Impact of di-n-butyl phthalate on reproductive system development in European pikeperch (*Sander lucioperca*)

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Abstract

Phthalic acid, di-n-butyl ester known as di-n-butyl phthalate, is an organic chemical compound that belongs to the group of endocrine disruptor compounds that have a documented negative impact on mammalian endocrine systems. Di-n-butyl phthalate is used widely as a plasticizer in the manufacture of artificial materials, which is why it is found in all types of environmental samples including those from water basins. The aim of the study was to describe the impact of di-n-butyl phthalate on the development of the reproductive system of European pikeperch (*Sander lucioperca*) during the sex differentiation period (age 61–96 days post hatch). A total of 240 fish were divided into 6 groups (40 fish per tank). Treatments consisted of a control group (0 g di-n-butyl phthalate·kg^{-1} feed) and five trial groups with 0.125, 0.25, 0.5, 1, and 2 g di-n-butyl phthalate·kg^{-1} feed, respectively. Histological changes of the fish gonads, sex ratio, survival and growth of fish were evaluated. Di-n-butyl phthalate seriously disturbed sex differentiation process of pikeperch. Histopathological analyses revealed that the administration of 2 g di-n-butyl phthalate·kg^{-1} significantly affected the sex ratio. The feminization process (intersex gonads) at concentrations of 1 g and 2 g di-n-butyl phthalate·kg^{-1} were observed. All analyzed concentrations delayed testicular development. Phthalate did not have a significant impact on the survival or growth rates of the pikeperch. This is the first report of disruption sex differentiation processes in fish by di-n-butyl phthalate.

Xenobiotics, EDCs, phthalates, gonadal development, fish

Phthalates, which are classified as endocrine disruptor compounds (EDCs), were first created in the 1920s for commercial applications, and they comprise a vast group of additives, or plasticizers, used in the manufacture of synthetic materials. The most commonly used phthalate plasticizers are those with a diester structure, i.e., di-n-butyl phthalate (DBP). Fish are exposed to phthalates present in the water column and sediments. The physicochemical properties of phthalates and the life strategies of fish can influence the bioavailability of these compounds in fish (Huang et al. 2008). The concentration of DBP in surface waters ranges widely from nanograms to micrograms per litre.

To date, a large number of studies have focused on the endocrine effects of phthalates on mammalian reproductive systems. Male rats exposed to DBP at the moment of reproduction system differentiation exhibit the underdevelopment or absence of testes, as well as hypospadias and cryptorchidism (e.g., Kim et al. 2004). However, there are new reports on the negative effects of these compounds on the reproductive systems of lower vertebrates, i.e. fish and amphibians.

Phthalates have begun to be categorized as "environmental oestrogens" since their weak oestrogenic activity has been documented in *in vitro* studies with mammalian and fish oestrogen screens (e.g., Jobling et al. 1995). Thus far, there is little evidence of the oestrogenic activity of phthalates in *in vivo* assays. There is strong support for the hypothesis that they act as an anti-androgen in mammals, and, more recently, also in fish (Oehlmann et al. 2009; Uren-Webster et al. 2010; Aoki et al. 2011). The mechanism

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by which DBP exerts its anti-androgenic action on reproduction is still unclear, but it does not appear to be mediated by androgen receptors (Wolf et al. 1999).

The aim of this study was to determine the impact of DBP on the development of the reproductive system (during the sexual differentiation period) and on the growth of European pikeperch (*Sander lucioperca*). This species is constant in terms of sexual function with direct sexual differentiation (Demska-Zakęś and Zakęś 1995).

Materials and Methods

The experimental material comprised juvenile European pikeperch obtained through artificial, out-of-season reproduction (Zakęś 2007). A sample of 240 fish with body weights from 0.80 to 2.00 g (Table 1) were chosen at random, and 40 fish were stocked into each of six circulation tanks with working volumes of 28 dm^3. The physicochemical properties of the water measured at the rearing tank outflows were: temperature – 22.0 ± 0.3 °C; oxygen content – 7.49 ± 0.09 mg $O_2 \cdot dm^{-3}$; total ammonium nitrogen (TAN = NH_4^+-N + NH_3-N) and nitrite (NO_2^--N) – 0.041 ± 0.017 mg TAN·dm^{-3} and 0.021 ± 0.011 NO_2^--N mg·dm^{-3}, respectively. Water pH ranged from 7.5 to 7.9.

The ten-week-long experiment was divided into two stages of five weeks each. During the first stage (61–96 days post hatch - DPH), fish were fed the base feed (NUTRA, Nutreco Aquaculture, France) with the addition of di-n-butyl phthalate (Sigma-Aldrich, Poland). Treatments consisted of a control group (0 g DBP·kg^{-1} feed) and five trial groups with 0.125, 0.25, 0.5, 1.0, and 2.0 g DBP·kg^{-1} feed, respectively. A measured quantity of the master solution (phthalate and 96% ethyl alcohol) was dissolved in 6 ml of 96% ethyl alcohol and distributed in 100 g of feed. The DBP solution was added to the feed under pressure with a vacuum device (AGA Labor, Poland), and dried for 24 h at room temperature. The control feed was the base feed with the addition of solvent only. In the second stage of the experiment (97–132 DPH), the fish were fed only commercial feed. The feed was delivered by automatic band feeders 18 h per day. The feed ration was reduced successively from 5 to 2% of the stocking biomass. Fish mortality was monitored daily. At the end of both stages of the experiment, the fish were weighed (± 0.01 g) and measured (± 0.1 cm). The data obtained were used to calculate the fish condition coefficient CF = (W·100)·Lt^{-3}; where: W is body weight (g), and Lt is total length (cm).

At the beginning of the experiment (60 DPH) and after the first and second stages, the gonads of 15 individuals from each group were collected for histological analyses during necropsies (the procedures described by Demska-Zakęś and Zakęś 1995). The degree of gonad development, histological changes, and sex ratio were also evaluated.

Leven's test, ANOVA single factor variance of analysis, and Tukey's *post hoc* test were used to compare the mean values of the rearing indexes. The sex ratio was verified with a test of two structure indicators, while the dependence between phthalate concentration and the number of males was verified with Spearman's rank correlation coefficient. All of the tests were verified at a level of significance of $P < 0.05$ using the STATISTICA 8.0 program.

Results

Di-n-butyl phthalate was not observed to impact fish survival or growth rates ($P > 0.05$; Table 1). Prior to the beginning of the experiment, the pikeperch aged 60 days post hatch

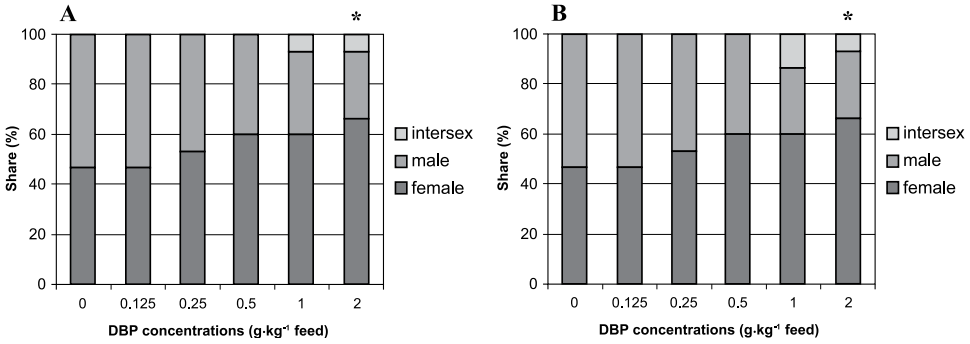

Fig. 1. Sex ratio of pikeperch in the control group and the groups exposed to di-n-butyl phthalate

A - after feeding with different doses of di-n-butyl phthalate in the diet (first stage of experiment - 96 DPH), B - at the end of the experiment after feeding commercial feed (second stage of experiment - 132 DPH), *significant differences in the sex ratio (n = 15; $P < 0.05$) DPH - days post hatch; DBP - di-n-butyl phthalate

Table 1. Rearing indicators (body weight, total length, condition factor) of juvenile pikeperch at the beginning of the experiment, after feeding with different concentrations of di-n-butyl phthalate in the diet (after 96 days post hatch) and at the end of the experiment after feeding commercial feed (after 132 days post hatch)

	Di-n-butyl phthalate concentrations (g·kg^{-1}feed) (mean ± SD)					
	0	0.125	0.25	0.5	1.0	2.0
Initial body weight (g)	1.73 ± 0.29	1.60 ± 0.41	1.63 ± 0.35	1.62 ± 0.38	1.68 ± 0.36	1.68 ± 0.37
Body weight after 96 DPH (g)	4.98 ± 1.14	5.35 ± 0.91	5.58 ± 1.48	5.87 ± 1.48	4.75 ± 0.96	4.35 ± 1.20
Body weight after 132 DPH (g)	13.29 ± 3.31	11.27 ± 4.44	12.68 ± 3.61	15.33 ± 3.26	13.47 ± 4.31	10.55 ± 4.12
Initial total length (cm)	5.82 ± 0.45	5.73 ± 0.48	5.75 ± 0.42	5.73 ± 0.49	5.85 ± 0.44	5.84 ± 0.39
Total length after 96 DPH (cm)	8.53 ± 0.74	8.82 ± 0.53	8.73 ± 0.79	8.86 ± 0.81	8.53 ± 0.53	8.38 ± 0.78
Total length after 132 DPH (cm)	11.86 ± 0.85	10.99 ± 1.31	11.58 ± 1.18	12.30 ± 0.83	11.95 ± 1.16	11.07 ± 1.54
Initial condition factor (CF_i)	0.88 ± 0.12	0.84 ± 0.10	0.84 ± 0.10	0.85 ± 0.10	0.83 ± 0.08	0.84 ± 0.11
Condition factor (CF_f) after 96 DPH	0.80 ± 0.08	0.77 ± 0.05	0.82 ± 0.06	0.83 ± 0.06	0.76 ± 0.04*	0.72 ± 0.05*
Condition factor (CF_f) after 132 DPH	0.78 ± 0.06	0.81 ± 0.07	0.80 ± 0.09	0.81 ± 0.05	0.76 ± 0.04	0.74 ± 0.07

DPH - days post hatch, *significant differences in comparison to the control group ($P < 0.05$)

(DPH) exhibited no signs of differentiation. After 96 DPH, females from the control group had paired cystic ovaries with well-developed oviducts. The ovarian lamellae were filled mainly with oogonia and oocytes in prophase I of meiotic division. The male gonads comprised gonocytes, numerous seminal vesicles, spermatogonia, and weakly developed sperm ducts. The sex ratios in the control group and in the groups treated with 0.125 and 0.25 g DBP·kg^{-1} were about 1:1 (Fig. 1A). Only about 50% of male individuals had gonads that were developed to the same degree as those in the control group. The testes of the remaining fish were smaller, with reduced numbers of seminal vesicles and spermatogonia. As the higher concentration of DBP was used in other groups, the percentage of males decreased (r_s = - 0.9856; $P < 0.05$). Significant differences in sex ratios were noted in the group treated with 2.0 g DBP·kg^{-1} ($P < 0.05$). In groups treated with 1.0 and 2.0 g DBP·kg^{-1}, 6.7% of the individuals had intersex gonads. Additionally, some fish classified as females (from 11 to 22%) from the group exposed to the three highest concentrations of DBP had ovaries that were similar in size to the testes. Significantly, these gonads had reduced oviducts. In total, 132 females treated with DBP comprised about 50% of the population (Fig. 1B) and had distinctly formed oviducts and relatively large ovaries with numerous oocytes and oogonia in the differentiated ovarian lamellae (Plate I, Fig. 2A). The males had weakly differentiated sperm ducts and pear-shaped testes filled with numerous seminal vesicles with spermatogonia and spermatocytes I (Plate I, Fig. 2B). Ovary development was normal in all females fed the feed with the addition of 0.125 and 0.25 g DBP·kg^{-1}, and in 80–89% of females in groups treated with 0.5, 1.0, and 2.0 g DBP·kg^{-1}. Oviducts were missing in 11–20% of females, and their gonads were small and filled tightly with few previtellogenic oocytes (Plate I, II, Fig. 2C, D). The opposite was observed in the males; disturbances in testicular development were noted in 80% (group treated with 1.0 g DBP·kg^{-1}) to 37.5% (group treated with 0.125 g DBP·kg^{-1}) of the

individuals. This was manifested as reduced numbers of seminal vesicles and germ cells that occurred mostly in the spermatogonium stage (Plate II, Fig. 2E). The percentage of males decreased with increasing concentrations of DBP used for treatment (r_s = -0.9710; $P < 0.05$); while there were 27% of males in groups treated with 1.0 and 2.0 g DBP·kg^{-1}. Finally, in group treated with 2.0 g DBP·kg^{-1} the sex proportion was significantly affected ($P < 0.05$; Fig. 1B). Ovo-testes were noted in 13.3% and 6.7% of the individuals from groups treated with 1.0 g DBP·kg^{-1} and 2.0 g DBP·kg^{-1}, respectively. Single previtellogenic oocytes occurred among the reduced number of seminal vesicles and germ cells (Plate II, Fig. 2F). Greater amounts of connective tissue were also noted.

Discussion

Di-n-butyl phthalate slowed testis development at all of the concentrations analyzed. No negative changes were observed in the gonads of females. Intersex specimens appeared at concentrations of 1.0 and 2.0 g DBP·kg^{-1} feed, and the sex ratio in the fish treated with the highest dose of phthalate was seriously affected. Presumably, DBP doses of 0.5 g·kg^{-1} feed can reverse the sex of genetic males, which was indicated by the atypical ovaries that were close to testes in size, but had either weakly developed oviducts or a complete lack of them.

Similar results were obtained by Ohtani et al. (2000). They exposed genetic Japanese wrinkled frog (*Rana rugosa*) males to DBP during the gonad differentiation period (19–23 DPH). Di-n-butyl phthalate dissolved in water at concentrations of 0.28 and 2.8 mg·dm^{-3} induced either partial or total ovarian development in 7% and 17% of the males, respectively. The histological examination of Atlantic salmon (*Salmo salar*) gonads that had been exposed to another phthalate, di-2-ethylhexyl phthalate (1.5 g DEHP·kg^{-1}), exhibited a 3% incidence of intersex fish (Norman et al. 2007). A similar dietary study on Atlantic salmon exposed to 1.5 g DEHP·kg^{-1} after yolk-sac resorption resulted in a significantly higher proportion of phenotypic females (64%) (Norrgren et al. 1999).

The recent findings strongly support the hypothesis that DBP acts as an anti-androgen compound in fish. Sexually mature male three-spined sticklebacks (*Gasterosteus aculetaus*) exhibited significantly lowered concentrations of spiggin, a protein produced by the kidneys during the breeding season and a sensitive biomarker of androgenic disruption, after exposure to 35 mg DBP·dm^{-3} (Aoki et al. 2011). Other phthalates also cause typical anti-androgenic effects in fish. Exposing male zebrafish (*Danio rerio*) to 5000 mg DEHP·kg^{-1} disrupted spermatogenesis and reduced the proportion of spermatozoa (Uren-Webster et al. 2010). Other oesters, such as diethyl phthalate (DEP), inhibited testicular growth in carp (Barse et al. 2007), and butyl benzyl phthalate (BBP) induced changes in sperm motility in zebrafish (Oehlmann et al. 2009).

The latest *in vitro* research indicates that DBP at the lowest analyzed dose of 0.1 mg·dm^{-3} caused a 50% reduction in concentration of testosterone (Mankidy et al. 2013). The activity of enzymes involved in the synthesis and metabolism of sex hormones is thought to be crucial to phthalate-induced anti-androgenic disruption. Thibault and Porte (2004) demonstrated in *in vitro* studies of the microsomes of the testes of carp (*Cyprinus carpio*), that DBP inhibited 5α-reductase, which participates in the conversion of testosterone to 5α-dihydrotestosterone. *In vivo* studies of rodents have also indicated that DBP can affect the expression of genes involved in cholesterol transport and steroidogenesis, which leads to reduced testosterone production in foetal testes (Lehmann et al. 2004). Interrupted testosterone synthesis disrupts differentiation of androgen-dependent tissues. By blocking endogenous androgen action, anti-androgens can create "an oestrogenic environment", which exhibits symptoms of oestrogen exposure. Thus, lowered testosterone concentrations could explain the delay in spermatogenesis, the occurrence of intersex fish, and affect the sex ratios observed in the pikeperch in the present study. To the best

of the authors' knowledge, the current study is the first to report that DBP disrupts sex differentiation in fish. In natural waters this phthalate probably does not cause negative changes in gonochoristic fish, because it acts during the relatively short period of sex differentiation. Di-n-butyl phthalate is sufficiently metabolized at lower, environmentally-relevant concentrations; thus, endocrine effects might not occur. Further research is needed to establish the consequences for fish of chronic DBP exposure, especially in males. Multi-generational studies are also required.

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References

Aoki KA, Harris CA, Katsiadaki I, Sumpter JP 2011: Evidence suggesting that di-n-butyl phthalate has antiandrogenic effects in fish. Environ Toxicol Chem **30**: 1338-1345

Barse AV, Chakrabarti T, Ghosh TK, Pal AK, Jadhao SB 2007: Endocrine disruption and metabolic changes following exposure of *Cyprinus carpio* to diethyl phthalate. Pestic Biochem Physiol **88**: 36-42

Demska-Zakęś K, Zakęś Z 1995: Sex differentiation in pikeperch (*Stizostedion lucioperca* (*L.*)). Arch Pol Fish **3**: 51-57

Huang PC, Tien C J, Sun Y.M, Hsieh CY, Lee CC 2008: Occurrence of phthalates in sediment and biota: relationship to aquatic factors and the biota-sediment accumulation factor. Chemosphere **73**: 539-544

Jobling S, Reynolds T, White R, Parker MG, Sumpter JP 1995: A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. Environ Health Perspect **103**: 582-587

Kim HS, Kim TS, Shin JH, Moon HJ, Kang IH, Kim IY, Oh JY, Han SY 2004: Neonatal exposure to di(n-butyl) phthalate (DBP) alters male reproductive-tract development. J Toxicol Environ Health **67**: 2045-2060

Lehmann KP, Phillips S, Sar M, Foster PM, Gaido KW 2004: Dose-dependent alterations in gene expression and testosterone synthesis in the fetal testes of male rats exposed to di (n-butyl) phthalate. Toxicol Sci **81**: 60-68

Mankidy R, Wiseman S, Ma H, Giesy JP 2013: Biological impact of phthalates. Toxicol Lett **217**: 50-58

Norman A, Borjeson H, David F, Tienpont B, Norrgren L 2007: Studies of uptake, elimination and late effects in Atlantic salmon (*Salmo salar*) dietary expose to di-2-ethylhexyl phthalate (DEHP) during early life. Arch Environ Contim Toxicol. **52**: 235-242

Norrgren L, Blom A, Andersson PL, Borjenson H, Larsson DGJ, Olsson PE 1999: Effects of potential xenoestrogens (DEHP, nonylphenol and PCB) on sexual differentiation in juvenile Atlantic salmon (*Salmo salar*). Aquat Ecosyst Heath Manage **2**: 311-317

Oehlmann J, Schulte-Oehlmann U, Kloas W, Jagnytsch O, Lutz I, Kusk KO, Wollenberger L, Santos EM, Paull GC, Van Look KJW 2009: A critical analysis of the biological impacts of plasticizers on wildlife. Philos Trans R Soc Lond, B, Biol Sci **364**: 2047-2062

Ohtani H, Miura I, Ichikawa Y 2000: Effects of dibutyl phthalate as an environmental endocrine disruptor on gonadal sex differentiation of genetic males of the frog *Rana rugosa*. Environ Health Perspect **108**: 1189-1193

Thibault R, Porte C 2004: Effects of endocrine disrupters on sex steroid synthesis and metabolism pathways in fish. J Steroid Biochem Mol Biol **92**: 485-494

Uren-Webster TM, Lewis C, Filby AL, Paull GC, Santos EM 2010: Mechanisms of toxicity of di(2-ethylhexyl) phthalate on the reproductive health of male zebrafish. Aquat Toxicol **99**: 360-369

Wolf C, Lambright C, Mann P, Price M, Cooper RL, Ostby J, Gray LE 1999: Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p,p'-DDE, and ketoconazole) and toxic substances (dibutyl and diethylhexyl phthalate, PCB, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. Toxicol Ind Health **15**: 94-118

Zakęś Z 2007: Out-of-season spawning of cultured pikeperch [*Sander lucioperca* (L.)]. Aquacult Res **38**: 1419-1427

Plate I
Jarmołowicz S.: Impact of... pp. 197-201

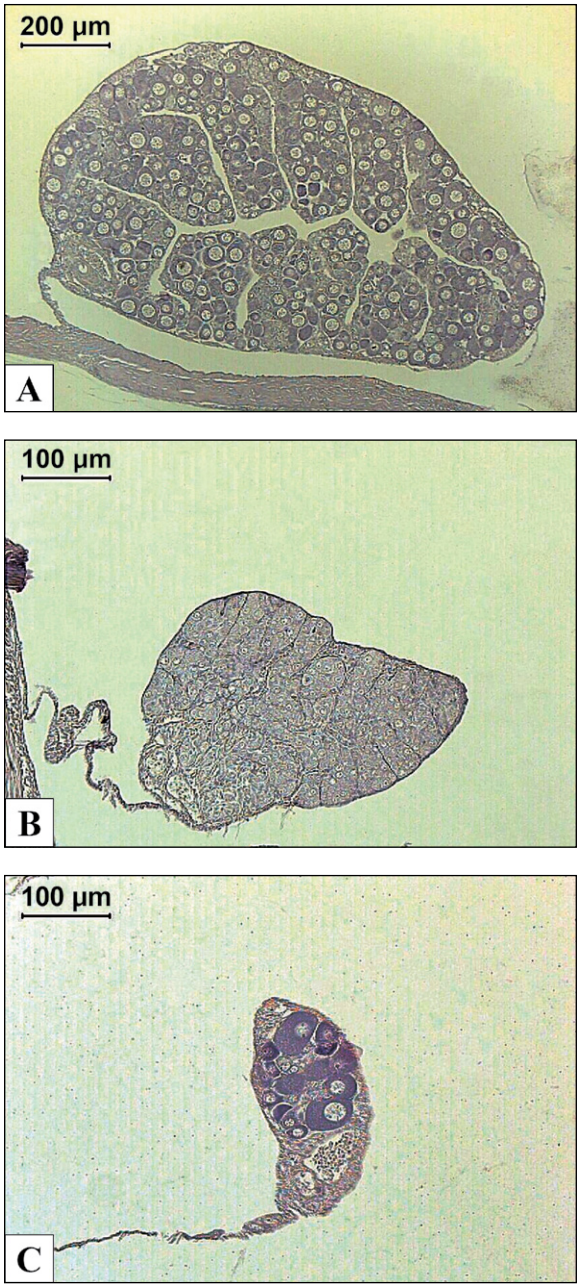

Fig. 2. Cross-section of pikeperch gonads at the end of the experiment (second stage of experiment - 132 days post hatch).

A - control group, ovary with developed oviducts and previtellogenic oocytes; B - control group, testicle with developed seminal vesicles; C - atypical ovary from group fed 2.0 g DBP·kg^{-1}

Plate II

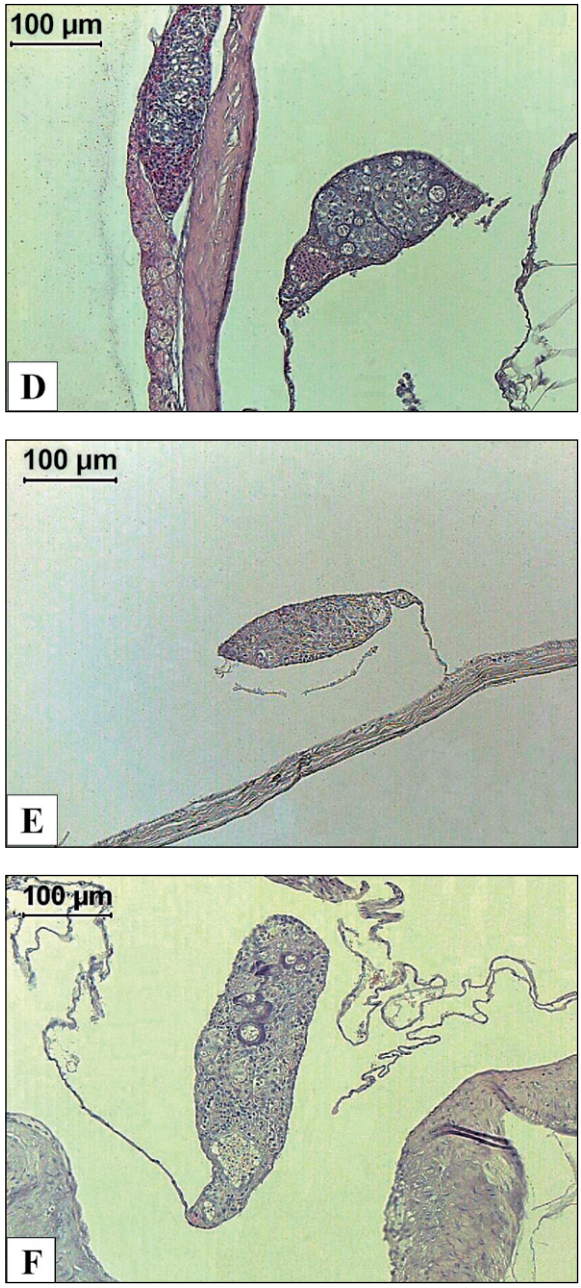

Fig. 3. Cross-section of pikeperch gonads at the end of the experiment (second stage of experiment - 132 days post hatch).

D - atypical ovary from group fed 2.0 g DBP·kg^{-1}; E - testicle from group fed 1.0 g DBP·kg^{-1}; F - ovo-testes from group fed 1.0 g DBP·kg^{-1}