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INDUCTION OF TESTIS-OVA IN PIKEPERCH (*SANDER LUCIOPERCA* (L.)) EXPOSED TO 4-NONYLPHENOL

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ABSTRACT. The aim of the study was to evaluate the effect of nonylphenol (NP) administered in feed (trout pellets) on the growth rate, mortality, and morphological and histological gonad development of pikeperch. The oral administration of NP (0.0, 0.1, 1.0, 10.0, or 100.0 mg kg⁻¹ feed) to pikeperch juveniles (initial body weight of approximately 0.3 g; fish age 28 days post hatch (28 DPH)) for 7 to 63 days (35-91 DPH) did not affect fish mortality, growth, or condition. Histological and morphological studies revealed that the administration of NP (time of exposure over 28 days (fish age above 63 DPH)) at a dose 0.1-100 ppm significantly decreased the percentage of males and produced intersex fish. Abnormal gonads contained numerous small spaces resembling the ovocell of ovaries. Testis-ova contained both female and male germ cells. An increase in treatment dose and time of duration increased the percentage of intersex fish and decreased the percentage of males.

Key words: PIKEPERCH (*SANDER LUCIOPERCA*), 4-NONYLPHENOL, TREATMENT DURATION, SEX DIFFERENTIATION, TESTIS-OVA

INTRODUCTION

Various chemical compounds, such as alkylphenols, are commonly present in aquatic environments (Maguire 1999). The origin of these chemicals is primarily from anthropogenic sources. Alkylphenols (APs) enter aquatic ecosystems with domestic and agricultural effluents through their use in detergents, cosmetics, spermicides, pesticides, herbicides, plastics, and antioxidants as well as through the biodegradation of alkylphenol polyethoxylates (APEs). Alkylphenol polyethoxylates are used as industrial surfactants, and industrial sewage may also release large concentrations of these compounds (Renner 1997). In the environment, APEs are ultimately metabolized to the corresponding alkylphenols (Giger et al. 1984, Meldahl et al. 1996). Since nonylphenol polyethoxylate compounds include about 80% of the APEs on the world market

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(Renner 1997, Wheeler et al. 1997), nonylphenols (NPs) have been identified as the main aquatic contaminants (Blackburn and Waldock 1995).

Nonylphenols are likely to be more persistent in the environment, more lipophilic, and more easily accumulated than their parent compounds (nonylphenol polyethoxylate). They have been found in surface waters, sediments, algae, plants, and animals (Ahel and Giger 1985, Naylor et al. 1992, Blackburn and Waldock 1995, Lewis and Lech 1996). In addition, NPs have been described as estrogenic in that they compete with natural estrogen for binding to the estrogen receptor in *in vitro* cultured hepatocytes (Lewis and Lech 1996, Ren et al. 1996). A few studies showed that exposing juvenile sexually undifferentiated fish to an aqueous solution of nonylphenol disrupts sexual differentiation. Abnormal gonad development and changes of sex ratios were observed in Japanese medaka, *Oryzias latipes* (Temminck & Schegel) (Gray and Metcalfe 1997), mangrove rivulus, *Rivulus marmoratus* Poey (Tanaka and Grizzle 2002), and pikeperch, *Sander lucioperca* (L.) (Demska-Zakęś 2005). The adverse effect on sexual differentiation may be assigned via the food chain (Demska-Zakęś, unpublished data).

The present study is the first ever to focus on the effect that exposure time to 4-nonylphenol (added to food) has on the course of gonadal differentiation and sex ratio in the pikeperch.

MATERIALS AND METHODS

ANIMALS AND EXPERIMENTAL DESIGN

The study was conducted on juvenile pikeperch obtained by artificial spawning according the method described in Zakęś and Demska-Zakęś (2005). Newly hatched larvae (age 4 days post hatch – 4 DPH) were distributed into 200 l fiberglass tanks at a density of 20 individuals l⁻¹. The tanks were supplied with filtered, heated water at a rate of 3 l min⁻¹. Water temperature was 21 ± 0.5°C in the first 5 days of rearing; then it was increased to 22 ± 0.5°C. The fish were gradually trained to consume formulated feed by replacing *Artemia* sp. nauplii with an artificial diet (Proton, INVE Aquaculture, Belgium).

The pikeperch were reared for 28 days (28 DPH) under intensive culture conditions to an average body weight of approximately 0.3 ± 0.1 g. They were then divided into 5 treatments (in two replicates each). Additionally, due to the varied time expo-

sure, each feeding group was divided into 9 subgroups of 14 fish each, which were marked and transferred to tanks of 200 l ($9 \times 14 = 126$ fish per tank) supplied with aerated water at a temperature of $22 \pm 0.5^\circ\text{C}$ and a water flow rate of $4\text{-}5 \text{ l min}^{-1}$. Dissolved oxygen concentrations ranged between 7.8 and 8.2 $\text{mg O}_2 \text{ l}^{-1}$, and the pH ranged between 7.7 and 8.0. Total ammonia nitrogen ($\text{TAN}=\text{NH}_4^+\text{-N} + \text{NH}_3\text{-N}$) and nitrite concentrations ($\text{NO}_2\text{-N}$) at the outflow were lower than 0.20 mg TAN l^{-1} and 0.05 $\text{mg NO}_2\text{-N l}^{-1}$. The light intensity in the culture room was low at less than 50 lx, and a 24 h light and 0 h dark photoperiod was applied.

DIET PREPARATION AND FEEDING

Technical nonylphenol (NP) was obtained from Sigma Aldrich Chemical Company (Germany). The preparation consisted of 98% nonylphenol isomers (90% 4-nonylphenol) and 2% dinonylphenol. Artificial food was supplemented with NP using a vacuum chamber. The following doses of NP were tested: 0.0 (control group), 0.1, 1.0, 10.0, or 100.0 mg kg^{-1} . The fish were fed with a commercially available trout diet (NUTRA, NUTRECO Holland) at 5 min intervals for 16 h d^{-1} at a daily rate of 10% of the total fish weight per tank. The size of the granules was increased as the fish grew (from 0.8 to 1.7 mm).

Pikeperch from each experimental group were exposed to nonylphenol for nine different exposure time durations (from 7 to 63 days). Following the treatment period, all the fish from each variant were removed to a culture tank that corresponded to the treatment dose and were fed a „clean“ diet. Samples for histological assays were collected at the end of the experiment (91 DPH).

DATA COLLECTION AND ANALYSIS

The survival factor was determined at the end of the experiment (91 DPH). Then, 14 fish from each group were sacrificed with an overdose of anesthetic (Propiscin) (Kazuń and Siwicki 2001), measured (total body length, $\text{TL} \pm 0.1 \text{ cm}$), weighed ($\text{BW} \pm 0.01 \text{ g}$), and placed in Bouin's fluid. After dehydration in graded ethanol and embedding in paraffin, serial sections ($5 \mu\text{m}$ thick) were cut and stained with hematoxylin and eosin. The preparations were analyzed for any modifications in the macro- and microscopic structure of the reproductive system and sex ratio.

One-way analysis of variance (ANOVA), and Duncan's multiple range test were used to compare the mean values of the factors measured (Steel and Torrie 1960). The mean percent of the sex ratio was *arcsine* transformed. The level of significance was accepted at $P < 0.05$.

RESULTS

Sporadic fish deaths were noted during the experiment in all groups and occurred in the first two days of cultivation and at the beginning of the post-treatment phase. A summary of survival factor, mean length, weight, and condition throughout the experiment is presented in Table 1. The analysis of variance indicated that there were no significant differences in these parameters between NP-treated groups and the controls ($P > 0.05$).

TABLE 1

Effects of 4-nonylphenol (NP) on survival, growth, and condition factor of juvenile pikeperch
(means \pm SD) of two replicate groups)

Exposure time (day)	NP dose (mg kg ⁻¹ diet)	Survival (%)	Total length TL (cm)	Body weight BW (g)	Condition factor*
1	2	3	4	5	6
0	0.0	89.5a \pm 10.5	10.83a \pm 0.69	9.33a \pm 1.21	1.19a \pm 0.15
7	0.1	89.5a \pm 10.5	9.85 a \pm 0.38	8.50a \pm 0.60	1.08a \pm 0.04
	1.0	85.5a \pm 6.5	10.99a \pm 0.50	9.46a \pm 1.07	1.15a \pm 0.09
	10.0	86.0a \pm 0.0	10.38a \pm 0.39	8.95a \pm 0.73	1.14a \pm 0.06
	100.0	82.5a \pm 3.5	10.91a \pm 0.71	9.21a \pm 1.13	1.09a \pm 0.04
14	0.1	89.5a \pm 10.5	11.18a \pm 0.89	9.57a \pm 1.31	1.17a \pm 0.13
	1.0	82.5a \pm 3.5	11.05a \pm 0.79	9.45a \pm 1.28	1.08a \pm 0.03
	10.0	86.0a \pm 0.0	11.51a \pm 0.91	9.76a \pm 1.52	1.17a \pm 0.12
	100.0	85.5a \pm 6.5	10.76a \pm 0.54	9.21a \pm 1.05	1.05a \pm 0.02
21	0.1	93.0a \pm 7.0	10.32a \pm 0.31	8.95a \pm 0.51	1.08a \pm 0.03
	1.0	82.5a \pm 3.5	10.99a \pm 0.54	9.32a \pm 0.96	1.16a \pm 0.13
	10.0	86.0a \pm 0.0	11.09a \pm 0.76	9.51a \pm 1.24	1.08a \pm 0.04
	100.0	89.5a \pm 10.5	10.23a \pm 0.42	8.89a \pm 0.61	1.16a \pm 0.11
28	0.1	85.5a \pm 6.5	10.21a \pm 0.36	8.77a \pm 0.45	1.08a \pm 0.02
	1.0	89.5a \pm 10.5	11.28a \pm 0.74	9.62a \pm 1.45	1.10a \pm 0.07
	10.0	82.5a \pm 3.5	11.90a \pm 1.00	10.01a \pm 2.01	1.09a \pm 0.04
	100.0	82.5a \pm 3.5	10.29a \pm 0.38	8.95a \pm 0.67	1.15a \pm 0.09
35	0.1	89.5a \pm 10.5	11.07 a \pm 0.77	9.55a \pm 1.38	1.12a \pm 0.07
	1.0	85.5a \pm 6.5	11.01a \pm 0.86	9.66a \pm 1.42	1.09a \pm 0.03
	10.0	86.0a \pm 0.0	10.27a \pm 0.33	8.75a \pm 0.69	1.18a \pm 0.13
	100.0	86.0a \pm 0.0	11.29a \pm 0.61	9.76a \pm 1.17	1.08a \pm 0.04

cont. TABLE 1

1	2	3	4	5	6
42	0.1	86.0a ± 0.0	10.47a ± 0.51	8.99a ± 0.92	1.13a ± 0.08
	1.0	82.5a ± 3.5	10.98a ± 0.71	9.26a ± 1.23	1.17a ± 0.12
	10.0	85.5a ± 14.5	11.03a ± 0.85	9.57a ± 1.34	1.08a ± 0.02
	100.0	86.0a ± 0.0	11.00a ± 0.54	9.55a ± 1.29	1.19a ± 0.13
49	0.1	89.5a ± 10.5	10.37a ± 0.29	8.89a ± 0.61	1.08a ± 0.04
	1.0	89.5a ± 10.5	10.99a ± 0.82	9.22a ± 1.21	1.15a ± 0.11
	10.0	85.5a ± 6.5	9.86a ± 0.30	8.38a ± 0.36	1.15a ± 0.09
	100.0	82.5a ± 3.5	10.98a ± 0.55	9.32a ± 0.96	1.07a ± 0.01
56	0.1	86.0a ± 0.0	11.07a ± 0.86	9.55a ± 1.24	1.10a ± 0.07
	1.0	85.5 a ± 6.5	10.65a ± 0.37	8.95a ± 0.56	1.06a ± 0.02
	10.0	86.0 a ± 0.0	10.97a ± 0.49	9.32a ± 0.96	1.12a ± 0.07
	100.0	82.5 a ± 3.5	11.12a ± 0.96	9.71a ± 1.44	1.09a ± 0.04
63	0.1	86.0 a ± 0.0	10.34a ± 0.31	8.75a ± 0.63	1.19a ± 0.15
	1.0	85.5 a ± 6.5	10.95a ± 0.73	9.33a ± 1.19	1.08a ± 0.04
	10.0	82.5 a ± 3.5	10.25a ± 0.43	8.89a ± 0.61	1.15a ± 0.09
	100.0	89.5 a ± 10.5	10.99a ± 0.91	9.43a ± 1.42	1.17a ± 0.13

*Condition factor $K = (BW (g) \times 100) TL^{-3}(cm)$

Means with the same letters in the same column are not significantly different with respect to the control ($P > 0.05$)

The morphological and histological studies of gonads indicated that gonadal development in the control group was typical. At the end of the experiment (91 DPH), the ovaries were characterized by a medially located ovocell and contained oogonia, meiotic oocytes, and oocytes in the previtellogenesis stage. The males had relatively smaller lobate gonads containing spermatogonia and spermatocytes. The male to female sex ratio in the control group was 0.5 : 0.5 (Fig. 1). A similar sex ratio was noted in the experimental groups fed the diet containing 4-nonylphenol at a dose of 0.1 – 100 ppm from days 7 to 28. The gonads appeared to be normal both morphologically and histologically. However, after 35 days of NP exposure in the all NP-treated groups, three types of gonads were observed: ovaries, testes, and gonads with abnormal morphology (Fig. 2). Histological examination revealed that the typical ovaries and testes resembled those of the control group. Abnormal gonads contained numerous small spaces resembling the ovocell of ovaries. Testis-ova contained both female and male germ cells. An increase in NP treatment dose and exposure time enhanced the percentage of intersex fish and decreased the percentage of males (Fig. 1). This tendency occurred in the groups fed the diet supplemented with xenobiotic from 28 to 63-84 DPH. Extending exposure time to 63 days (from 28 to 91 DPH) did not have a statistically significant influence on the sex ratio.

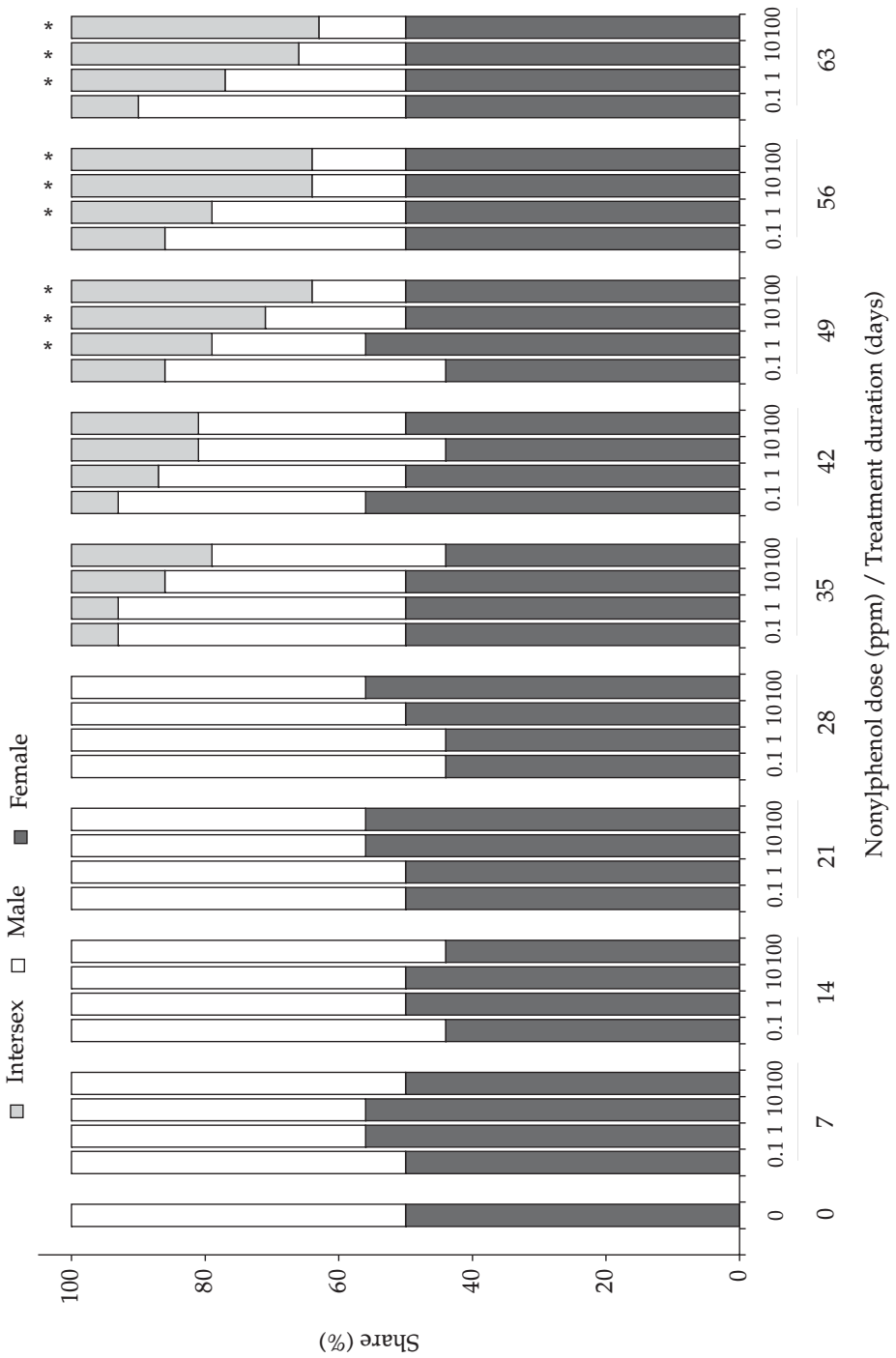


Fig. 1. Sex structure of juvenile pikeperch in the control group and in groups exposed to 4-nonylphenol for 7 to 63 days (fish age from 28 to 91 days post hatch). Asterisks indicate significant differences in the proportion of treated groups with respect to the control ($P < 0.05$).

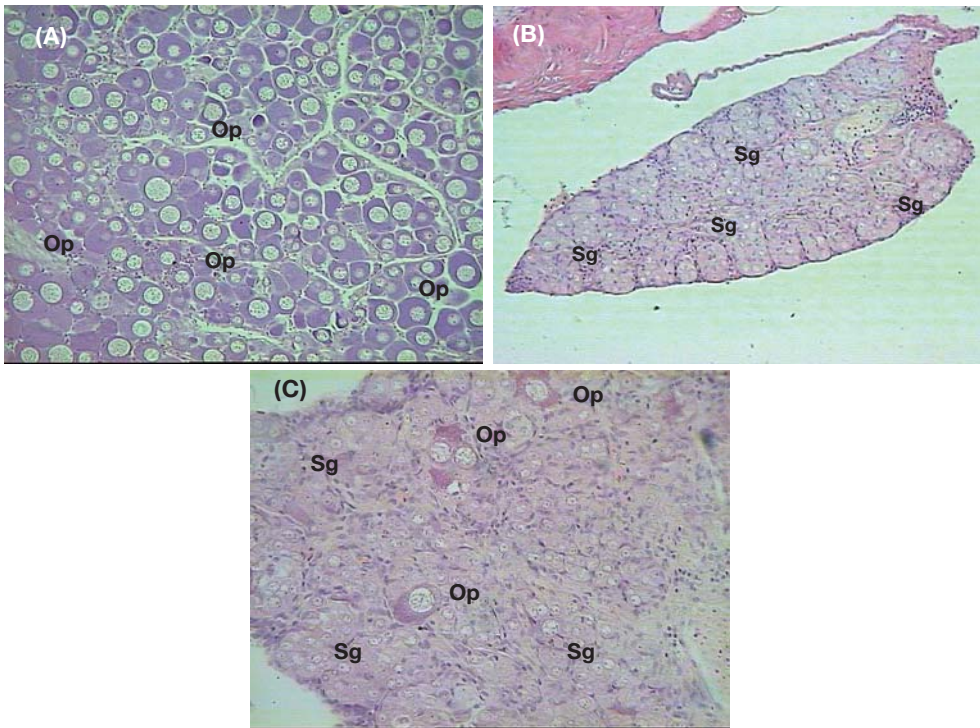


Fig. 2. Gonads of a pikeperch exposed for 35 days to 100 ppm nonylphenol. A – ovaries with previtellogenic oocytes; B – testis with spermatogonia and spermatocytes; C – feminised testis contained spermatogonia and previtellogenic oocytes. Op – previtellogenic oocytes, Sg – spermatogenic seminiferous lobules.

DISCUSSION

Gonadal differentiation can take various forms in fish and can range from species in which individuals develop as males or females and remain the same sex throughout life (gonochoristic species) to hermaphroditic species that contain functional male and female tissues at different or at the same times during life (Devlin and Nagahama 2002). In mammals, gonadal differentiation proceeds down a single developmental pathway to yield completely differentiated ovaries or testes (Capel 1998). In contrast, the development of fish gonads can be influenced by fluctuations in internal and external environmental factors. The disruption of these conditions leads, for example, to intersex gonad production in typically gonochoristic fishes. The induction of abnormal gonads is usually achieved through the manipulation of genetic or endocrine conditions (Lepori 1980, Devlin and Nagahama 2002). In gonochoristic fish, testis-ova are

often induced following injections or implants of steroids or dosing via food or the water phase under both laboratory and aquaculture conditions (Devlin and Nagahama 2002).

Some authors (Lepori 1980, Yano 1995) are of the opinion that abnormal hermaphroditism occurs occasionally in gonochoristic fishes under natural conditions. Schultz (1996) reported that intersexes were identified at a low frequency (about 2%) in wild populations. However, in the last decade more and more incidences of intersex fish have been observed (Purdom et al. 1994, Jobling et al. 1997). These fish were collected from polluted reservoirs where pseudo-estrogenic substances, such as alkylphenols, were detected (Lye et al. 1997). The induction of intersex and neofemale (genetically male) fish can be achieved under experimental conditions by exposing juvenile fish to an aqueous solution of 4-nonylphenol (Gray and Metcalfe 1997, Tanaka and Grizzle 2002, Demska-Zakęś 2005).

The current results indicate that it is possible to alter gonadal differentiation in pikeperch through NP oral treatment during the early stage of ontogenesis. Even a low NP dose (0.1 mg kg^{-1} diet) induced the formation of intersex gonads, but only when the treatment phase started in fish at age 28 DPH and lasted for more than 28 days (>56 DPH). An increase in the nonylphenol dose and exposure time reduced the percentage of males and increased the percentage of intersex fish. In contrast, no alterations in the phenotypic sex ratio were observed when NP treatment started in fish at age 28 DPH and lasted from 7 to 28 days (fish age from 35 to 56 DPH). The present data concur with the results obtained in previous studies by Demska-Zakes and Zakes (1997, 1999). The sex reversal effect in pikeperch was achieved when androgen (17 α -methyltestosterone or 11 β -hydroxyandrostenedione) treatment started at 56-63 DPH (initial body weight above 2 g). Testis-ova formation can be induced by 4-nonylphenol in fish provided the treatment takes place during sexual differentiation (Demska-Zakęś and Zakęś 1995).

The present data show that a permanent feminizing effect was not achieved by the oral administration of 4-nonylphenol. However, neofemales have been experimentally induced following NP exposure via the water phase (Gray and Metcalfe 1997, Tanaka and Grizzle 2002, Demska-Zakęś 2005). It is probable that gonad sensitivity to 4-nonylphenol administration depends not only on dose and exposure time but also on fish species and the method of 4-nonylphenol application. Exposure to the NP levels

employed in the present study did not cause mortality, but it did affect fish growth. Similar observations were made in NP-treated pikeperch by Demska-Zakęś (2005) and in Japanese medaka by Gray and Metcalfe (1997).

The effects of endocrine disruptor chemicals, such as alkylphenols, detected to date in fish range from disturbed gonadal maturation and abnormal levels of vitellogenin in adult plasma to intersex gonads (Kinnberg et al. 2000, Weber et al. 2002, Demska-Zakęś et al. 2005). The intersex fish had deformed reproductive systems, which might have prevented the production of normal ova and milt. However, the chronic activity of 4-nonylphenol can lead to negative changes in the sexual structure, and, consequently, the disappearance of natural fish populations.

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STRESZCZENIE

JAJNIKO-JĄDRA U SANDACZA (*SANDER LUCIOPERCA* (L.)) EKSPONOWANEGO NA DZIAŁANIE 4-NONYLOFENOLU

Wzrost zanieczyszczenia środowiska różnego rodzaju związkami chemicznymi, a zwłaszcza tzw. steroidami środowiskowymi może prowadzić do negatywnych zmian w strukturze populacji poszczególnych gatunków ryb, a nawet do ich wyginięcia. Celem niniejszych badań było określenie wpływu nonylofenolu podawanego w pokarmie na tempo wzrostu, śmiertelność oraz rozwój układu płciowego sandacza. Materiał badawczy stanowiły niezróżnicowane płciowo sandacze, które podchowiano w obiegu recyrkulacyjnym. Począwszy od 28 dnia po wykluciu (średnia masa jednostkowa ryb 0,3 g) ryby żywiono paszą sztuczną zawierającą nonylofenol (dawka ksenobiotyku 0,0, 0,1, 1,0, 10,0 lub 100 ppm). Czas ekspozycji wynosił od 7 do 63 dni. Stwierdzono, że nonylofenol nie wpłynął istotnie na tempo wzrostu, kondycję i śmiertelność sandacza (tab. 1). Powodował natomiast zaburzenia w przebiegu dyferencjacji płci, przyczyniając się do powstania osobników biseksualnych, co z kolei wpłynęło istotnie na zmiany w strukturze płci w grupach doświadczalnych (rys. 1, 2). Zakres tych zmian zależał od czasu ekspozycji ryb na działanie ksenobiotyku oraz jego stężenia.