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Promotion by 17β-estradiol and β-hexachlorocyclohexane of hepatocellular tumors in medaka, *Oryzias latipes*

J.B. Cooke, D.E. Hinton *

Department of Anatomy, Physiology and Cell Biology, School of Veterinary Medicine, University of California-Davis, Davis, CA, 95616, USA

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Abstract

A feature common to many laboratory and field studies with various fish species is a higher prevalence of hepatocellular neoplasia in females than in males. During female sexual maturation, endogenous estrogens stimulate substantial increases in synthetic activity, including production of vitellogenin and choriogenin and proliferation of hepatocytes. We tested the hypothesis that estrogens, either natural or xenobiotic, promote growth of hepatic preneoplastic lesions and tumors. Medaka (*Oryzias latipes*) were exposed to a low dose of the carcinogen diethylnitrosamine (DEN; 200 μg l^{-1} aqueous bath for 24 h) at 3 weeks of age, then fed purified casein-based diet or the same diet containing 17β-estradiol (E2; 0.01–10.0 μg g^{-1} dry diet) or a xenoestrogen, β-hexachlorocyclohexane (βHCH, 0.01–100.0 μg g^{-1} dry diet) daily from 1 to 7 months of age. Livers were removed, embedded in glycol methacrylate, step-sectioned, stained with hematoxylin and eosin, and examined for foci of cellular alteration (FCA) and hepatocellular tumors. E2 increased prevalences of hepatocellular adenoma or carcinoma (26% in DEN plus 10 ppm E2 group versus 4.6% in DEN only group, $P < 0.01$). With increasing level of E2, average numbers of basophilic FCA (BF) rose and numbers of eosinophilic FCA (EF) sharply declined. There were greater numbers of tumors in most and greater numbers of BF in all of the DEN plus βHCH treatment groups, but statistical analyses indicated no significant elevation in tumor or BF prevalence relative to treatment with DEN only. In all DEN-treated groups, BF were more common in female medaka and EF more common in males. No tumors were found in fish fed E2 or βHCH without DEN exposure. Among control medaka, liver weights were significantly larger in females, but treatment with 0.1, 1.0 or 10.0 ppm E2 elevated liver weights in males similar to that in females. βHCH had no effect on liver weights. This data shows E2 is a tumor promoter in medaka. Because tumor increases were not statistically significant, βHCH was considered a weakly positive modulator. E2 particularly promoted tumor development in male medaka, indicating xenobiotics with mechanism of action like that of E2 may escalate growth in wild fish of previously initiated cells into tumors.

Keywords: Tumor promotion; Hepatocarcinogenesis; Medaka; Estradiol; β-Hexachlorocyclohexane

* Corresponding author. Tel.: +1 530 7526413; fax: +1 530 7529692; e-mail: dehinton@ucdavis.edu

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1. Introduction

Production of hepatic tumors by single chemical agents has been studied in a variety of fish species (Metcalfe, 1989; Hawkins et al., 1995). Modulation of tumor prevalence, though, has been investigated primarily in rainbow trout (*Oncorhynchus mykiss*). Extensive information has been generated for this one species regarding modulation by factors of steroid hormones, antioxidants, diet components and chlorinated hydrocarbons (Bailey et al., 1987; Goeger et al., 1988; Orner et al., 1995). To expand knowledge of tumor development in fish, we examined tumor promotion using the medaka (*Oryzias latipes*) hepatocarcinogenesis model. Medaka differ from rainbow trout in that they are a warm, fresh and brackish water species with much shorter periods of tumor latency and time to reproductive maturity. Because of their small size, we are able to dedicate large numbers of medaka to each exposure level while generating minimal waste water and can examine livers thoroughly for histological alterations. Our laboratory has identified steps occurring during hepatic neoplasia in medaka following exposure to single carcinogens (Hinton et al., 1988). The following paper details the first time a two-step, initiation-promotion assay, in which a sub-carcinogenic dose of initiating carcinogen is followed by continuous administration of a tumor promoter (Farber, 1980), has been applied to the medaka model.

Hepatic tumors have been found in medaka following exposure to a variety of chemical carcinogens (Ishikawa et al., 1975; Aoki and Matsudaira, 1977; Egami et al., 1981; Hatanaka et al., 1982; Hinton et al., 1984; Hawkins et al., 1988, 1990; Hinton et al., 1988; Teh and Hinton, 1993). In most investigations, the effect of gender on tumor susceptibility was not examined. When results were separated by sex of fish, however, females were consistently shown more susceptible to tumor development than males (Masahito et al., 1989, Hinton et al., 1994; Teh, 1996). Similar susceptibility of females to hepatic neoplasia has been observed in other fish species including zebra danio (*Brachydanio rerio*) after laboratory exposure (Stanton, 1965), and in field surveys of common dab (*Limanda limanda* L.; Vethaak and van der Meer, 1991), European flounder (*Platichthys flesus*; Vethaak and Jol, 1996) and winter flounder (*Pseudopleuronectes americanus*; Murchelano and Wolke, 1991). Hepatic tumors occur more often in sexually mature female trout without overt carcinogen exposure (Takashima and Hibiya, 1972; Takashima, 1976) and following treatment with DDT (Hendricks, unpublished, in Nuñez et al., 1988). Sinnhuber et al. (1974) postulated that sex steroids stimulated growth of hepatic tumors in female rainbow trout exposed to aflatoxin M1.

The effects of steroid hormones on normal liver physiology suggest that endogenous estrogens may contribute to the increased susceptibility of female medaka to hepatic neoplasia. 17β-Estradiol (E2) controls the hepatic production and release of vitellogenin (VG), a phospholipoglycoprotein precursor of yolk protein (Ng and Idler, 1983). During sexual maturation, female liver undergoes substantial morphological and molecular alterations (Callard et al., 1980; van Bohemen et al., 1981; Selman and Wallace, 1983; Mommsen and Walsh, 1988). Elevated liver weights are characteristic of sexually-maturing female fish (Aida et al., 1973; van Bohemen et al., 1981; Ho, 1987; Mommsen and Walsh, 1988). Unlike females, liver participation in gonadal maturation of males is minimal and liver ultrastructure during this time is constant (Aida et al., 1973).

E2 has positively modulated tumor formation in the rainbow trout hepatocarcinogenesis model. E2 significantly promoted tumor incidence when administered after low doses of aflatoxin B1 (Nuñez et al., 1989). Post-carcinogen feeding of diet containing 20 ppm E2 also elevated hepatic tumor incidence relative to that in rainbow trout exposed to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) alone (Nuñez et al., 1988). To test the hypothesis that estrogen is a promoter of hepatic neoplasia in medaka, we exposed larvae to the initiating carcinogen DEN, then fed 1/2 of them continuously with diet containing E2. In their work with trout, Nuñez and colleagues did not record tumor incidences separately for males and females. A question left unanswered by the Nuñez studies is whether E2 augmented the tumor incidence in males to equal that in females exposed to

carcinogen alone. In the following study, association between tumor prevalence and gender was a central focus.

Recent literature documents the potential of aquatic species for exposure to environmental contaminants having estrogen-like properties (Sumpter, 1995). In the present study, tumor modulating effects of E2 were compared with those of a non-steroidal estrogenic compound, β-hexachlorocyclohexane (βHCH). Estrogen-like effects, such as testicular atrophy and suppression of ovulation, resulted from oral administration of 250 mg βHCH kg^{-1} diet to young rats (van Velsen et al., 1986). Similarly, after juvenile medaka were exposed for 3 months to 0.32 mg l^{-1} βHCH, in vivo estrogenic effects included oocyte development in testes and production of VG by both sexes (Wester and Canton, 1986).

This study was conducted to determine the effects of E2 and βHCH on neoplastic development in medaka. For this analysis, both hepatic foci of cellular alteration (FCA) and hepatic tumors were considered critical endpoints. FCA are small groups of hepatocytes, differing from surrounding parenchyma by staining, that appear during the promotion phase of development, between the time of carcinogen exposure and detection of tumors (Farber and Sarma, 1987). FCA are considered to be precursors of hepatic tumors in rodent (Popp and Goldsworthy, 1989; Bannasch and Zerban, 1992) and teleost (Sinnhuber et al., 1977; Hendricks et al., 1984; Hinton et al., 1988) models. Yield of FCA is therefore useful for additional quantification of promoting ability (Williams, 1982).

2. Materials and methods

Medaka ($n = 2300$) were raised in our laboratory breeding facility. Larvae, which hatched over a 5 day period, were placed in tanks on a recirculating water system and fed a purified, casein-based (PC) diet (DeKoven et al., 1992).

When fish were 3 weeks old, DEN exposure was begun. Medaka were transferred to a carcinogen-approved glove box and placed in gently aerated, covered, glass aquaria holding an aqueous bath of 200 ppm DEN (CAS No. 55-18-5; Isopak™ from Sigma Chemical, St. Louis). Each 5 l bath held 550 larvae. Fish remained in the exposure bath under static conditions for 24 h. Following exposure, medaka were held in a static, fresh-water bath for 24 h. Control fish were held under identical temperature and static water conditions without exposure to DEN. Fish were not fed during the entire 48 h. After the clean water bath, all fish were moved to a limited access room for maturation. Each randomly selected group of 100 fish was released into a separate tank and fed basal PC diet.

The promotion phase of the experiment was conducted in individual, static, 38 l tanks containing foam biological filters and aeration. Tanks were seated in a water bath equipped with heaters and circulating pumps to maintain a uniform temperature (24 ± 2°C). To ensure an uncontaminated water supply, well water was completely deionized and reconstituted using purified salts (US EPA guidelines for moderately hard water; Horning and Weber, 1985).

Fish were monitored daily and moribund or dead fish were removed, fixed in formalin and recorded. Maximum and minimum ambient air temperatures and water temperature were also recorded. Lights were maintained on a 16 h light and 8 h darkness cycle. Tanks were siphoned daily and algae removed as needed. Water quality tests for pH and nitrogen compounds were performed on selected tanks every 2 weeks. Conditions consistently met acceptable values of pH 6.5–7.5, 0 ppm ammonia, 0 ppm nitrite and 0–10 ppm nitrate. On a wekly basis, 1/4 of water in each tank was removed and replaced weekly. Waste water from tanks of fish fed βHCH was filtered through two large columns of activated carbon. Filtered water was held in a large drum until analysis by gas chromatography and mass spectrometry verified no detectable βHCH, then discarded.

E2 and βHCH were incorporated during PC diet production. Specific diet preparations contained 0.01, 0.10, 1.0 or 10.0 ppm E2 (CAS No. 50-28-2; Sigma Chemical, St. Louis) or 0.01, 0.10, 1.0, 10.0, or 100.0 ppm βHCH (CAS No. 319-85-7, Chem. Service, West Chester, PA) by weight of

dry ingredients and oil. Measured amounts of E2 or βHCH stock solutions were diluted in acetone and thoroughly mixed with the dextrin component of the diet. Acetone was removed by cold evaporation under vacuum. The remaining dry ingredients were mixed into dextrin, and oils and then water added. Diets were pelleted by extruding the dough through the smallest die of a commercial pasta maker (Kitchen Aid, St. Joseph, MI.), freeze drying for 24 h using a LabConco drier, and pressing dried diet through stainless steel sieves (US Standard Tyler Sieves, No. 35–60). Final concentrations of βHCH were measured by GC-MS after addition of water and freeze-drying and were found to be 0.02, 0.08, 0.86, 14.3, and 137 ppm βHCH. Final concentrations of E2 in the diets were not determined. Diets were prepared once at the start of the experiment and stored at −80°C.

Groups of DEN-exposed fish were fed one of the nine test diets or basal PC diet, for a total of ten groups. Similarly, ten groups of carcinogen controls (0 ppm DEN) fish were fed one of the test diets or basal PC diet. Feeding of diets containing βHCH or E2 began 8 days after start of DEN exposure. Assignment of diet treatment was done randomly. Because of the large number of test diets, there were no replications. The total amount of food fed per day was approximately 3% of average biomass per tank.

A total of five medaka per group were sampled at 0.5, 1, 2, 3 and 4 months after start of DEN exposure. The 5 month sampling contained eight individuals from each group. Fish were selected randomly from each tank for sampling. Following a period of 6 months after DEN exposure, all remaining fish were killed. A single sampling protocol was followed for each time point. Fish were killed by an overdose of MS-222, (3-aminobenzoic acid ethyl ester methane sulfonate; CAS No. 886-86-2), blotted dry and weighed. The peritoneal cavity of small medaka (body weight >50 mg) was opened and these fish were fixed whole in 10% buffered formalin. Livers of larger medaka were resected, weighed and fixed separately from the carcasses. Small fish and livers were fixed individually in 10% buffered formalin. Bodies of larger fish were decalcified in Bouin's fixative prior to being processed and embedded in paraffin. Livers were embedded in glycol methacrylate and sectioned at a thickness of 4 μm, using a LKB Historange microtome and glass knives. At least 15 liver sections, 24–40 μm apart, were stained with Mayer's hematoxylin and aqueous eosin (HE) and examined for tinctorially-altered FCA and for neoplasms. Preparation of stain solutions and duration of staining were standardized to ensure uniform coloration of foci.

Hepatic neoplasms and FCA were classified according to the identification system previously published by this laboratory (Hinton and Laurén, 1990; Hinton, 1994; Okihiro, 1996). This system is similar to standards recently adopted by the National Toxicology Program (NTP) for evaluation of hepatoproliferative lesions in rats (Maronpot et al., 1986), mice (Maronpot et al., 1987) and medaka (Boorman et al., 1997). Unlike the NTP medaka system, amphophilic foci in this study were categorized separately from basophilic foci. Prevalence of each type of tumor was defined as the ratio of medaka with one or more tumors to the number of medaka in the treatment group, expressed as a percentage.

Four phenotypes of FCA were recorded: clear cell (CCF), amphophilic (AF), eosinophilic (EF) and basophilic (BF). These types have been previously identified in DEN-induced medaka (Hinton et al., 1988; Bunton, 1990; Hinton and Laurén, 1990; Okihiro, 1996). Clear cell FCA were comprised of hypertrophic hepatocytes with homogeneous, clear cytoplasm that was free of vacuoles. These were differentiated at high magnification from hepatocytes containing rounded vacuoles with distinct margins, which were fat vacuoles. Further differentiation was enabled by staining a subset of foci with periodic acid-Schiff reagent. A positive reaction in clear cell FCA without vacuoles indicated the presence of glycogen. Prevalence of each type of FCA was defined as the ratio of medaka with one or more foci to the number of medaka in the treatment group, expressed as a percentage. Numbers of FCA profiles in each section of liver were also recorded. Because each liver was not completely step-sectioned, foci in the uncut portion and small foci located entirely between step sections were not counted. Data is

presented as average number of focus profiles per 15 sections of each liver. Placement and appearance of foci were carefully compared between step sections to avoid counting a single focus more than once.

Hepatosomatic index (HSI) was defined as the ratio of liver weight to total body weight, expressed as a percent. Prevalence data were compared using the Pearson χ^2-test and Fisher's Exact test. Significance of average numbers of foci and tumors and of liver weights was determined by ranking data and analyzing by one-way ANOVA, followed by post hoc comparisons using the Tukey-Kramer honestly significant difference test. All tests except Fisher's Exact were calculated with use of the computer program JMP (Sas Institute, 1989, vers. 2.0.1). Unless otherwise noted, values were considered statistically different if $P \leq 0.05$.

3. Results

Histological examination of a subset of fish that died between samplings showed the probable cause of death was mycobacterial infection. Mortalities ranged from 6 to 44 medaka per tank and were not associated with exposure to DEN, treated diets, or DEN plus treated diet. The average mortality rate was 18.75.

3.1. Normal liver

Normal liver tissue, in control medaka and surrounding hepatic lesions in treated medaka, did not differ from published descriptions (Hinton et al., 1984). Hepatocytes contained moderate to large amounts of glycogen. Generally, hepatocytes in mature female medaka had less glycogen and were more basophilic than those in males.

3.2. Characterization of hepatocellular tumors

Three types of liver tumors, hepatocellular adenoma (HA), hepatocellular carcinoma (HC) and cholangiocellular adenoma (CA), were present in fish exposed to DEN. Characteristics of these tumors were similar to neoplasms found in other fish and to findings in previous exposures of medaka to DEN (Hinton, 1994).

Hepatocytes of most HA were basophilic and glycogen depleted. Very rarely, medaka given DEN plus E2 or DEN plus βHCH exhibited basophilic, glycogen-enriched HA. A total of 18% of HA in the study were eosinophilic and occurred in fish from DEN plus E2 or DEN plus βHCH treatments. Multiple HA occurred only in fish receiving DEN plus E2 or βHCH.

3.3. Tumor prevalences

3.3.1. Den only

The percentage of medaka exposed to DEN alone that developed HA was 4.6. No HC were observed in this group.

3.3.2. DEN plus E2

Prevalences of HA and HC in medaka exposed to DEN and fed E2 are presented in Fig. 1. E2 at the highest dietary concentration of 10 ppm significantly ($P < 0.01$) increased the number of medaka that developed HA. The only HC was observed in a fish treated with DEN and 0.01 ppm E2. CA were present in medaka from DEN only and from DEN plus E2 treatment groups. Formation of CA was not associated with either level of E2 or with gender.

3.3.3. DEN plus βHCH

Prevalences of HA and HC in medaka exposed to DEN and fed βHCH are shown in Fig. 2. Dietary levels of 0.01, 0.1 and 100 ppm βHCH elevated tumor prevalences, but statistical analyses indicated there was no significant elevation in tumor prevalence in βHCH-exposed fish. The prevalence of medaka with HA or HC fed 100 ppm βHCH was 3.2 times the prevalence of the DEN-only group. Prevalence of HC increased from 0% in each of 0, 0.01, and 10.0 ppm βHCH groups, to 2% in 0.1 and 1.0 ppm groups and 6% in the 100 ppm group. CA were also seen in fish from DEN plus βHCH groups, but no concentration-dependent effects were identified.

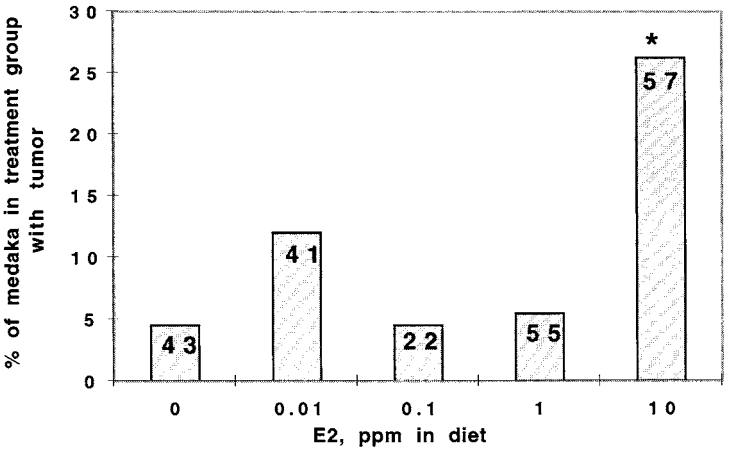

Fig. 1. Prevalence of hepatocellular adenoma and carcinoma in medaka exposed to diethylnitrosamine (200 ppm for 24 h) at 3 weeks of age and fed control diet or diet containing 17β-estradiol (E2) for 6 months. Tumors were counted on 15 step sections (approximately 30 μm apart) per liver. *N*, number on column; *, significantly different from 0 ppm E2 group ($P < 0.01$).

3.3.4. Treatments lacking DEN

Medaka fed normal PC diet but not exposed to DEN failed to develop FCA or tumors. Also, no tumors were seen in those groups fed E2 or βHCH, but not exposed to DEN.

3.4. Characterization of FCA

FCA in medaka exposed to DEN and fed E2 or βHCH were similar to those in fish exposed to DEN only. No new phenotypes were encountered with E2 or βHCH treatment. In all types of foci, the normal tubular architecture of the liver (Hampton et al., 1985) was unaltered. Tubules in foci merged with tubules of surrounding parenchyma and did not cause compression, but staining difference between focal hepatocytes and surrounding liver provided an obvious border. Mitotic figures were rarely seen in FCA. Each type of FCA is described in Table 1. Hepatocytes in EF and BF were similar in staining and morphology to hepatocytes in eosinophilic and basophilic HA, respectively. FCA with two or more cell types (mixed cell foci) were not seen.

3.5. Time to FCA appearance

Time to appearance of FCA in medaka exposed to DEN only was longer than 1 month, since liver sections from fish sampled at 0.5 and 1 month contained no FCA. At 2 months, CCF were the only focal phenotype present in fish treated with DEN alone. At 2 months, AF as well as CCF were present in medaka given DEN plus E2.

3.6. Numerical estimation of FCA profiles

In the following sections and figures, 'number of foci' refers to numbers of focus profiles counted in 15 liver sections. FCA were counted only in fish sampled at 6 months.

3.6.1. DEN only

Average numbers in medaka treated with DEN only of CCF, AF, EF and BF were 0.61, 0.09, 0.86 and 0.39 foci per liver, respectively. Focal prevalence, the percent of fish with one or more foci of a particular type, was greatest for CCF (51%) followed by EF (35%), BF (23%) and AF (9%). Examination of the numbers of foci in individual fish showed that medaka with EF tended to have at least two of the eosinophilic type, and that most medaka with CCF had only one. Average numbers of FCA were used for comparison with results in DEN-exposed and E2 or βHCH-fed groups (see Figs. 3–6).

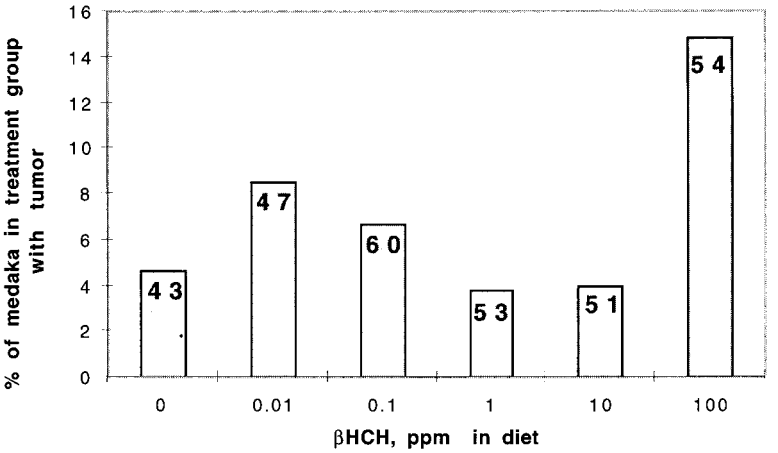

Fig. 2. Prevalence of hepatocellular adenoma and carcinoma in medaka exposed to diethylnitrosamine (200 ppm for 24 h) at 3 weeks of age and fed control diet or diet containing β-hexachlorocyclohexane (βHCH) for 6 months. Tumors were counted on 15 step sections (approximately 30 μm apart) per liver. *N*, number on column.

3.6.2. DEN plus E2

Mean profile numbers of CCF and AF, and of EF and BF from DEN plus E2 treatment groups are presented in Fig. 3 and Fig. 4 respectively. Significant differences in numbers of FCA per fish were found between DEN alone and DEN plus E2 groups. Average numbers of CCF were lower for all groups treated with DEN and E2 and were significantly reduced after feeding 0.01 and 1.0 ppm E2 relative to DEN alone (Fig. 3). The AF phenotype was least encountered. Groups fed 0.01 or 1.0 ppm E2 had slightly higher numbers of AF (0.27 and 0.22 AF per fish) than did other treatment groups (0.09 AF per fish in DEN only), but fluctuations in AF number were not associated with level of E2 (Fig. 3). Numbers of EF were inversely correlated with concentration of E2 (Fig. 4). Average number of EF in the DEN plus 10 ppm E2 group was significantly less than that in the DEN-only group (0.09 and 0.86 EF per fish, respectively). BF were positively correlated with concentration of E2 (Fig. 4). Numbers of BF increased significantly, from 0.40 in the DEN-only group to 1.61 BF per fish in the DEN plus 10 ppm E2 fish. Also in the 10 ppm E2 group, numbers of EF were significantly less than numbers of BF.

Table 1
Characteristics of hepatic foci of cellular alteration

Cell type	Characteristics
Clear cell	Cytoplasm clear or with pale, lacy network Hepatocytes hypertrophic Nucleus often at periphery of cell
Eosinophilic	Granular, eosinophilic cytoplasm with some glycogen Hepatocytes of normal size or slightly hypertrophic
Basophilic	Basophilic cytoplasm, either grainy or homogeneous Generally glycogen depleted Hepatocytes of normal size or slightly hypertrophic
Amphophilic	Gray or gray-brown granular cytoplasm Color was neither eosinophilic nor basophilic Hepatocytes of normal size or slightly hypertrophic

3.6.3. DEN plus βHCH

Average numbers of CCF and AF, and of EF and BF from DEN plus βHCH-treated medaka are displayed in Fig. 5 and Fig. 6 respectively. Significant differences between DEN-only and DEN plus βHCH groups were apparent for several types of FCA. CCF increased, then decreased as concentration of βHCH increased (Fig. 5).

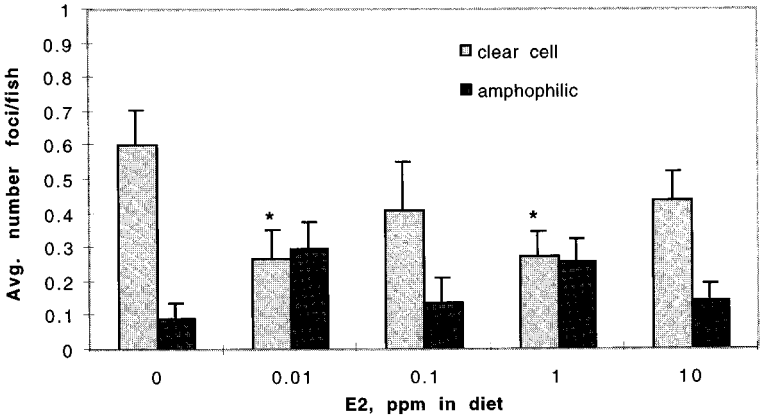

Fig. 3. Average number of clear cell and amphophilic foci of cellular alteration (FCA) per medaka exposed to diethylnitrosamine (200 ppm for 24 h) at 3 weeks of age and fed control diet or diet containing 17β-estradiol (E2) for 6 months. FCA were counted on 15 step sections per liver. *, significantly different than clear cell FCA in 0 ppm E2 group.

Average numbers of CCF in groups fed 10.0 or 100.0 ppm βHCH (0.25 and 0.29 CCF per fish, respectively) were significantly lower than those in the DEN-only group (0.60 CCF per fish). Average numbers of AF were small (range 0.06–0.17 AF per fish) and did not correlate with level of βHCH (Fig. 5). Average numbers of BF were elevated in all groups that were fed βHCH, but a relationship between diet level and response was not apparent (Fig. 6). BF were significantly more numerous in the DEN plus 0.01 ppm βHCH group than in the DEN alone group (0.66 versus 0.23 BF per fish). Growth of EF varied between groups fed βHCH, but numbers of foci were not altered significantly from those in the DEN-only treatment and did not exhibit any concentration-dependent trend (Fig. 6).

3.6.4. Treatments lacking DEN

Among fish fed E2 or βHCH, but not exposed to DEN, numbers of foci were extremely small. In fish fed E2 only, two FCA were found, one EF and one CCF, each from different fish of the 1.0 ppm E2 group. In groups fed only βHCH, two individuals from each of the 0.01 and 0.1 ppm βHCH groups and one fish from the 1.0 ppm group had a single EF. One fish in each of the 1.0 and 10 ppm βHCH groups had single CCF.

3.7. Gender-associated differences in tumors and FCA

Sex of each fish was identified by observation of fin shape (Egami, 1975) and verified by histological examination of the gonads. Exposure to higher concentrations of E2 resulted in abnormal gonad development in some individuals. Two fish fed 1.0 ppm E2 diet and ten fed 10 ppm E2 were intersexes with testicular and ovarian tissue in a single gonad. Gonads of 12 fish in the 10 ppm E2 group were termed degenerate, being comprised primarily of connective tissue with a small number of immature oocytes. Only three fish of the 10 ppm E2 group had developing testes and could be histologically classified as males. Liver lesions of intersex/degenerate medaka from groups fed E2 were reported separately from normal males and females. Two medaka fed low levels of βHCH exhibited abnormally immature or degenerate gonads.

3.7.1. Tumor prevalences

Tumor prevalences analyzed with respect to gender are presented in Table 2, for E2 treatments and in Table 3 for βHCH treatments. In the DEN-only group, the two HA developed in female fish. Combining tumors in medaka exposed

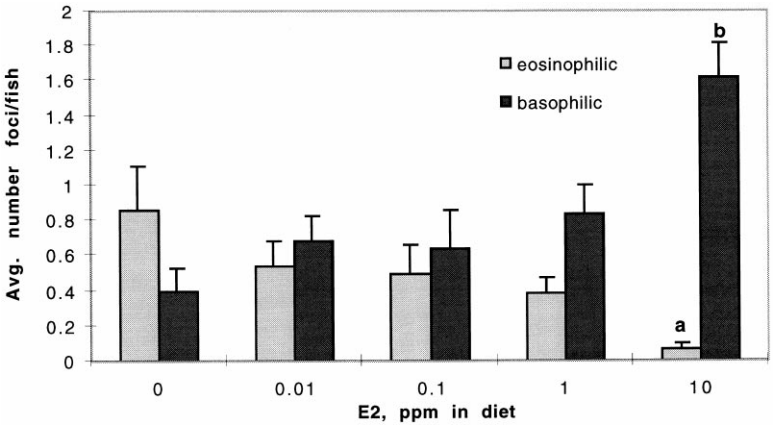

Fig. 4. Average number of eosinophilic and basophilic foci of cellular alteration (FCA) per medaka exposed to diethylnitrosamine (200 ppm for 24 h) at 3 weeks of age and fed control diet or diet containing 17β-estradiol (E2) for 6 months. FCA were counted on 15 step sections per liver. (a) Significantly different than eosinophilic foci in 0 ppm E2 group. (b) Significantly different than basophilic foci in 0 ppm E2 group.

to DEN plus any E2 diet, 68% of HA and the single HC were found in females and 24% of HA were present in fish with degenerate or intersex gonads. Among fish treated with DEN plus βHCH, 71% of HA and 60% of HC were found in females. In medaka fed either treated diet, most tumors in females were basophilic, while those in males were eosinophilic.

3.7.2. FCA prevalences

FCA were also separated by sex of medaka. Average numbers of FCA per fish and prevalences of fish with one or more foci were calculated for males, females and fish with abnormal gonads. Increases or decreases in FCA number generally paralleled changes in prevalence. Distinct differences in occurrence of EF and BF were apparent between males and females. Prevalence of EF was greater in males than females, for all groups except DEN plus 1.0 or 10.0 ppm E2. BF prevalence, conversely, was significantly greater in females than males also excepting the 10 ppm E2 group.

3.7.3. Numbers of FCA, DEN plus E2 treatments

A rise in E2 concentration corresponded to a decline in number of CCF similarly in males and females. Numbers of AF were slightly higher in medaka of either sex given DEN and any of the E2 diets, relative to DEN alone, but no concentration-associated trend was present. Average numbers of FCA profiles for EF and BF separated by sex of medaka are presented in Fig. 7 and Fig. 8, respectively. Number of EF remained small in females as dietary E2 concentration increased (range: 0.06–0.31 EF per fish) and was also very low in fish with degenerate/intersex gonads (0.09 EF per fish). Number of EF in males decreased significantly with increasing concentration of E2, from 1.4 EF per fish in the DEN-only group to 0 EF per fish in the phenotypic males from the 10 ppm E2 treatment (Fig. 7). Numbers of BF in males increased from 0.08 to 1.67 BF per fish as concentration of E2 increased from 0 to 10 ppm (Fig. 8). Numbers of BF in females increased more gradually with increasing E2 level, from 0.83 to 1.53 BF per fish. In medaka with degenerate/intersex gonads from the 10 ppm E2 group, BF numbered 1.73 BF per fish.

3.7.4. Numbers of FCA, DEN plus βHCH treatments

Average numbers of CCF were similar in males and females fed βHCH after DEN exposure. The two highest concentrations of βHCH produced approximately 1/2 as many CCF per liver as treatment with DEN only. There were no significant differences in numbers of AF between males and females of any βHCH-treated group. Average numbers of FCA profiles for EF and BF from

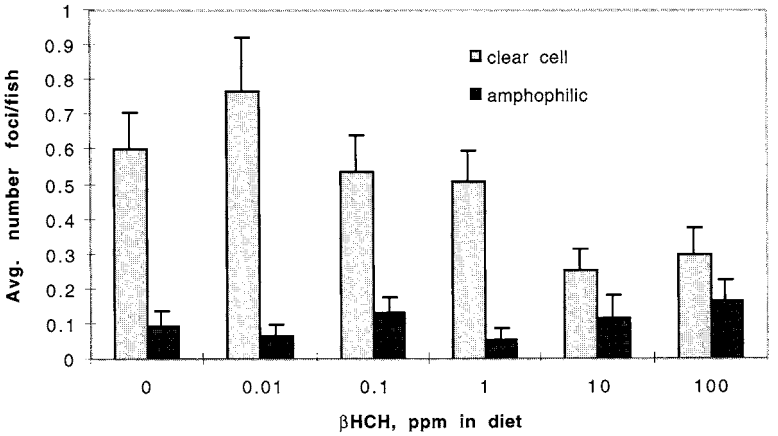

Fig. 5. Average number of clear cell and amphophilic foci of cellular alteration (FCA) per medaka exposed to diethylnitrosamine (200 ppm for 24 h) at 3 weeks of age and fed control diet or diet containing β-hexachlorocyclohexane (βHCH) for 6 months. FCA were counted on 15 step sections per liver.

βHCH-fed medaka, separated by sex, are presented in Fig. 9 and Fig. 10, respectively. In males, numbers of EF fluctuated between 0.74 and 1.62 EF per fish, but were not correlated with βHCH concentration (Fig. 9). Numbers of EF in females were significantly smaller than in males, starting at 0.17 EF per fish in the DEN-only group, increasing to 0.42 EF per fish in the group fed 0.1 ppm βHCH diet, and declining to 0.08 EF per fish in females fed 100 ppm βHCH. Numbers of BF were consistently greater in females than in males (Fig. 10). Relative to the DEN-only group, numbers of BF were higher in all groups fed βHCH and increases were statistically significant for all except the 1.0 ppm βHCH group. BF in males fed βHCH (range: 0.26–0.82 BF per fish) were also more numerous than BF from the DEN-only group (0.08 BF per fish).

3.8. Liver weights

Liver weights expressed as a percentage of total body weight (hepatosomatic index; HSI) are displayed in Fig. 11 for E2 treatments and in Fig. 12 for βHCH treatments. Male medaka at maturity have significantly smaller liver weights and HSI than do female medaka. This is also true of medaka exposed to DEN (Teh and Hinton, 1998).

3.8.1. E2 treatments

In this study, administration of E2 increased liver weights and HSI of male fish. HSI of males fed any concentration of E2 was not significantly different from females from the same treatment group (Fig. 11). At 10 ppm E2, HSI of males and females were 2.9 and 1.4-times higher, respectively, than HSI of corresponding males and females fed no E2. HSI of intersex fish was also highly elevated. In medaka exposed to DEN and E2, HSI displayed a similar trend with increasing concentration of E2. Significant difference between HSI of DEN-treated males and females was seen in 0 and 0.01 ppm E2 groups, and not in those fed higher levels of E2 (data not shown).

3.8.2. βHCH treatments

Consumption of βHCH did not increase liver growth in males, and HSI of females remained higher (Fig. 12). Significant differences between HSI of males and females were also present in medaka exposed to DEN and βHCH (data not shown).

4. Discussion

When administered after the carcinogen in an initiation-promotion assay, E2 significantly increased the number of fish per group with hepato-

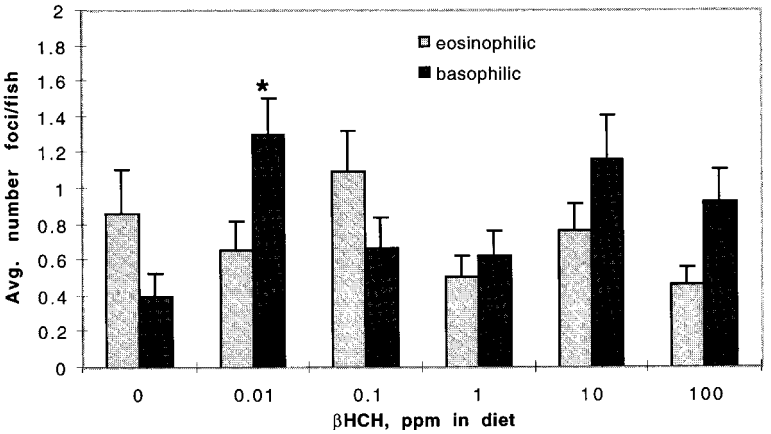

Fig. 6. Average number of eosinophilic and basophilic foci of cellular alteration (FCA) per medaka exposed to diethylnitrosamine (200 ppm for 24 h) at 3 weeks of age and fed control diet or diet containing β-hexachlorocyclohexane (βHCH) for 6 months. FCA were counted on 15 step sections per liver. *, significantly different from basophilic foci in 0 ppm E2 group.

cellular adenoma or carcinoma. Compounds are classified as promoters by either elevating tumor prevalence or reducing the tumor latency period, or both (Peraino et al., 1983). The increase in tumor yield is evidence that E2 is a hepatic tumor promoter in medaka. Although tumor prevalence was higher in groups that received DEN and bHCH than in the DEN alone group, the increase was not statistically significant. At this time, we classify βHCH as a weakly positive modulator (positive only at levels above environmental relevance) of tumorigenesis in the medaka model.

The few previous attempts to modulate chemical carcinogenesis in medaka were conducted with adults. Medaka, 1 year old were used by Aoki and Matsudaira (1981) to show that bath exposure to 30 ppm caffeine for 7 days enhanced the prevalence of hepatic carcinomas to 1.5 times the

Table 2
Prevalence of hepatocellular adenoma and carcinoma in female, male and intersex/degenerate medaka after single exposure to diethylnitrosamine (200 ppm bath for 24 h) and feeding of control diet or diet containing estradiol (E2) for 6 months

E2	Females		Males		Unknown[a]	
(ppm)	(%)		(%)		(%)	
0	11	(2/18)	0	(0/25)*	NA	NA
0.0	22	(4/18)[b,c]	4	(1/23)*	NA	NA
0.10	0	(0/14)	12.5	(1/8)	NA	NA
1.00	9	(3/32)	0	(0/21)*	0	0/2
10.00	34	(11/32)	33	(1/3)	14	3/22[c]

[a] Fish that were either intersexes or had degenerate, fibrotic gonads with immature oocytes. The sexual genotype, although probably male, could not be verified.
[b] A female in the 0.01 ppm E2 group developed the only hepatocellular carcinoma.
[c] Multiple adenomas were present in one female in the 0.01 ppm E2 group and in two fish with degenerate gonads from the 10 ppm E2 group.
*, Significantly ($P<0.02$) smaller prevalence in males fed 0, 0.01, or 1.0 ppm E2 when compared with females fed 10 ppm E2.

Table 3
Prevalence of hepatocellular adenoma and carcinoma in male and female medaka after exposure to diethylnitrosamine (200 ppm bath for 24 h) and feeding of control diet or diet containing β-hexachlorocyclohexane (βHCH) for 6 months

βHCH	Females		Males	
(ppm)	(%)		(%)	
0	11	(2/18)	0	(0/25)
0.01	12	(3/25)	4.5	(1/22)
0.10	0	(0/26)	12	(4/34)
1.00	6.7	(2/30)	0	(0/23)
10.00	10.5%	(2/19)	0	(0/32)
100.00	23	(6/26)[a]	7	(2/28)

[a] One fish with multiple adenomas was found in the DEN plus 100 ppm βHCH group.

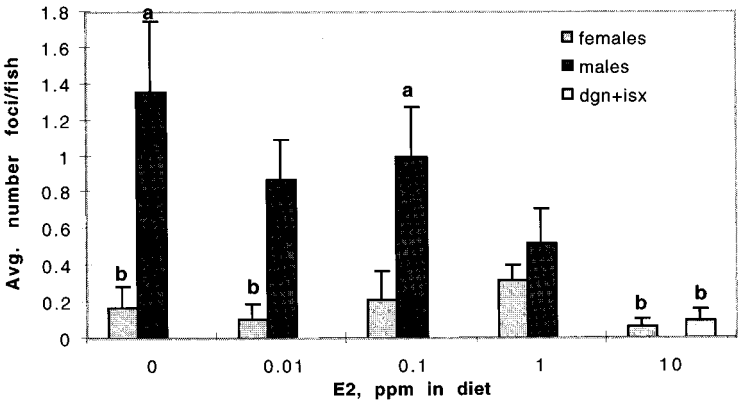

Fig. 7. Average number of eosinophilic foci, separated by sex of medaka. Medaka were exposed to diethylnitrosamine and fed control or diet containing 17β-estradiol (E2) for 6 months. '*dgn + isx*'—Medaka with degenerate or intersex gonads. (a) Significantly different than females fed 0 ppm E2. (b) Significantly different than males fed 0 ppm E2.

prevalence with methylazoxymethanol acetate alone. Tumor prevalence in medaka was also enhanced by post-initiation administration of physical agents including elevated temperature (Kyono, 1978), partial hepatectomy (Kyono-Hamaguchi, 1984), and X-ray irradiation (Aoki and Matsudaira, 1981). The positive effects of E2 and βHCH on tumor production show that juvenile medaka are responsive in a multiple-agent tumor formation assay. These results further establish medaka as a useful surrogate species for environmental tumorigenesis studies.

This study is the first to examine effects of tumor modulators on hepatic FCA in medaka. Numbers of preneoplastic FCA and rates of focal growth are used in rodent tests to determine promoting efficacy (Farber, 1980; Schröter et al., 1987). Extensive evaluation by Hinton and colleagues (Hinton et al., 1992; Teh and Hinton, 1993) of early changes in medaka tumorigenesis has shown FCA develop prior to tumors and certain types, namely eosinophilic and basophilic foci, seem to be preneoplastic. Basophilic foci are considered immediate precursors of HA in other species, including rainbow trout (Hendricks et al., 1984) and rat (Bannasch and Zerban, 1992). In this study, numbers of basophilic foci were increased by post-initiation treatment with either E2 or βHCH. These modulators also reduced growth of eosinophilic foci. Although eosinophilic foci were found in medaka from all treatment regimens, high concentrations of both E2 and βHCH decreased average numbers of this phenotype. A few eosinophilic adenomas did form, suggesting that select eosinophilic foci may have developed into adenomas independently of a growth stimulus caused by the modulators.

The fate of eosinophilic foci in medaka is currently uncertain. The eosinophilic phenotype is common in carcinogen-exposed male medaka. Most hepatic adenomas, however, are basophilic. Transformation from an eosinophilic to a basophilic phenotype, as is believed by Bannasch and colleagues to occur in the rat model (Bannasch and Zerban, 1992), may also occur in medaka. At this time, however, we lack direct evidence for this conversion. Other potential endpoints for eosinophilic foci are regression with infiltration by inflammatory cells as has been suggested in rainbow trout (Hendricks et al., 1984), persistence as the same phenotype, or progression to tumor (Farber and Sarma, 1987). Evidence of transformation in medaka, in the form of single lesions containing eosinophilic and basophilic cell types, is rarely found (Nakazawa et al., 1985, Okihiro, 1996). The probability of detecting transitional phenotypes of FCA may be limited, because of the uneven ratios of eosinophilic foci to basophilic foci in both sexes (ratio is large in male and quite small in female medaka) and because of infrequent sampling times. Increasing the prevalence of EF, such as occurred in males exposed to

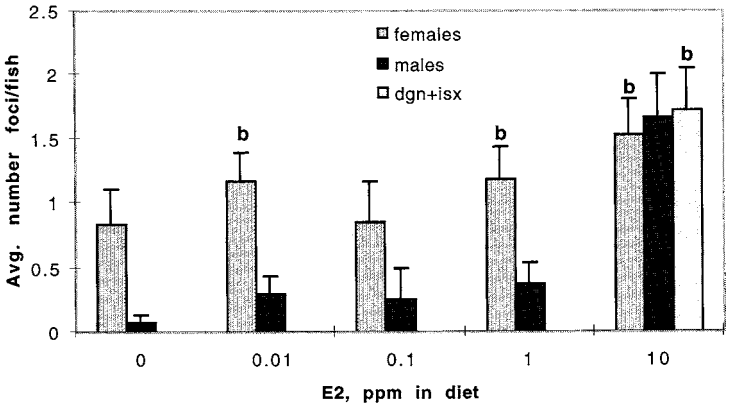

Fig. 8. Average number of basophilic foci, separated by sex of medaka. Medaka were exposed to diethylnitrosamine and fed control or diet containing 1β-estradiol (E2) for 6 months. '*dgn + isx*'—medaka with degenerate or intersex gonads. (b) Significantly different than males fed 0 ppm E2.

DEN and fed low doses of E2, could facilitate detection of transitional foci should they be present.

Study of the fate of focus phenotypes in fish has relevance to field situations. For example, Myers et al. (1987) studying English sole (*Parophrys vetulus*) in the Puget Sound saw high prevalences of the eosinophilic variant and suggested they were preneoplastic. Eosinophilic, as well as basophilic foci were observed in livers from European flounder (Vethaak and Wester, 1996) and common dab (Kranz and Dethlefsen, 1990). FCA are frequently described, but are not considered definitive biomarkers of environmental carcinogen exposure to fish because there is a lack of detailed information about the relationship of each phenotype to tumor development (Hinton et al., 1992; Myers et al., 1992; ICES, 1996). Future investigations with carcinogen only and carcinogen plus promoter regimes, followed by multiple sampling points during the early stages of hepatic tumor development would enable a more thorough evaluation of the role of these lesions in neoplasia. Although useful in establishing numbers and extent of hepatic alterations, the hematoxylin and eosin staining procedures used herein are insufficient to identify subcellular alterations occurring in FCA. Enzyme histochemistry, immunohistochemistry and electron microscopy should also be used to characterize altered biology during promotion. Such studies would bring fish models to a level near their rodent counterparts (Bannasch et al., 1997, Ruebner et al., 1997).

The present study also raises the question of whether higher than expected numbers of basophilic foci in male fish indicate promotion by an estrogenic compound. The degree to which focal growth patterns vary with type of promoter is unknown for fish. Further research with other classes of tumor promoters is necessary to maximize the utility of FCA in identifying carcinogen and modulator exposures.

No tumors were seen in medaka fed E2, but not exposed to DEN, indicating neither test compound acted as a complete carcinogen. This result is consistent with evidence assembled from other species. E2 alone did not produce tumors in rainbow trout (Nuñez et al., 1988). Conclusions drawn from numerous mammalian studies indicate estrogens act as promoters, not as complete hepatocarcinogens (Yager, 1983).

A mechanism of action proposed for estrogen and other promoters in mammals is stimulation of cell proliferation (Yager, 1983; Schulte-Hermann, 1985). E2 and synthetic estrogens do increase the mitotic index of rat hepatocytes (Ochs et al., 1986). As shown byNuñez et al. (1989), E2 increased hepatocyte DNA synthesis in rainbow trout. Supporting evidence of a proliferative stimulus is provided in this study by the induction of liver hypertrophy at concentrations greater than

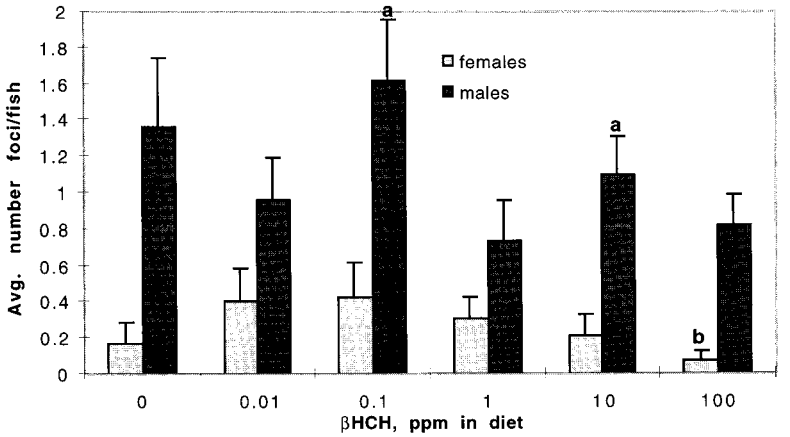

Fig. 9. Average number of eosinophilic foci, separated by sex of medaka. Medaka were exposed to diethylnitrosamine and fed control or diet containing β-hexachlorocyclohexane (βHCH) for 6 months. (a) Significantly different than females fed 0 ppm E2. (b) Significantly different than males fed 0 ppm E2.

0.01 ppm E2. Liver weights and HSI of E2-treated males were elevated to levels similar to those of females. Interestingly, average liver weight in intersex medaka, which were presumed to be genotypic males substantially altered by E2, was slightly higher than in females from the same E2 treatment group. Similarly, exogenous estrogens increased HSI in rainbow trout (van Bohemen et al., 1982) and salmon (*Oncorhynchus kisutch*; Donaldson et al., 1979).

βHCH administered alone produced no tumors and very few FCA. These results confirmed expectations that βHCH is not an initiating carcinogen. βHCH is negative in several short-term mutagenicity tests (van Esch, 1992) and does not induce liver tumors in rats (Ito et al., 1975; Schröter et al., 1987) or mice (Ito et al., 1973). Evidence for hepatic promotion by βHCH in rats includes enhancement of numbers and profile areas of FCA induced by *N*-nitrosomorpholine (Schröter et al., 1987).

The molecular mechanism of action of promotion by βHCH is likely not the same as E2. βHCH neither displaced E2 from estrogen receptors of human mammary tumor cells (Coosen and van Velsen, 1989) nor induced production of VG in channel catfish (*Ictalurus punctatus*; Nimrod and Benson, 1997). On an organ level, βHCH did not increase HSI of male medaka to equal that of females. βHCH alone did, however, slightly increase HSI above that in controls for both sexes. Liver enlargement produced by βHCH in rats results from both hyperplasia and hypertrophy (Schröter et al., 1987).

Several results from the DEN plus βHCH treatments appeared non-linear with respect to dose. Tumor prevalences were greater in medaka fed 0.01 and 0.1 ppm βHCH after DEN exposure than in those fed higher doses of 1.0 or 10 ppm βHCH. Also, numbers of basophilic foci were higher in medaka fed 0.01 βHCH than the next highest doses of 0.1 and 1.0 ppm βHCH. Although tumor promotion by E2 increased, then decreased with level of E2 in this study, higher mortality in one of the DEN plus E2 groups made interpretation difficult. The βHCH effects may be similar to non-linear responses seen after exposure of male mice to diethylstilbestrol (DES) or E2 (vom Saal et al., 1995, 1997). Exposure starting with very low doses produced inverted U-shaped dose-response curves for prostate size (DES and E2) and male territorial behavior (DES). The phenomenon of U-shaped dose-response curves has implications for assessment of risk of estrogenic compounds (Calabrese, 1996).

A particular goal of this work was to determine the effects of E2 on tumor prevalence as related to sex of medaka. Hepatic tumor prevalence was higher in males promoted by E2 than in males treated with DEN only. Tumor prevalence in

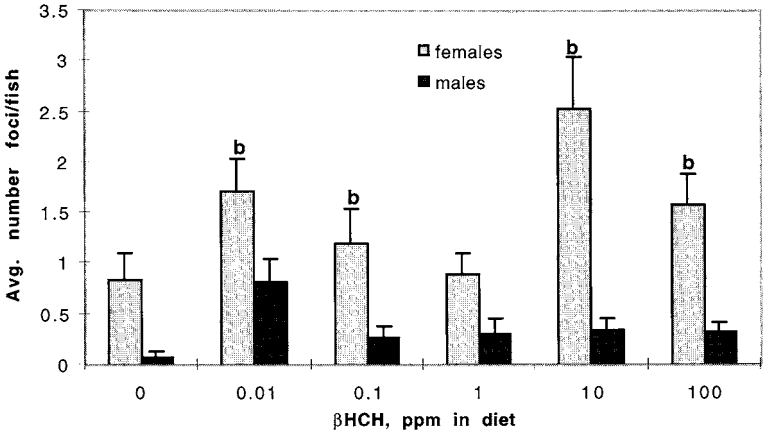

Fig. 10. Average number of basophilic foci, separated by sex of medaka. Medaka were exposed to diethylnitrosamine and fed control or diet containing β-hexachlorocyclohexane (βHCH) for 6 months. (b) Significantly different than males fed 0 ppm E2.

E2-promoted males, however, was less than the prevalence in females given DEN only. At concentrations of E2 from 0.01 to 1.0 ppm, tumor prevalences in males were considerably lower than in females from the same treatment (prevalences in the 10 ppm E2 group were equal between the sexes, but very few fish were histologically identified as males). Additionally, the eosinophilic color that is characteristic of normal male liver was retained both in non-focal parenchyma and in most FCA from liver of males promoted with E2. Thus, it seems a fundamental difference between male and female liver was not eliminated by treatment with E2. Germ cell differentiation is already beginning around the time of hatching (Satoh and Egami, 1972), with development in females occurring slightly before males (Yamamoto, 1975). By the start of E2 feeding at 4 weeks of age, liver changes attendant upon vitellogenesis may already have begun. Indeed, as early

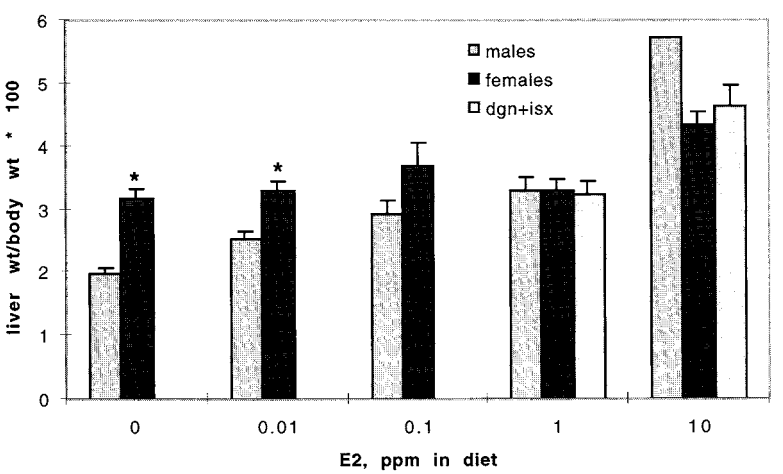

Fig. 11. Hepatosomatic indices (HSI) of medaka fed basal diet or diet containing 17β-estradiol (E2) starting at 1 month of age for 6 months. HSI was calculated by dividing liver weight by whole body weight and multiplying by 100. Each bar represents an average of 39 54 fish, depending upon treatment group. HSI of males and females within each diet group were compared for statistical significance. '*dgn + isx*'—fish with degenerate or intersex gonads, which were found only in groups fed 1.0 or 10.0 ppm E2. *, female significantly different from male of same treatment group.

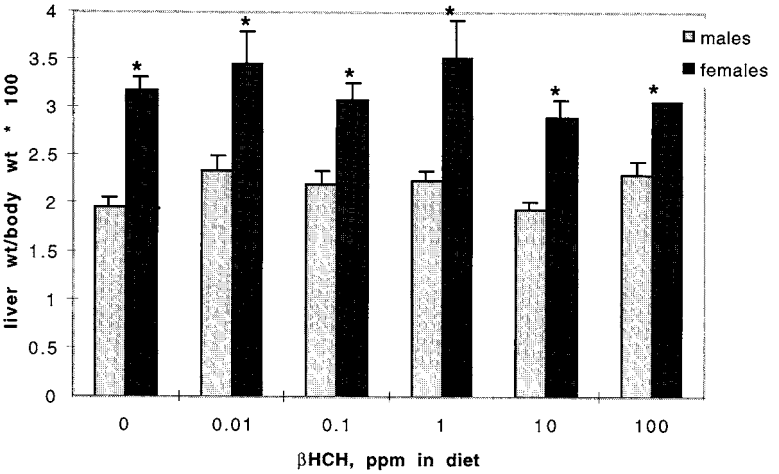

Fig. 12. Hepatosomatic indices (HSI) of medaka fed basal diet or diet containing β-hexachlorocyclohexane (βHCH) starting at 1 month of age for 6 months. HSI was calculated by dividing liver weight by whole body weight and $\times 100$. Each bar represents an average of 30–61 fish, depending upon treatment group. *, female significantly different from male of same treatment group.

as 7 weeks of age, HSI of female medaka is significantly greater than that of males (Teh, 1996). Genotypic males would likely produce more basophilic foci per fish and a higher prevalence of HA if medaka were exposed earlier to DEN and E2.

Two conclusions may have broad applicability to studies of fish carcinogenesis. First, these data indicate that there may be variations in prevalence, time of appearance and rate of growth of tunors in laboratory-exposed fish and in wild fish that are mediated through exposure to endogenous estrogens. Second, exposure of wild fish to endocrine disrupting compounds may affect tumor prevalences in contaminated environments.

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