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Studies of Uptake, Elimination, and Late Effects in Atlantic Salmon (*Salmo salar*) Dietary Exposed to Di-2-Ethylhexyl Phthalate (DEHP) During Early Life

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Abstract. The phthalate esters are a group of industrial chemicals considered to have endocrine-disrupting properties. The most common tonnage product among these, di-2-ethylhexyl phthalate (DEHP), is widely spread in the environment. The objectives with the present work were to study uptake and metabolism of orally administered DEHP and its major metabolite mono-2-ethyl hexyl phthalate (MEHP) and to evaluate the impact of early life exposure on sex differentiation in Atlantic salmon. The feeding with contaminated diet started immediately after yolk sac resorption and continued for 4 weeks. Nominal concentrations of DEHP in the diet were 400 (measured 359), 800 (measured 827), and 1500 (measured 1648) mg DEHP/kg and a control group was fed food mixed with solvent. After the exposure period, fish were fed non-contaminated diet until final sampling 4 months post-exposure. There were no effects on growth or survival of the fish and no late effects on hepatosomatic index or sex ratio. However, the histological examination of gonads from fish exposed to 1500 mg DEHP/kg revealed a small but significant incidence (3%) of intersex fish (ovo-testis). Chemical residues of DEHP and MEHP were analyzed weekly during the first 3 months of the post-exposure period. Both DEHP and MEHP were rapidly eliminated to near background levels within one week post exposure. The study indicates that exposure of Atlantic salmon to relatively high concentrations of DEHP during a sensitive part of the life cycle may interfere with gonad differentiation.

Environmental pollutants that can interfere with endocrine functions in animals have raised concern during the last decades. These so-called endocrine-disrupting chemicals include compounds with the ability to mimic natural hormones, block the action of natural hormones, or interfere with the synthesis and/or degradation of natural hormones (Soto *et al.* 1995). A group of widely used industrial chemicals considered to have endocrine-disrupting properties are the phthalate esters. These chemicals mainly serve as important additives in polyvinylchloride (PVC) resins and are used as plasticizers in various products, *e.g.*, industrial hardware, food packaging, medical products, and toys (Staples *et al.* 1997; Balafas *et al.* 1999; Wahl *et al.* 1999; Earls *et al.* 2003). Since the phthalate esters are not chemically bound to the plastic material, they can leach into the environment (Page and Lacroix 1995). The most common tonnage product among the phthalate esters, di-2-ethylhexyl phthalate (DEHP), has been found in both fresh and marine water environments (Giam *et al.* 1975; Tan 1995) as well as in food (Page and Lacroix 1995) and considerable concern has been focused on its potential risk to human and wild life health.

DEHP, via its metabolite mono-2-ethyl hexyl phthalate (MEHP), has been shown to be a peroxisome proliferator (PP) by activating peroxisome proliferator-activated receptors in rodents (PPARs) (*e.g.*, Moody and Reddy 1978; Lapinskas *et al.* 2005) and long-term exposure of rodents to DEHP has resulted in development of hepatocarcinomas (Reddy and Lalwani 1983). In fish, peroxisome proliferation has been reported after exposure to phthalates (*i.e.*, dibutylphthalate) (Ortiz-Zarragoitia and Cajaraville 2000) and PPARs with similar sequence and distribution as in mammals have recently been found in a few fish species, including Atlantic salmon (*Salmo salar*) (Ruyter *et al.* 1997; Andersen *et al.* 2000). Effects of DEHP on reproduction and development have been shown in several vertebrates but the mechanism of action remains unclear. The estrogenic activity of DEHP has been tested in different *in vitro* assays showing weak or no affinity to the estrogen receptor (ER) at high concentrations (Harris *et al.* 1997; Blom *et al.* 1998; Zacharewski *et al.* 1998; Metcalfe *et al.* 2001). In rat, maternal exposure to DEHP resulted in lowered testosterone levels and reproductive tract malformations in androgen-dependent tissues in male offspring (Gray *et al.* 1999; Parks *et al.* 2000). These anti-androgenic effects appear to be

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mediated independent of the androgen receptor (AR) (Gray *et al.* 1999; Parks *et al.* 2000). In a study with female rats, DEHP exposure resulted in decreased serum levels of 17β-estradiol (E_2), prolonged estrous cycle, and anovulation (Davis *et al.* 1994). *In vitro* studies on rat granulosa cells have shown that MEHP decreases aromatase RNA message and protein levels in a dose-dependent manner (Lovekamp and Davis 2001), which might explain the lowered serum E_2 levels observed *in vivo*. Recent studies suggest that the inhibitory effect of peroxisome proliferators on aromatase activity in mammalian granulosa cells is mediated via the PPARs (Mu *et al.* 2000). Anti-estrogenic effects have been reported in a study on Japanese medaka (*Oryzias latipes*) water exposed to DEHP for three months post-hatching (Kim *et al.* 2002). Female fish treated with 1–50 µg DEHP/L showed reduced vitellogenin levels and retardation of oocyte development whereas no adverse effects were observed in males. Metcalfe *et al.* (2001) observed no skewed sex ratio or intersex gonads in Japanese medaka water exposed to 5000 µg DEHP/l. In a dietary study on Atlantic salmon, exposure to a nominal concentration of 1500 mg DEHP/kg food during the first four weeks after yolk-sac resorption resulted in a significantly higher proportion of phenotypic females (64%) and in an elevated hepatosomatic index (Norrgren *et al.* 1999). However, actual exposure levels were not measured either in food or fish, which makes it difficult to establish a lowest effect concentration. The major aim of the present study was to study uptake and elimination of DEHP and MEHP in Atlantic salmon after exposure to contaminated diets during the first four weeks after yolk-sac resorption. In order to study possible late effects, fish were examined after a four-month post-exposure period with focus on growth, relative liver weights, and gonad morphology.

Materials and Methods

Fish and Exposure

The fish used in the present study originated from feral Atlantic salmon (*Salmo salar*) from River Dalälven, Sweden. Hatching and subsequent rearing were conducted at the National Board of Fisheries, Älvkarleby, Sweden. Stock solutions of DEHP (Neste-Oxo AB, Sweden) were prepared in 99.5% ethanol. The ethanol solutions (0.5l) were sprayed 5–6 times onto 1 kg of granulated start feed (Aller Aqua, SGP 514, Denmark, 14% lipid content), to obtain nominal concentrations of 0, 400, 800, and 1500 mg DEHP/kg. The food was allowed to dry in fume hoods for one hour and manually mixed thoroughly between each spraying. The control diet was prepared in the same way with pure ethanol. The treated food was kept in fume hoods until the ethanol had evaporated completely (one week). At the start of the study, each experimental group consisted of approximately 1000 individuals originating from a mix of ten family groups. Each group was kept in a trough (1 m^3) individually supplied with water from River Dalälven at a flow rate of 10 l/min. Light conditions in the rearing hall followed natural fluctuations. The water temperature ranged between 6.5 and 23.6°C during the experimental period from June to November, following the river water temperature. Dissolved oxygen and pH was measured regularly. Feeding with experimental diets started immediately after yolk-sac resorption, *i.e.*, four weeks post hatch, and continued for four weeks. At the start of exposure, the mean weight of the fry was approximately 0.2 g. Fish were fed approximately 2% of their body weight daily using automatic feeders delivering food four times per day. After the exposure period, feeding continued with non-contaminated food for four months.

Chemical Analysis

Aliquots of the four diets were taken for DEHP analysis at the start and the end of the exposure period. In addition, sub-samples of fish were taken for analysis of DEHP and the metabolite mono-2-ethyl hexyl phthalate (MEHP) at weekly intervals from the first day post-exposure and for the following three months. The chemical analyses were made at the Research Institute for Chromatography, Belgium.

DEHP in diets were analyzed in duplicate by first extracting approximately 100 mg of sample with 10 ml of cyclohexane in an ultrasonic bath for one hour. The extract was then centrifuged and analyzed by GC-MS in selected ion mode (m/z 149). Analysis of DEHP in fish tissue involved two sequential extractions of approximately 3–15 g of macerated fish taken from 2–8 pooled individuals (in order to obtain a similar fat amount in all samples) with 10-ml aliquots of cyclohexane for 30 min in an ultrasonic bath. After centrifugation, the combined cyclohexane fractions were evaporated to dryness. The resulting residue was weighed to provide a determination of the lipid content of the samples. The fat residue was then re-dissolved in a sufficient volume of dichloromethane to yield a fat concentration of approximately 100 mg/ml. Deuterium labeled d_4-DEHP (m/z/153) internal standard was added to 1 ml of the fat extract, which was then subjected to clean-up using gel permeation chromatography. The fraction containing DEHP was collected, evaporated to dryness under a gentle stream of nitrogen, re-dissolved in 0.1 ml of cyclohexane, and then analyzed using GC-MS.

For MEHP analysis, 1 ml of the dichloromethane extract described above was spiked with $^{13}C_4$-MEHP as an internal standard. The extract was evaporated to dryness with nitrogen. One milliliter of buffer (methanol/ammonium acetate, 25/75% by volume) was added to the remaining residue and vortexed for 10 sec. The extract was held in an ultrasonic bath for 15 min and then centrifuged during 10 min at 800 rpm. The supernatant was passed through a C18 silica gel solid phase cartridge, which was then eluted with a 75/25% methanol/ammonium acetate buffer. An aliquot of this elute was then analyzed by HPLC-MS using negative ion monitoring chemical ionization in the selection ion monitoring mode (m/z 277 for MEHP and m/z 281 for $^{13}C_4$-MEHP). Further analytical details and method validation results are provided by Tienpont *et al.* (in prep.).

Biological Analysis

Sampling of fish for biological measurements was conducted four months post-exposure, *i.e.*, at the end of October/beginning of November. Between 199 and 207 individuals per group were examined. Fish were anesthetized with MS222 and externally inspected. Individual body length and weight were recorded. After decapitation, the liver was dissected and weighed to calculate the hepatosomatic index (HSI = liver weight/total body weight × 100). The gonads were dissected and fixed in *toto in* phosphate-buffered formalin. After dehydration in a graded series of ethanol, the gonads were embedded in paraffin blocks. From each gonad, between 5 and 10 longitudinally 3–5-µm-thick step-sections were cut and placed on glass slides. The sections were stained with eosin-hematoxylin and examined under a light microscope. The histological evaluation included gonadal differentiation and presence of ovo-testis.

Table 1. Concentrations of DEHP (mg/kg) in diet measured at start and termination of the exposure and mean tissue concentrations of DEHP and MEHP (mg/kg) in whole Atlantic salmon at termination of the 4-week dietary exposure to DEHP

Nominal and measured, mean concentrations of DEHP in diet (mg/kg) at start/termination of exposure			
Nominal	Measured mean concentrations	Measured mean concentration of DEHP in fish (mg/kg_{wet})	Measured mean concentration of MEHP in fish (mg/kg_{wet})
0	2/2	0.016 [0.78]	0.020 [0.99]
400	346/371	0.656 [28.77]	0.222 [9.73]
800	825/829	1.318 [37.55]	0.502 [14.31]
1500	1661/1634	2.551 [85.84]	0.762 [25.63]

For the chemical analysis of the fish, 2–8 individuals were pooled to get an equal amount of fat in each sample. Values in [] denote concentrations expressed on a lipid normalized (mg/kg_{fat}) basis.

Statistics

A one-way analysis of variance (ANOVA) test was used to examine whether HSI, weight, and length of fish differed significantly ($p < 0.05$) between groups. The mortality was analyzed using a chi square test for significant ($p < 0.05$) differences between groups. For statistical analysis of the sex ratios, fish were classified as females, males, or intersex individuals. The number of fish with intersex gonads was compared with the total number of fish in each group. The number of males with precocious gonads, *i.e.*, individuals with running milt, was compared with the total number of males in each group. Sex ratios, intersex ratios, and precocious ratios were analyzed using Fisher's exact test for significant ($p < 0.05$) differences between groups.

Results

Chemical Analysis

The procedure used for analyzing DEHP in diets was found to give mean recoveries ranging from 87–111%. All groups were exposed to DEHP-concentrations that were close to nominal values. The measured mean concentrations of DEHP in the diet at start/finish of the exposure were 2/2, 346/371, 825/829, and 1661/1634 mg/kg.

In fish tissue, the recoveries of DEHP ranged from 89–94% with a limit of quantification that was determined by the procedural blank of 0.001 mg/kg_{wet} for a fish with a lipid content of 5%. The overall recoveries for the MEHP analysis in fish tissue ranged from 94–97% with a limit of quantification of 0.005 mg/kg_{wet} for a fish with a lipid content of 5%. Tissue concentrations of both parent and metabolite compounds were shown to exhibit a dose-dependent relationship at the end of the exposure period. Fish fed with diets that were not artificially contaminated with DEHP still showed some background levels of both DEHP (0.016 mg/kg fish) and MEHP (0.020 mg/kg fish) (Table 1). At the end of the four-week exposure period, tissue concentrations of DEHP were approximately three times higher than MEHP (Table 1). During a subsequent depuration phase, DEHP and MEHP were rapidly eliminated from fish tissue by the first sampling time (one week) to near background levels observed in controls (Fig. 1).

Biological Analysis

The mortality in all groups was low, *i.e.*, 3 to 4%. No dose-dependent difference in weight was observed four months post-exposure. The overall means were 11.7 ± 1.6 g (weight), 10.1 ± 0.5 cm (length), and the HSI ranged from 1.1 to 1.2. The histological evaluation of the gonads revealed no skewed sex ratios (Fig. 2). The proportion of females ranged from 49 to 51%. The majority of the females had lamellae-structured ovaries dominated by oocytes in the perinucleolar stage characterized by a relatively large centrally located nucleus with numerous peripherally nucleoli (Fig. 3). A small number of the fish in each group also defined as females had less developed ovaries not differentiated to lamellar structures with a relatively low number of perionucleolar oocytes. Testes were, in general, anatomically thread-like structures in an early stage of differentiation into a tubular system (Fig. 4). However, in the groups exposed to nominal concentrations of 800 and 1500 mg DEHP/kg food, 1% of the fish (2 out of 199 and 3 out of 202, respectively) had relatively large testes with running milt (not statistically significant). Histologically, these were characterized by well-differentiated testicular tissue containing germ cells at different stages of maturation from spermatogonia to spermatozoa (Fig. 5). In the groups exposed to 800 and 1500 mg DEHP/kg feed, individuals with the simultaneous presence of both spermatozoa and oocytes (ovo-testis) were observed (Fig. 2). Oocytes were found both in fish with poorly differentiated testes with only a few spermatozoa (1 in the 800-group, and 3 in the 1500-group) and in males with well-developed testis and running milt (1 in the 800-group, and 3 in the 1500-group) (Fig. 5). The only significant difference was recorded in the group exposed to 1500 mg DEHP/kg, where 6 out of the 202 exposed fish had ovo-testis ($p = 0.014$).

Discussion

According to model calculations, exposure via diet serves as the major route of uptake for lipophilic compounds with log $K_{ow} > 5.0$ (Thomann 1989). The water solubility of DEHP is approximately 3 μg/L and the log K_{ow} is around 7 (Staples *et al.* 1997), which supports the use of dietary administration.

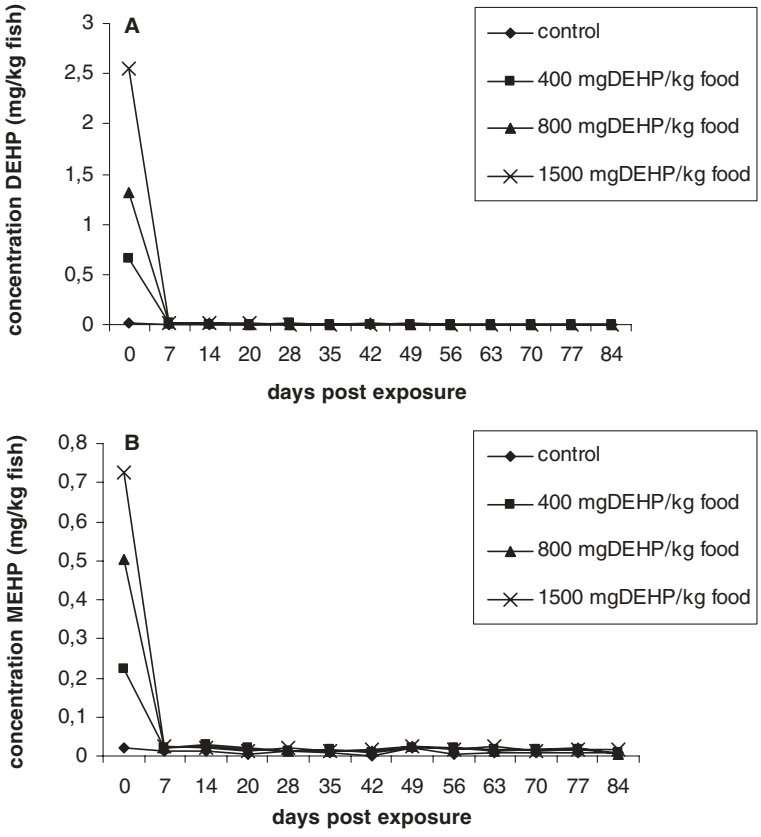

Fig. 1. DEHP and MEHP concentrations (mg/kg fish) in fish sampled at termination of the four-week exposure to DEHP and regularly during the post-exposure period

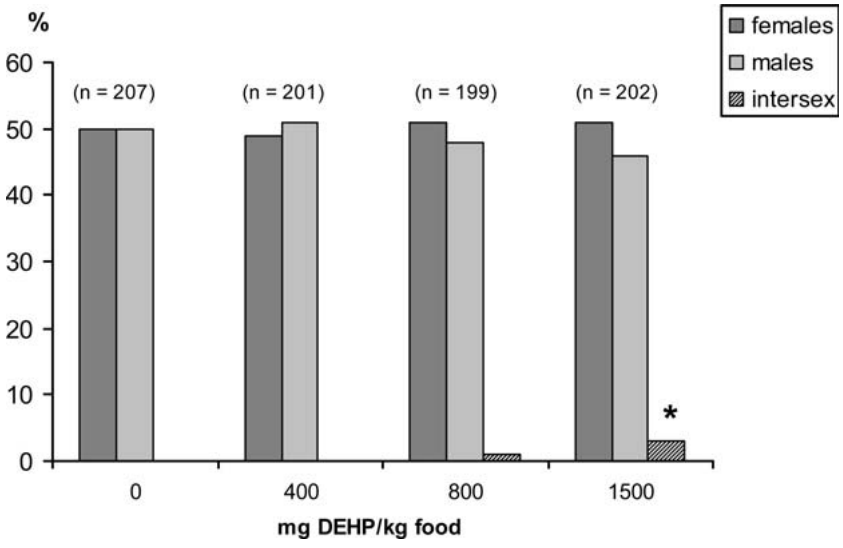

Fig. 2. Percentage of females, males, and individuals with intersex (ovo-testis) sampled four months after termination of exposure to different nominal concentrations (mg/kg) of DEHP in diets. *Significant ($p < 0.05$) difference between the control and the exposed group

Although DEHP is highly lipophilic, the bioaccumulation in fish has been reported to be low, which can be explained by an effective biotransformation (Barron *et al.* 1989). The metabolism of DEHP in fish is similar to mammals but appears to be dependent on species and exposure route (Barron *et al.* 1995). The first metabolic step in the biotransformation of DEHP is formation of mono-ethylhexyl phthalate (MEHP), followed by oxidation of MEHP by cytochrome P450, and excretion (Barron *et al.* 1995). In the present dietary study on Atlantic salmon, there was a rapid elimination of both DEHP and MEHP to near background levels within one week post-exposure, which suggests a low risk of bioaccumulation.

The sex differentiation in many fish species is labile and susceptible to several environmental factors, such as temperature, pH, density, and social hierarchy (Devlin and Nagahama 2002). Also, administration of hormones during sensitive parts of the life cycle is known to affect the sex differentiation in fish. The sensitive period for hormonal sex

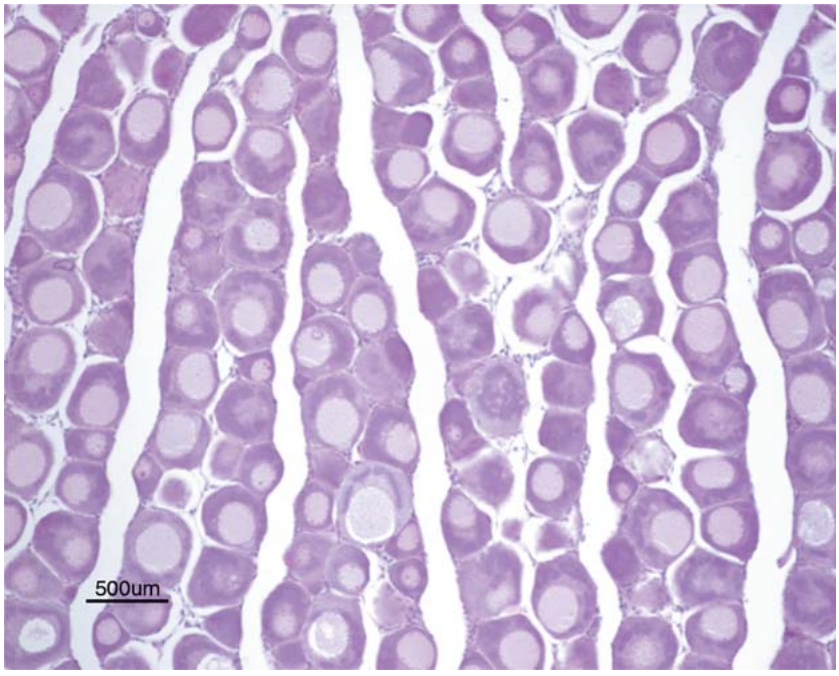

Fig. 3. Ovary from a control fish. The oocytes are in an early perinucleous stage and arranged in a lamellae-like structure (H&E stain)

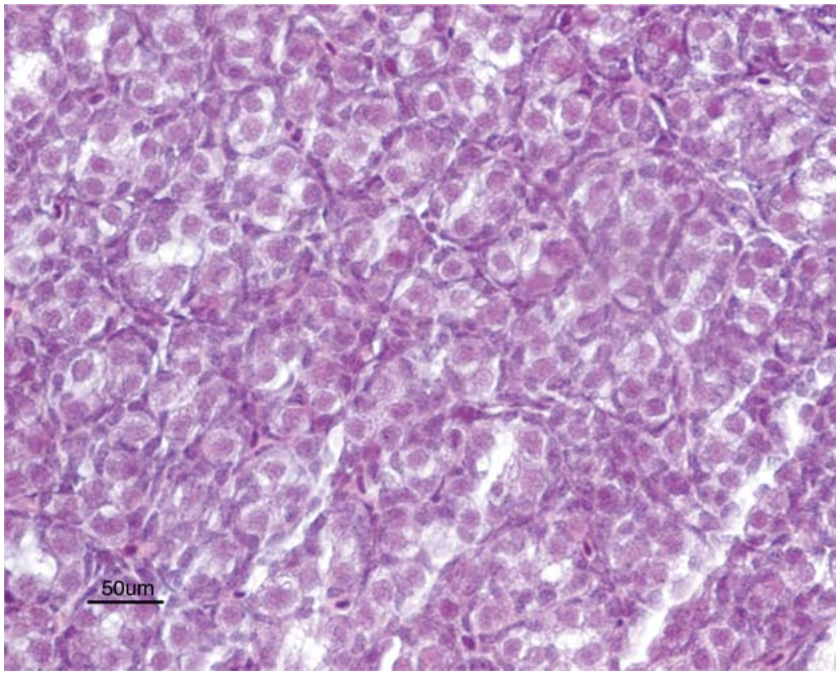

Fig. 4. Testis from a control fish. The tubular system has started to differentiate (H&E stain)

reversal in salmonids seems to vary between species (Pandian and Sheela 1995). In Atlantic salmon, exposure to 17β-estradiol (E_2) via immersion of eyed eggs and alevins in combination with exposure via food (20 mg E_2/kg) during the 21 first days of feeding resulted in all-female stocks (Johnstone *et al.* 1978). Exposure to E_2, via food only, from day 0 to 80 or from day 15 to 45 of the first feeding period also resulted in 100% females. Sower *et al.* (1984) exposed Atlantic salmon via the food to 20 mg E_2/kg for 60 days following yolk-sac resorption, which resulted in a skewed sex ratio (85% females) and in intersex fish (17%) nine months post-exposure. In a study by Norrgren *et al.* (1999), dietary exposure of Atlantic salmon to 30 mg E_2/kg for 30 days following yolk-sac resorption resulted in 100% females. In the same study, exposure of Atlantic salmon to 1500 mg DEHP/kg food (nominal concentration) resulted, contradictory to the present study, in significantly female-biased sex ratio (64% females) (Norrgren *et al.* 1999). The exposure period was the same as in the present study. In both studies, food was prepared in the same way with DEHP delivered from the same producer but from different batches. Also the food was delivered from the same supplier but from different batches.

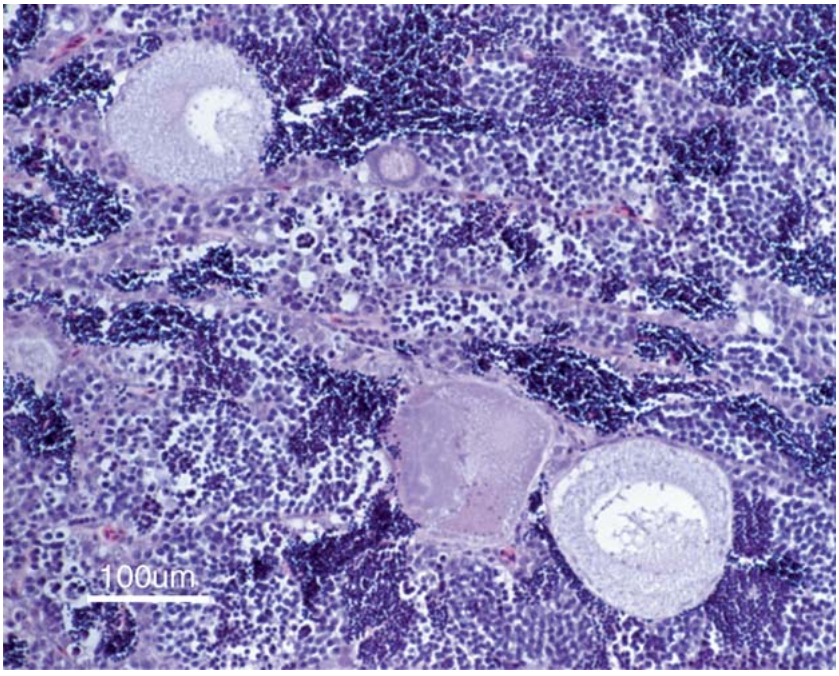

Fig. 5. Ovo-testis in an Atlantic salmon four months post-exposure to 1500 mg DEHP/kg food. Well-differentiated testicular tissue including spermatozoa interspersed with oocytes in the perinucleolar stage (H&E stain)

However, in the study by Norrgren *et al.* (1999), no chemical analysis of the food was performed and chemicals known to have endocrine disrupting effects, *i.e.*, nonylphenolethoxilate (NPEO) and E_2 were tested parallel to the DEHP. In both studies, the river Dalälven Atlantic salmon stock was used and fish were kept under similar rearing conditions concerning photoperiod, stocking density, and water supply. Since river water was used, small variations in composition and temperature between the studies cannot be fully avoided. A slightly higher mean temperature (+1°C) was observed in the present study during the experimental feeding phase, which might affect the distribution and elimination of DEHP in the fish. In a study with rainbow trout, the capacity for elimination of DEHP increased linearly with increasing temperature (Barron et al. 1987). However, the capacity to store DEHP was even greater, which resulted in an increased potential for biological persistence and bioaccumulation with increased temperature. The exposure of the highly perfused tissues appeared to decrease with increasing temperature, while the exposure of poorly perfused tissues increased (Barron *et al.* 1987). This may be of toxicological importance since changes in temperature may alter the amount of DEHP entering target tissues, such as the liver and the gonads.

Although no skewed sex ratios were found in the present study, histological examination of the gonads four months post-exposure revealed a significant incidence of individuals with ovo-testis in the group exposed to1500 mg DEHP/kg. This dose level is approximately 1000 times higher than measured levels in aquatic prey (David and Sandra 2001; Vethaak *et al.* 2002). To our knowledge, juvenile hermaphroditism has not been observed in Atlantic salmon and no case of ovo-testis was found among the 207 individuals examined in the control group. Intersex may be induced in fish as a result of exposure to hormones during sensitive periods (Koger *et al.* 2000). In the UK rivers, high incidences of intersex (ovo-testis) in roach (*Rutilus rutilus*) have been associated with effluents from sewage treatment works (STW) known to contain estrogenic compounds (Jobling *et al.* 1998; Larsson *et al.* 1999). Furthermore, signs of feminization, *i.e.*, ovo-testis and induction of vitellogenin in males, have been described in a number of fish species from industrialized estuaries containing endogenous hormones but also potential EDCs, such as nonylphenol and DEHP (Matthiessen *et al.* 2002). In Coho salmon (*Oncorhynchus kisutch*), lower concentrations of E_2 increased the proportion of intersex fish compared to higher concentrations, which resulted in complete sex reversal (Goetz *et al.* 1979). Furthermore, lower concentrations of E_2 have been reported to cause intersex in Atlantic salmon (Sower *et al.* 1984), whereas complete sex reversal has been observed after exposure to higher concentrations (Norrgren *et al.* 1999) or after a longer exposure period (Johnston *et al.* 1978). Since the actual DEHP dietary concentrations in the study by Norrgren *et al.* (1999) were not measured, an accurate comparison with the present study is difficult to perform. The lack of complete sex reversal and hence skewed sex ratios in the present study might be explained by the fact that the actual dose of DEHP was lower than in the study by Norrgren *et al.* (1999). The mechanism by which DEHP exerts its effects on development and reproduction in vertebrates is still unclear. However, most *in vitro* and *in vivo* studies suggest that the effects are not mediated via the androgen or estrogen receptors. Studies on rat indicate that maternal exposure to DEHP disrupts fetal male sexual differentiation by reducing testosterone to female levels (by an AR-independent mechanism) (Parks *et al.* 2000). If this is the case also for Atlantic salmon, lowered testosterone levels could explain the presence of intersex observed in the present study and the skewed sex ratio described in the study by Norrgren *et al.* (1999). In order to understand how DEHP and its metabolites act on the fish reproductive system, there is a need for more mechanistic studies and alternative test models based on species with short life cycles and well-characterized biology.

Enlarged liver has been observed in rodents after exposure to DEHP (Moody and Reddy 1978). Isenberg *et al.* (2001) demonstrated enhanced peroxisome proliferation and increased liver weight in rodents while exposed to DEHP but two weeks post-exposure the effects were reversed. In fish, increased relative liver weights have been reported after exposure to gemfibrozil, a known rodent peroxisome proliferator (Scarano *et al.* 1994) but also after exposure to estrogenic compounds (*e.g.*, Haux and Nordberg 1985). A significant ($p < 0.01$) increase in relative liver weight (HSI) was seen in Atlantic salmon exposed to 1500 mg DEHP/kg (nominal concentration) four months post-exposure in the study by Norrgren *et al.* (1999) but not in the present study, which indicates different exposure conditions.

In summary, dietary exposure of Atlantic salmon to DEHP caused no late effects on growth, survival, or hepatosomatic index. Ovo-testis was observed in 3% of the fish exposed to a nominal concentration of 1500 mg DEHP/kg but no complete sex reversal resulting in skewed sex ratios was found. The environmental significance of the mild histological changes observed is lowered by the relatively high dose level used. The rapid elimination of both DEHP and MEHP to near background levels one week post-exposure suggests a short half life and a low risk of bioaccumulation in Atlantic salmon. However, it cannot be excluded that chronic exposure to DEHP, in contrast to the short-term exposure performed in the present study, may cause more pronounced effects.

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