammals (e.g. Oakley et al., 2009), and similar functions have recently been suggested for telosts (Nocillado et al., 2007; Filby et al., 2008; Martinez-Chavez et al., 2008; Kitahashi et al., 2009). Kisspeptin is released by specific neurons in the hypothalamus, where it binds to its receptor, G-protein-coupled-receptor 54 (GPR54) on the surface of gonadotropin-releasing neurons. These neurons secrete gonadotropin-releasing hormone (GnRH) which acts on the pituitary, stimulating the release of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), which act on the ovaries and testes, triggering gonad maturation, gametogenesis, and steroidogenesis. The HPG axis has been shown to be extremely sensitive to the effects of endogenous steroids, especially during fetal and perinatal life (Tena-Sempere, 2010). Thus, it has been proposed that reproductive dysfunction later in life may be caused by exposure to EDCs and other chemicals during development of the HPG axis. Interestingly, APs can affect the HPG system via induction/inhibition of gonadotropins (Harris et al., 2001; Yadetie and Male, 2002; Maeng et al., 2005; Vetillard and Bailhache, 2006; Rhee et al., 2008, 2009) and can also directly affect steroidogenesis (Yokota et al., 2005; Arukwe, 2005; Kortner et al., 2009b).

It has been suggested that early-life exposure to EDCs from environmental pollutants may be a possible cause of the earlier onset of puberty in girls, recently reported in studies from both the USA, Europe and Asia (Herman-Giddens, 2007; Golub et al., 2008; Aksglaede et al., 2009; Chen et al., 2009; Mouritsen et al., 2010; Wang et al., 2005). Recently, it has been suggested that EDCs may act on the kisspeptin/GPR54 system, and that this may explain the observed effects on the timing of puberty (Navarro et al., 2009; Tena-Sempere, 2010). Bellingham et al. have shown that fetuses from sheep grazed on sewage-treated pastures had reduced kisspeptin mRNA expression in the hypothalamus, and had fewer kisspeptin positive cells in the pituitary. This important study clearly demonstrated that the developing fetus is sensitive to environmentally relevant concentrations of EDCs, and that exposure to these chemical substances can cause alterations in kisspeptin/GPR54 expression levels that may have serious consequences for the future development of the reproductive system (Bellingham et al., 2009). It has been known for many years that nonylphenol (NP) can influence timing of puberty and the development of secondary sexual characteristics in rats (Chapin et al., 1999; Laws et al., 2000; Nagao et al., 2001). A pubertal onset assay, which measures the timing of vaginal opening in female rats, has been suggested to be one of the most sensitive bioassays for the detection of oestrogenic EDCs in rodents (Kim et al., 2002, 2005; Willoughby et al., 2005).

Previous studies from our laboratory have shown that oral administration of a mixture of four APs (4-*tert*-butylphenol (4-*tert*-BP), 4-*n*-pentylphenol (4-*n*-PP), 4-*n*-hexylphenol (4-*n*-HP) and 4-*n*-heptylphenol (4-*n*-HEPP)) to cod, disrupted the endocrine system resulting in an induction of Vtg in male cod, a decrease in the levels of sex steroids in the blood of both females and males, and a delay in ovary and testis development (Meier, 2007; Meier et al., 2007). In these previous studies we detected effects even at the lowest AP dose used in the experiment, which corresponded to a nominal body burden of 20 μg/kg. The purpose of the present study was to examine the biological effects of even lower doses of APs and real PW. The study was designed to further investigate the mechanisms behind the observed endocrine disruption in female cod, including the potential effects on age at onset of puberty and the delay in gonad development of maturing females. We have therefore studied the mRNA expression levels of pituitary gonadotropin genes, the mRNA expression and activity levels of aromatase in the ovary, and the plasma levels of sex steroids, 17β-oestradiol (E_2) and testosterone (T) in female cod. In addition, testis development and sperm quality were studied in male cod.

2. Materials and methods

2.1. Chemicals and PW

The APs used in the exposure experiment; 4-*tert*-BP, 4-*n*-PP, 4-*n*-HP and 4-*n*-HEPP, and 17-β-oestradiol (E_2) were purchased from Sigma–Aldrich (Oslo, Norway). Equal proportions of each of these four APs (subsequently referred to as the AP mixture) were combined with fish feed-paste as described in Section 2.3. The following deuterium-labelled APs from Chiron (Trondheim, Norway) or C/D/N Isotopes (USA) were used as internal standards in the GC–MS analysis: phenol (d5), p-cresol (d8), 2,4-dimethylphenol (d3), 4-ethylphenol (d9), 4-*n*-propylphenol (d12) and 4-*n*-nonylphenol (d5). [1β-3H]-4-androstene-3,17-dione (15–30 Ci/mmol; 1 mCi/ml) and Ultima Gold liquid scintillation cocktail was from Perkin Elmer Life Sciences (Boston MA, USA). Unlabelled 4-androstene-3,17-dione was from Fluka Chemie GmbH (Buchs, Switzerland). Trichloroacetic acid (TCA) and dithioerythritol (DTE) was obtained from Merck (Darmstadt, Germany). Nicotinamide adenine dinucleotidephosphate reduced form tetrasodium salt (NADPH) and activated charcoal (NORIT CA1, 100–400 mesh) were obtained from Sigma Chemical Co. (St. Louis, MO, USA), and Dextran 70 was obtained from Amersham Biosciences (Uppsala, Sweden).

In 2004, real PW was obtained from the Oseberg C oil production platform (StatoilHydro, Norway) located in the North Sea off the west coast of Norway (Sundt et al., 2009b). PW was collected in 5 L glass containers from the last sampling point before discharge, degassed for 5 min to remove hydrogen sulphide, and immediately transported to land by boat. The PW was transferred to 100 ml bottles, frozen and stored at −20 °C until use in the fish exposure studies.

2.2. Fish

Atlantic cod (mean weight 0.586 ± 0.080 kg), expected to spawn for the first time in the following season, were used in this experiment. The fish came from a strain of Norwegian coastal cod produced at the Institute of Marine Research (IMR), Bergen, Norway. Normally, farmed cod mature significantly earlier than wild cod (Svåsand et al., 1996). This is partly due to the intensive feeding in aquaculture which results in very high energy stores (high condition factor and large liver). For this study we tried to produce farmed cod that better resemble the wild population by restricting feeding throughout their lifetime. The fish were divided into nine groups of approximately 55 fish each, and transferred to 20 m^3 indoor tanks (in July 2004) prior to the start of the AP exposure experiment (Kjesbu et al., 1996). At the start of the experiment (in October 2004), when the fish were 20 months old, it was still not possible to tell the sex of the cod. However, since it is known that male cod are generally slightly smaller than females, we used this information to try and ensure that each experimental group consisted of a male:female ratio as close to 1:1 as possible by selecting fish from across the full size-range of the population. Each individual fish was weighed and tagged with internal *Trovan* electronic tags. Fish were then divided into experimental groups such that the size distribution (from 400 to 800 g) in each group was the same. During the experiment, the (artificial) light was controlled in day-length to follow the natural diurnal rhythm for Bergen (64°N:

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