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Experimental exposure to treated municipal effluent affects papillomatosis intensity in male roach

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Running title: Effluent exposure and fish papillomatosis

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Abstract

Connection between contaminants and papillomatosis in fish has been observed in field studies but experimental evidences are scarce. We studied changes in intensity of epidermal papillomatosis in roach (*Rutilus rutilus* L.) after exposure to treated pulp mill and municipal effluents in two experiments (1 and 2). Moreover, relative amount of heat shock protein 70 (HSP70) was analyzed in Experiment 2 to evaluate cellular level stress response in fish. At the Experiment 2 the highest papillomatosis intensity change was in male roach exposed to 15 % concentration of treated municipal effluent and was significantly higher than in 1.5 % effluent exposed male, but not significantly higher than in 0 % effluent exposed control male. Interestingly, the increasing effect of effluents seemed to be more pronounced in male fish than in females in all the experiments conducted. This could indicate endocrine disrupting substances in the effluents. There were no differences in HSP70 expression between exposure groups. Present results indicate that treated municipal effluents can promote papillomatosis in fish in environmentally relevant concentrations.

Keywords: roach, disease, HSP70, sewage effluents, environmental stress, sex differences

Introduction

Several studies have established that pulp and paper mill and sewage effluents affect organisms at biochemical, physiological and population levels, including immunosuppressing effects of effluents (e.g. Wester et al., 1994; Price et al., 1997; Aaltonen et al., 2000; Karels et al., 2001; Oakes, et al., 2004). In recent years there has been concern of substances in effluents called "endocrine disrupters", which mimic or antagonise steroid hormones. For example, treated sewage effluents including these substances have shown to cause intersexuality, reproductive and development disturbances in roach *Rutilus rutilus* L. (Jobling, et al 1998; Liney, et al 2005; Rodgers-Gray, et al 2001). Moreover, these artificial or natural endocrine disrupting chemicals present in effluents may hormonally interfere for example with the immunity of fish (Savino and Dardenne 1995; Slater, et al 1995; Watanuki, et al 2002) and cause possible outbreak of fish diseases.

Papillomatosis is rather common disease in several fish species and the disease has presumably viral etiology (for reviews see: Anders and Yoshimizu 1994; Getchell, et al 1998). It has been suspected that development of papillomatosis in fish is multifactorial and is affected by environmental stress and / or seasonal changes in endocrine activity by the impairment of immunity of the fish, and / or direct carcinogenic effects of substances (Baumann 1998; Baumann, et al 1996; Lee and Whitfield 1992; Anders and Yoshimizu 1994; Sano, et al 1993). Field studies have demonstrated higher papilloma prevalence in fish populations exposed to environmental contaminants, including industrial and municipal effluents (Baumann, et al 1996; Hayes, et al 1990; Kortet, et al 2002; Mikaelian, et al 2000; Premdas, et al 1995; Smith, et al 1989; Vethaak, et al 1992). Therefore, papillomatosis of fish has been proposed as an indicator of contaminated waters (e.g. Baumann, et al 1996; Korkea-aho, et al 2006a). Papilloma prevalence increased in caged black bullheads (*Ictalurus melas*) in final oxidation pond of domestic chlorinated wastewater (Grizzle, et

al 1984). However, experimental studies on the effects of industrial or municipal effluents on the development of papillomatosis have not been conducted since.

It has not been previously studied whether the stressed fish would show both enhanced development of papillomatosis and increased expression on cellular level stress. We wanted to further explore possible cellular level stress in fish exposed to treated municipal effluent by measuring Heat shock protein 70 (HSP70) expression in gills of fish. HSP70 family (68 - 73 kDa) is one of the most extensively studied heat shock proteins. The HSP70 has constitutive function in animal cells, but it is also known to be up-regulated in cells which are exposed, as first discovered, to heat shock and several other abiotic and biotic stressors (for reviews see: Iwama, et al 1998; Basu, et al 2002). Moreover, some studies have shown fish exposed to bleached kraft pulp mill effluent (BKME) having the enhanced expression of HSP70 protein in tissues (Janz, et al 1997; Vijayan, et al 1998). However, the use of HSP70 as indicator of environmental stress is not always straightforward (Iwama, et al 2004). For example, Porter & Janz (2003) did not notice difference in HSP70 protein levels in longear sunfish (*Lepomis megalotis*) between municipal sewage treatment effluent impacted site and reference site.

Papillomatosis has been proposed as indicator of environmental stress, which is often caused by anthropogenic effluents in aquatic environments (Baumann, et al 1996; Korkea-aho, et al 2006a). However, experimental studies of effluent exposure effects on fish papillomatosis are rare. Our aim was to explore the effect of environmentally relevant concentrations of pulp mill and municipal effluents on roach papillomatosis intensity in laboratory conditions. Beside of this organism level stress response -the change in the intensity of papillomatosis- we wanted to investigate the possible cellular level stress response in fish from the HSP70 protein expression levels in gills.

Materials and Methods

Fish and Effluents

Roach were exposed to treated municipal and pulp mill effluents in year 2005 (Experiment 1) and treated municipal effluents in year 2006 (Experiment 2). The experiments were performed with the permission of the National Laboratory Animal Centre, Finland (Permission No. STO222-99, 05-11 and STO222-99, 06-22, for years 2005 and 2006, respectively). Roach were collected from Lake Kallavesi by fish traps between 17th of February and 1st of March 2005, for Experiment 1 and by fish traps or ice-fishing between 24th and 31st of March 2006, for Experiment 2. After the fish were caught, they were transported to a laboratory tank with flow-through Lake Kallavesi water. Diseased (with visible papillomas) and sexually mature fish were transported to plastic tanks (70 L in volume, 60 cm in diameter), 15 fish for each tank. The tanks had standing water with varying dilutions of effluent and Lake Kallavesi water. Chemical properties of Lake Kallavesi water, pulp mill effluent and municipal effluent are given in Table 1. Aeration and biological filtering of water in the tanks were done by internal biological filter (Magic Jet 380, Resun®). The volume of water in tanks was set to 60 L and tanks were partly shaded from top to prevent direct light into the tanks. Fish were fed excess with commercial pellets (BioMar, Ecoline pellet) in all the experiments. Half of the water in the tanks was changed and filters rinsed with lake water once a week in Experiment 1 and every fourth day in Experiment 2. The treated effluents and their chemical characteristics (Table 1) were provided by Powerflute Oy Savon Sellu, which produces semi chemical pulp, and Lehtoniemi wastewater treatment plant, which treats municipal wastewaters from the city of Kuopio. Both plants treat the effluents mainly mechanically and biologically (clarification and activated sludge-treatment) prior to discharge into recipient water. At start of both experiments fish were anhestized with tricaine methanesulfonate 10 % solution (MS-222, Sigma). Fish were tagged by fin cutting, weighed, length measured and intensity of papillomatosis estimated as scale coverage (Korkea-aho, et al 2006b). At the end of all experiments fish were killed with blow on the head, they were weighted, length measured, intensity of

papillomatosis counted as in the beginning of experiments and sex determined from the gonads. At the Experiment 2 all gill filaments from the other side of fish were excised, immediately frozen into liquid nitrogen and stored in -70 °C freezer for HSP70 analyses.

Experiment 1

At the Experiment 1, two exposure trials were conducted: (a) exposure to pulp mill effluent was started 10th of March 2005, and spread for 19 days (b) treated municipal effluent exposure experiment started 30th of March 2005, and spread for 38 days. For both exposure trials two exposure groups were set up in two replicate tanks, with 15 fish in each, a total of 30 fish per exposure group: fish were kept in 10 % concentration of effluent and control group was kept in Lake Kallavesi water with 0 % concentration of effluent. The average length of fish (± SE) used in pulp mill effluent and municipal effluent exposure was 161.3 ± 2.3 mm and 161.3 ± 1.5 mm, respectively. Water oxygen level, temperature (OxyGuard® oxygen electrode) and pH (Consort P901 pH meter) were monitored three times a week for every tank. Average (±SD) temperature in pulp mill effluent exposure was 7.1±1.1 °C and 7.8±0.8 °C (n = 8 days), oxygen level ranged for 9.8±1.6 mgO_2/L and 9.8±2 mgO_2/L (n = 8 days) and pH value was 6.9±0.1 and 6.6±0.1 (n = 7 days) for 10 % pulp mill effluent exposure and control tanks, respectively. Average (±SD) temperature in municipal effluent exposure was 9.3±0.8 °C and 9.5±0.8 °C (n = 16 days), oxygen level ranged for 8.3±1.2 mgO_2/L and 8.9±0.8 mgO_2/L (n = 16 days) and pH value was 7.0±0.1 and 6.8±0.2 (n = 16 days) for 10 % municipal effluent exposure and control tanks, respectively.

Experiment 2

The Experiment 2 for the treated municipal effluent exposure started on 6th of April, 2006 and continued for 22 days. Three groups were set up, in the base of pilot study, with three replicate tanks and 45 fish per exposure and two replicate control tanks with 30 fish: 15 %-, 1.5 %- and

control group (n = 120 fish). The average length of fish (±SE) was 161.2 ± 1.5 mm. Oxygen level and temperature was measured (OxyGuard® oxygen electrode) every weekday and pH was measured (Consort P901 pH meter) twice a week. Average (±SD) temperature was 8.3±0.6 °C, 9.0±0.5 °C and 8.6±0.5 (n = 17 days), oxygen level was 9.6±1.5 mgO_2/L, 9.1±1.4 mgO_2/L and 9.9±1.2 mgO_2/L (n = 17 days), pH value was 7.3±0.2, 7.2±0.2 and 7.2±0.2 (n = 6 days) for 15 %-, 1.5 % municipal effluent exposure and control tanks, respectively.

Semi-quantative HSP70 analysis

Heat shock protein 70 (HSP70) was semi-quantifiably measured from roach gills by western immunoblot analysis following mainly procedure described by Vehniäinen, et al (2003). Prior the analysis gills were unfrozen and couple of arches were homogenized in a glass homogenizer on ice with potassium gluconate buffer (5mmol/l $MgSO_4$, 5mmol/l NaH_2PO_4, 40 mmol/l HEPES, 70 mmol/l potassium gluconate, 150 mmol/l sorbitol, pH 7.8). The homogenate was centrifuged at 1000 x g for 5 min. Total protein concentration was measured by DC Protein Assay (BioRad) according to manufactures standard assay protocol, absorbance of sample was read at 750 nm wavelength.

HSP70 was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with 25 µg protein per lane using a Mini-Protean II apparatus (Bio-Rad) (e.g. Laemmli 1970). Samples were diluted in buffer solutions 1:1 (0.125 mol/l Tris-HCl, 2 % SDS, 20 % glycerol, 0.02 % bromophenol blue, 5 % 2-mercaptoethanol), heated in 95 °C for 4 min and loaded onto gel. The positive control was cadmium exposed roach by intraperitoneal injection, which was shown to express HSP70 in previous analysis. Proteins were run at 200 V for 45 minutes on a SDS-PAGE gel. After that gels were equilibrated in blotting buffer (25 mmol/l, 192 mmol/l glysine, 20 % methanol, pH 8.3) and proteins were transferred to a nitrocellulose membrane (Hybond ECL, Amersham Biosciences) using a BioRad Mini-Protean II apparatus at 100 V for 70

min. To confirm successful protein transfer, membrane was stained with 0.2 % Ponceu S in 3 % trichloroacetic acid for 5 min after blotting.

Membrane was washed twice, for 5 min each, with Tris-buffered saline-Tween (TBST; 0.9% NaCL, 10 mmol/l Tris, 0.1 % Tween 20) and incubated in blocking buffer (3 % gelatine in TBST) either 1 hour in room temperature or over night at + 4°C. After this blots were reacted with HSP70 monoclonal antibody (MA3-006, Affinity BioReagents Inc.) with a dilution of 1:5000 in TBST with 1 % bovine serum albumine (BSA) added for 1 h. After the blot was washed with TBST (1 x 15 min, 3 x 5 min), it was reacted with goat anti-mouse total immunoglobulin G (IgG) peroxidase conjugate (DC08L, Calbiochem; 1:5000 in TBST). Blots were again washed (TBST, 1 x 15 min, 3 x 5 min), detected with ECL Plus Western Blotting detection reagents (Amersham Biosciences) and chemifluorescence visualized with Typhoon 9400 Imager (Amersham Biosciences) scanned with excitation at 450 nm and emission at 488 nm.

Semi-quantification of western blots was performed with image analysis software ImageQuant TL v2005 (Amersham Biosciences). Blots were compared to positive control by normalisation, where positive control band volume was set to 100 and the unknown bands were expressed as a percentage of the known positive control band.

Statistics

Differences in mortality of fish between the treatment groups were analyzed using χ^2-test. All the fish which died during the experiment were excluded from further statistical analyses. Also one fish in Experiment 2 was excluded, because sex was not determined. Male and female fish were tested separately by analysis of covariance (ANCOVA) for the change in the intensity of papillomatosis during the experiments since sex affects papillomatosis in roach (Korkea-aho, et al 2006a; Kortet, et al 2002). Data from replicate tanks within the same treatment were combined

prior to final analyses if the analysis of variance (ANOVA) revealed no differences between the tanks in fish length or change in the intensity of papillomatosis during the experiment.

The effects of treatments on the development of papillomatosis intensity were analyzed using ANCOVA with the change in the intensity of papillomatosis during the experiment as a response variable, treatment as a fixed factor and both fish length and intensity of papillomatosis in the beginning of the experiment as covariates. For the Experiment 2, differences between exposure groups were analyzed by Bonferroni post hoc test. The following transformations were made to meet the assumptions of ANCOVA in Experiment 1: Lg (papillomatosis intensity in the beginning), Lg (length) and for the municipal effluent experiment 2006: Lg (intensity change + 10), Lg (papillomatosis intensity in the beginning + 1) and Lg (length). At the Experiment 2, the differences in relative amount of HSP70 between exposure groups were analyzed by ANOVA for all the fish and separately for male fish. Female fish were not tested separately because of too low number of specimens per exposure group for statistical tests. The value for relative amount of HSP70 was logarithm transformed prior the analysis to meet the assumptions of ANOVA. All the statistical tests were performed using SPSS for windows 13.0 (SPSS Inc, USA).

Results

Effluent exposure

At the Experiment 1 between 0 % and 10 % concentrations of effluents, mortality was not significant between treatments in pulp mill or municipal effluent experiments. In both experiments two fish died. None of the fish died during the Experiment 2.

In the pulp mill effluent exposure at Experiment 1, change in intensity of papillomatosis was at the same level in 10 % and 0 % exposure groups in male roach while the mean change in intensity of papillomatosis was higher in 0 % than 10 % exposure group in female roach (Fig. 1a). However, the change in the intensity of papillomatosis was not statistically different

between 10 % and 0 % exposure groups ($F_{(1,44)}$ = 0.002, P = 0.967 and $F_{(1,14)}$ = 0.982, P = 0.345 for male and female roach, respectively) (Fig. 1a). In the municipal effluent exposure at the Experiment 1, the interaction between variable 'treatment´ and covariate 'papilloma intensity in the beginning' was significant in males (ANCOVA, P = 0.008). This interaction could skew further results of ANCOVA. However, the covariate ´papilloma intensity in the beginning´ did not affect significantly in the model in males (ANCOVA, P = 0.300), so it was excluded from further analyses to eliminate the interaction in the analyses. The mean papillomatosis intensity change was negative in 0 % exposure group and increased in 10 % exposure group in male roach, while female roach had on average higher papillomatosis intensity change in 0 % than in 10 % exposure group (Fig. 1b). However, again ANCOVA revealed that there were no statistical differences in papillomatosis intensity change between exposure groups during the experiment ($F_{(1,40)}$ = 2.747, P = 0.106 and $F_{(1,18)}$ = 0.491, P = 0.494 for male and female roach, respectively) (Fig. 1b).

In Experiment 2, the ANCOVA model including factor by covariate interaction revealed that the interactions between 'treatment*length' and 'treatment*papilloma intensity in the beginning' were not statistically significant neither in male roach ($F_{(2,79)}$ = 1.711, P = 0.188 and $F_{(2,79)}$ = 0.818, P = 0.446, respectively) nor in female roach ($F_{(2,40)}$ = 1.829, P = 0.177 and $F_{(2,40)}$ = 1.326, P = 0.280, respectively) . This indicated that the effects of covariates length and intensity of papillomatosis in the beginning of the experiment did not differ between the treatments neither in male nor female roach. Therefore, ANCOVA was continued with full factorial model where length ($F_{(1,79)}$ = 0.078, P = 0.780 and $F_{(1,40)}$ = 1.844, P = 0.183 for male and female roach, respectively) had no significant effect, but disease intensity in the beginning ($F_{(1,79)}$ = 92.529, P < 0.001 and $F_{(1,40)}$ = 39.508, P < 0.001 for male and female roach, respectively) had a significant effect on the change of papillomatosis intensity during the experiment for both male and female roach. For the female roach, the mean papillomatosis intensity change was very similar in every exposure group ($F_{(2,40)}$ = 0.158, P = 0.854) (Fig. 2). However, for the males highest mean papillomatosis intensity change

was noticed in 15 % exposure group and lower in 1.5 % and 0 % exposure groups (Fig. 2). ANCOVA revealed a statistically significant difference in the change of papillomatosis intensity between exposure groups among male roach ($F_{(2,79)} = 4.311$, $P = 0.043$). Further analysis with Bonferroni Post Hoc test showed that there was a significant difference in the change of papillomatosis intensity in males between 15 % exposure group and 1.5 % exposure group ($P = 0.014$), but the 0 % exposure group did not differ in papillomatosis intensity change from either 15 % exposure group ($P = 0.725$) or 1.5 % exposure group ($P = 0.354$) (Fig. 2).

HSP70 analysis

For some individuals western blot detected two HSP70 isoforms in gill tissue (Fig. 3). From the analysed individuals there were two roach in 15 % exposure group, two roach in 1.5 % exposure group and one roach in 0 % exposure group which expressed two isoforms of HSP70. The Heat Shock Protein 70 (HSP70) antibody detects proteins from ~70 kDa to ~78 kDa representing different members of the HSP70 family (MA3-006, Affinity BioReagents Inc. Product Data Sheet). Thus, both bands were used for comparison of immunoreactive bands volume in image analysis, when applicable. Although the mean relative amount of HSP70 was higher in 1.5 % and 15 % exposure groups than in 0 % exposure group (Fig. 4), the differences between exposure groups were not statistically significant (ANOVA, $F_{(2,24)} = 0.999$, $P = 0.384$). When only males were compared the highest mean relative amount of HSP70 were also in 15 % and 1.5 % exposure groups (137 ± 27, $N = 5$ and 137 ± 16, $N = 7$, respectively) and lowest in 0 % exposure group (117 ± 16, $N = 5$). Although there was no difference between exposure groups in males neither (ANOVA, $F_{(2,14)} = 0.287$, $P = 0.755$).

Discussion

At the Experiment 2 the highest average papillomatosis intensity change was observed in the male roach exposed to highest effluent concentration. The present result is in concordance with several field studies which have shown that the prevalence of papillomatosis in fish populations is increased by contaminants (Baumann, et al 1996; Hayes, et al 1990; Kortet, et al 2002; Mikaelian, et al 2000; Premdas, et al 1995; Smith, et al 1989; Vethaak, et al 1992). Most recently the connection was found between higher papillomatosis prevalence in roach populations and lake sites which were impacted by industrial and / or sewage effluents (Korkea-aho, et al 2006a). Beside the connection with effluents and fish papillomatosis found in the field studies, only Grizzle et al. (1984) has studied before this connection experimentally. They observed that papillomatosis was affected in black bullheads (*Ictalurus melas*) caged in final oxidation pond of domestic wastewaters and suspected that this was mainly due the chlorination of wastewater as after the amount of carcinogenic chlorine decreased in wastewater also prevalence of papillomas decreased (Grizzle et al. 1984). However, chlorination was not applied in the treatment of effluents used in the present experiment. Nowadays the chlorination is less applied wastewater treatments and basically the effluents are treated by activated sludge treatment.

In the present study, the papillomatosis intensity change was significant only between 1.5 % and 15 % exposure groups in male fish. The absence of statistical significance between 15 % and 0 % exposure group is difficult to explain. It is known that papillomas in fish are spontaneously regressing and proliferating (Premdas and Metcalfe 1994; Getchell, et al 1998), so the observed proliferation of papillomas also in 0 % exposure group in males is not a new phenomenon, especially for the feral fish transferred to laboratory. This spontaneous regression and prolifiration of papillomas can induce high variation in the change of papillomatosis intensity between individuals and makes it more difficult to analyse statistically. Moreover, papillomatosis in fish is known to fluctuate seasonally (for review see Getchell et al 1998) and seasonal changes in the prevalence and intensity of papillomatosis has also been noticed in roach (Kortet et al. 2002). The

experiments in the present study were done in different time during the spring and this further explains the differences in papillomatosis intensity in 0 % exposure groups between exposure trials and experiments. Furthermore, papillomatosis intensity change in roach is also affected by fish length (Korkea-aho, et al 2006b) and the connection between the initial papillomatosis intensity and the papillomatosis intensity change were noticed during the present experiment. These variables: fish length and initial papillomatosis intensity, were controlled in the statistical analysis used by using them as covariates.

In this study at the both experiments there were differences in papillomatosis intensity change between male and female roach in relation to exposure. It was almost like exposure to effluents did not affect average papillomatosis intensity in female fish, or was even suppressing the disease intensity in females at Experiment 1, but average papillomatosis intensity change was higher in all exposure groups compared to control group in male roach. Even though significant differences between groups were not always reached, the similar patterns observed in papillomatosis intensity change in all experiments strengthen the conclusions. Papillomatosis peaks during the spawning time of fish (Anders and Moeller 1985; Kortet, et al 2002; Anders and Yoshimizu 1994) and both prevalence and intensity of papillomatosis in roach has shown to be affected by sex, being higher in males (Korkea-aho, et al 2006a, b; Kortet, et al 2002). Furthermore, Kortet et al (2003) found higher testosterone levels in papilloma diseased male roach than healthy male roach. Although Premdas et al. (2001) did not found correlation between circulating steroid hormones and papillomatosis in white sucker (*Catostomus commersoni*) in field study; experimentally they induced papillomatosis by testosterone and 17β-oestradiol injections. Noticed sex-difference in the present research could indicate that there might be endocrine disrupting substances in the effluents. Endocrine disrupting substances are known to be present in different industrial and municipal effluents, acting both androgenic and estrogenic agonists and antagonist (Jobling, et al 1998; Parks, et al 2001; Tyler, et al 1998). Jobling et al (1998) have found

connection between estrogenic chemicals in sewage effluents and a high incidence of intersexuality in roach. Aaltonen et al (2000) exposed roach to primary and secondary treated kraft mill effluents in laboratory and noticed that inhibitory effect for immunity was more obvious in male than female roach. Also in rainbow trout (*Oncorhynchus mykiss*) and carp (*Cyprinius carpio* L.) immunity responses were suppressed when exposed to sewage effluents (Hoeger, et al 2005; Price, et al 1997), and interestingly female rainbow trout experienced more severe immunosuppression than males (Hoeger, et al 2005). Endrocrine disrupting substances in the effluent can also affect by suppressing immunity of fish (Savino and Dardenne 1995; Watanuki, et al 2002). Especially androgens are known to be immunosupressive (Slater, et al 1995) which might further explain the difference in disease response between sexes and why male roach seems to be more vulnerable for the disease. However, the immuno-endocrine interactions are complex and immunosupressive effect of steroids are not always evident (Law, et al 2001; Vainikka, et al 2004). Unfortunately there is no further evidence of possible presence of endocrine disrupting chemicals in the effluent used in this study and more research is needed to clarify the connection between papillomatosis induction in male roach and effluent exposure. Furthermore, we cannot exclude the possibility that there could be also tumor promoting genotoxic substances present in the effluents (White and Rasmussen 1998). Although this option would not explain the observed sex-difference in papillomatosis intensity change.

Environmental stress as such, caused by effluents in water, might be part of explanation between effect of effluents and papillomatosis intensity change in roach. The effluents might cause secondary response due to stress and effect on fish papillomatosis also by suppressing the immune system of fish (Anderson 1990). In the present experiment fish did not show any obvious signs of stress, such as increased mortality. Moreover, even though HSP70 expression levels were somewhat higher in 15 % and 1.5 % exposure groups than in 0 % exposure group the difference was not statistically significant so one might conclude that there were no obvious

differences in cellular level stress in fish between exposure groups. Though, immune system of fish has been shown to be affected when exposed to effluents (Aaltonen, et al 2000; Fatima, et al 2001; Hoeger, et al 2005) and possible immunosuppression could be also due the direct consequences of exposure to immunotoxic substances in the effluent (Price, et al 1997). Hence, HSP70 expression has been shown to be induced by several environmental contaminants in fish (e.g.Vijayan, et al 1998; Basu, et al 2002). Porter & Janz (2003) noticed impairment of reproductive endocrine homeostasis, for example increases in serum testosterone, in male longear sunfish (*Lepomis megalotis*) exposed to treated municipal sewage discharge in field, while HSP70 expression was not affected. Although sex-related differences has been noticed in HSP70 expression levels in bleached kraft mill effluent (BKME) exposed juvenile chinook salmon (*Oncorhynchus tshawytscha*) (Afonso et al. 2003) and in BKME exposed female white sucker had elevated plasma 17 β-estradiol and increase in HSP70 mRNA (Janz et al. 1997). Thus, in this experiment enhanced HSP70 expression levels in male fish were not noticed neither.

In the present experiment HSP70 expression was quite high also in fish at 0 % exposure group (control group), and lack of statistical significance between exposure groups do not necessarily mean lack of stress, but rather several other stressors might have contributed to HSP70 expression also in 0 % exposure group. The roach used in this study were collected from nature, and though, physiological stress such as handling should not affect levels of HSP70 expression (Basu, et al 2002), the history of previous exposures of caught fish is not known. Moreover, connection of fish diseases and expression of HSP70 has been noticed (Ackerman and Iwama 2001) and all the fish used in this study were affected by papillomatosis.

The interesting observation in the present experiment was expression of two isoforms of HSP70 in some roach, regardless of exposure group. These immunoreactive bands could probably be constitutive and inducible forms of HSP70. Both forms of HSP70 enhance their expression after heat shock in rainbow trout (*Oncorhynchus mykiss*) cells, while expression of

inducible form is more remarkable (Ojima, et al 2005). However, it is not possible to distinguish between these HSP70 forms with the present methods and specie used in this study.

The etiology of epidermal papillomatosis in feral fish is complex and present results suggest that papillomatosis in roach is also contributed by environmentally relevant concentrations of effluents. Especially observed difference in papillomatosis intensity change between male and female roach suggest that there could be endocrine disrupting agents present in the effluents. While major concern is the disrupting effect of estrogenic chemicals for maturation and reproduction of fish (Jobling et al., 1998; Folmar et al. 2001; Liney et al. 2005), also known ability of steroid hormones to suppress the immune system of fish (Savino and Dardenne 1995; Slater, et al 1995) might have population-level effects on outbreak of fish diseases. Biomarkers of such substances are needed to observe whole organism end points in fish (Hutchinson, et al 2006). Roach is widely distributed, easy to catch and rather sedentary species and papillomatosis does not effect on survival of roach (Kortet, et al 2003) which makes roach-papillomatosis system quite promising biomarker for estimation of organism end-point in natural fish populations. However, more experimental studies are needed to investigate the causal link between fish diseases and effluent exposure.

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Table 1. Chemical variables of the waters. ND = Not Detected, NA = Not Analysed

	Lake Kallavesi inflow water	Pulp mill effluent	Municipal effluent	
	2005	2005	2005	2006
pH	6.9	7.2	7.6	NA
Conductivity, mS/m	72	237	121	NA
BOD_7, mg/L	NA	NA	8	7,9
COD_{cr}, mg/L	12	390	NA	NA
Tot-N, mg/L	0.83	160	66	57
NH_4-N, mg/L	ND	200	61	47
Tot-P, mg/L	0.30	0.12	0.35	0.43

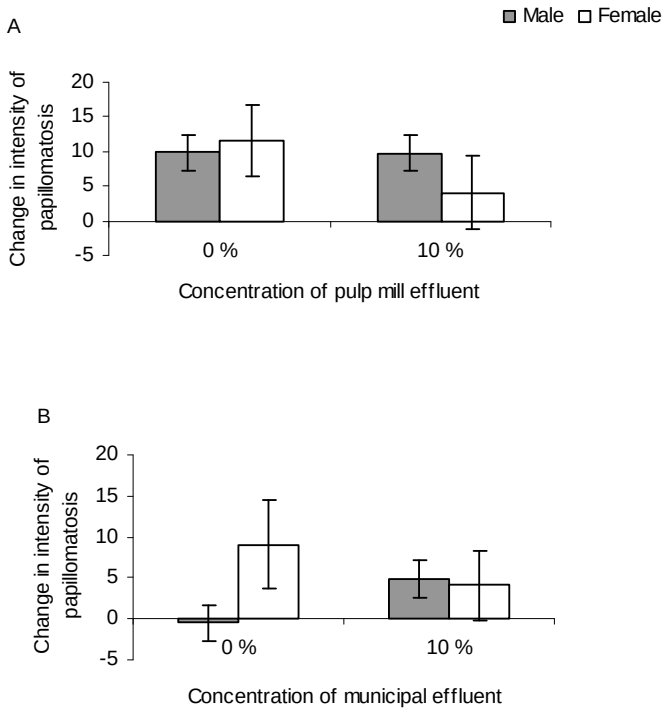
A
Male
Female
Change in intensity of papillomatosis
20
15
10
5
0
-5
0 %
10 %
Concentration of pulp mill effluent
B
Change in intensity of papillomatosis
20
15
10
5
0
-5
0 %
10 %
Concentration of municipal effluent

Fig. 1

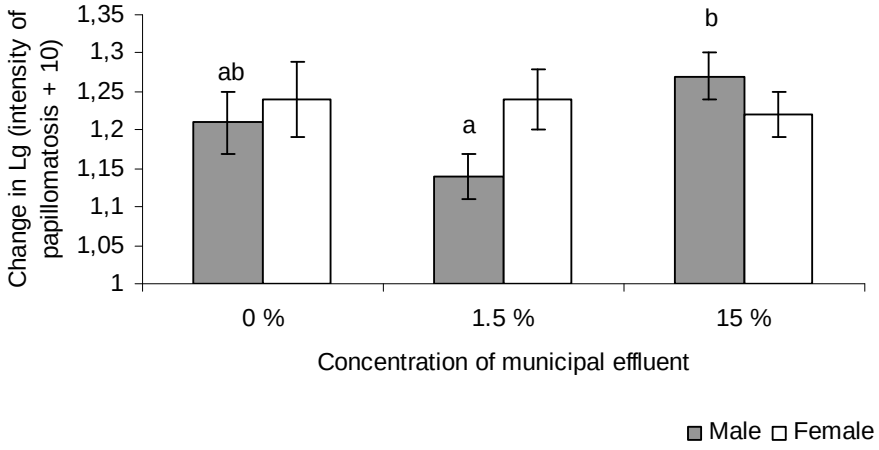
Change in Lg (intensity of papillomatosis + 10)
1,35
1,3
1,25
1,2
1,15
1,1
1,05
1
ab
a
b
0 %
1.5 %
15 %
Concentration of municipal effluent
Male
Female

Fig. 2

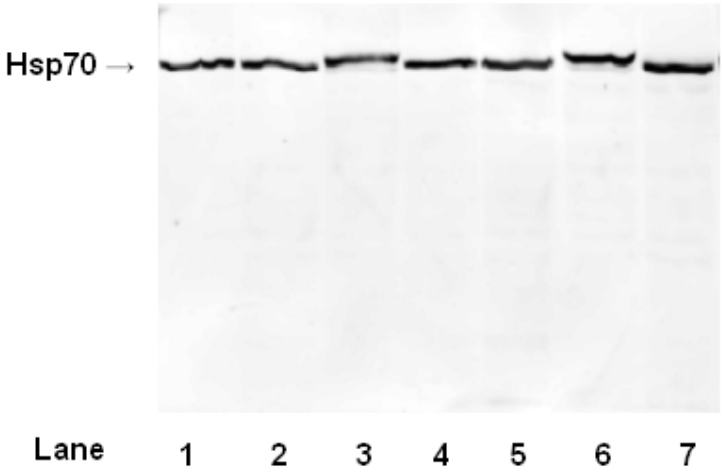

Fig. 3

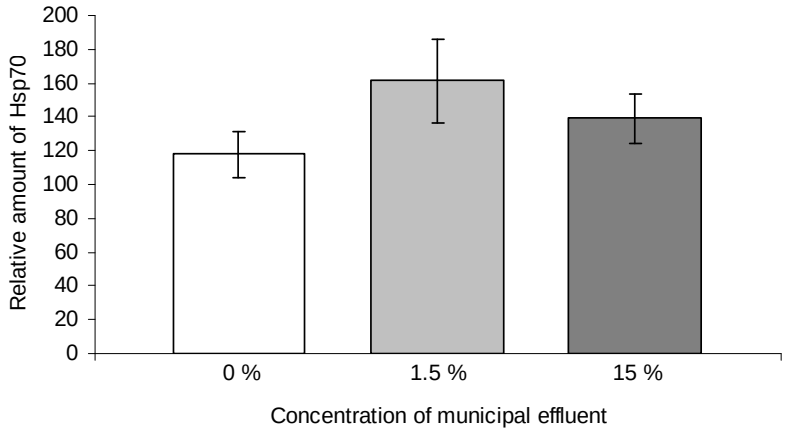

Fig. 4

Figure legends

Fig. 1. Change in intensity of papillomatosis in roach exposed to 10 % and 0 % concentrations of municipal effluent (A) and pulp mill effluent (B) in the effluent exposure pilot study in 2005 (Experiment 1).

Fig. 2. Change in intensity of papillomatosis (logarithm transformed) in roach exposed to 15 %, 1.5 % and 0 % concentrations of municipal effluent in 2006 (Experiment 2).

Fig. 3. Western blots of the HSP70 protein expression in gill tissue of seven roach individuals. Lanes 3 and 6 show expression of two isoforms of HSP70 family.

Fig. 4. Relative amount of HSP70 in 15 % (n = 9), 1.5 % (n = 10) and 0 % (n = 6) concentration of municipal effluent in 2006 (Experiment 2).