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Molecular and Cellular Markers of Toxicity in the Japanese Medaka Oryzias latipes

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Abstract

The Japanese Medaka (Oryzias latipes) has been recommended for use as a model organism to detect carcinogenic, teratogenic, cytotoxic, and genotoxic compounds in aquatic systems. Studies with the Medaka from other laboratories have focused on carcinogenesis with particular emphasis on the histopathological identification of neoplastic lesions in the tissues of animals exposed to carcinogens. Because a long latent period often occurs between initial contact with deleterious chemicals and subsequent expression of the pathology, we are investigating early biologically-relevant responses that can be used as genotoxicity markers of exposure and effect. This project focuses on the development of genotoxic bioassays and experimental protocols for exposing Japanese Medaka to genotoxic compounds.

Biomarker responses in the Japanese Medaka exposed to benzo[a]pyrene (BaP) were evaluated over a 16-d exposure period. Data were obtained on BaP uptake, DNA integrity (strand breaks, DNA distribution), detoxification enzymes, and reproductive and developmental parameters. Interpretation of the data obtained to date is consistent with the occurrence of a minor physiological response in the medaka to BaP shortly after exposure. This response was observed as an increase in fecundity and in the number of ovarian vitellogenic oocytes; however, we did not detect concomitant genotoxic effects as evidenced by DNA strand breaks.

Introduction

The Japanese Medaka (Oryzias latipes) has been recommended as a model organism to detect carcinogenic, teratogenic, cytotoxic and genotoxic compounds in aquatic systems (Donaldson and Scherer, 1983; Ishikawa et al, 1975). Previous studies with the medaka have focused on carcinogenesis with particular emphasis on the histopathological identification of neoplastic lesions in the tissues of animals exposed to carcinogens. Neoplastic histogenesis in medaka follows a course similar to that observed in rodents: tumor formation is preceded by preneoplastic lesions which appear as both eosinophilic and basophilic foci (Hawkins et al., 1988).

A number of biological endpoints must be evaluated to measure biomarkers of toxicity in an organism and to determine the degree of exposure the organism has had to xenobiotic compounds. Biological markers can be used to measure both the short- and long-term effects of exposure to toxic substances (Shugart et al., 1987). Early exposure to xenobiotic compounds may be detected via the interaction of such compounds with cellular macromolecules such as DNA, RNA and proteins (Kurelec, 1990), changes in the activities and levels of detoxification enzymes, the induction of mutations or repair of damaged DNA. Changes in DNA sequence can lead to both heritable mutations and carcinogenesis. The interactions of xenobiotics with cellular macromolecules can perturb metabolism and result in cytotoxic effects. Longer-term responses to toxic substances can be evaluated by studying

neoplastic transformation, developmental (teratogenic) abnormalities, survivability, fecundity, immunocompetence or pathology.

In the medaka, the establishment of experimental protocols for exposure to xenobiotic compounds is particularly important to the development of bioassays for measuring toxicity in this fish. Three cytotoxic and genotoxic compounds were selected for study; diethylnitrosoamine (DEN), benzo[a]pyrene (BaP), and acetylaminofluorene (AAF)(Taningher et al., 1990). Medaka are currently being exposed to these compounds either by injection or in water. This report will focus on recent experiments in which medaka were exposed to BaP dissolved in water.

Experiments and bioassays currently underway at this lab to assay exposure of the Japanese Medaka to benzo[a]pyrene are: 1) determination of the uptake of radiolabeled BaP from water and accumulation of radiolabel in the whole organism; 2) measurement of DNA damage and/or modification by quantitating DNA strand breakage, ^{5}m-deoxy-cytidine content, and DNA adducts; 3) toxicant-induced changes in reproductive physiology and fecundity; 4) teratogenic studies of the development and hatchability of embryos exposed to xenobiotics; 5) mixed-function oxidases and other detoxification enzyme assays; 6) flow cytometry and histopathology, and 7) evaluation and modification of current protocols and development of new bioassays. The sections that follow will discuss DNA damage (alkaline unwinding), reproductive and developmental effects of BaP

exposure and to determine the rate of BaP accumulation in the medaka during aqueous exposure.

Materials and Methods

Uptake and Accumulation of Radiolabeled BaP.

Twenty-five fish (Japanese Medaka; 15 female and 10 male) were maintained in one 20-L aquarium. Water in the aquaria was amended with [^{14}C]-BaP + unlabeled BaP in 1% Tween 80 (Kennedy et al., 1989) on days 0, 2, 4 and 6 of an eight day exposure. The stock solution of [^{14}C]-BaP was prepared in toluene (1 mCi/ml; 1 μl = 1 μCi = 21 ng BaP = 2.2 x 10^9 dpm). To prepare BaP + [^{14}C]-BaP saturated water, 30 μl of [^{14}C]-BaP stock in toluene was dried and dissolved in 100% methanol, the [^{14}C]-BaP was purified by HPLC on a Varian 5000 Liquid Chromatograph equipped with a 250 x 4.6 mm C18 column. Fluorescence was detected with a Schoeffel FS-970 fluorometer with an excitation monochromter set at 246 nm and a 370 nm emission filter. An isocratic flow system using 100% methanol as solvent was used to elute purified BaP. The [^{14}C]-BaP eluted from the C18 column was dried and dissolved in 7.5 ml of 1% Tween 80 containing 40 μg/ml unlabeled BaP; 250 μl of this solution was added per liter of aquaria water (per liter: 1 μCi [^{14}C]-BaP/21 ng [^{14}C]-BaP/10 μg unlabeled BaP).

Medaka were exposed for 8 days. Each day, 10 ml of water were removed and extracted 2X with 1 ml of methylene chloride. The extract was centrifuged, the methylene chloride layer separated,

dried by evaporation and resuspended in 1 ml of methanol. Replicate samples were analyzed by HPLC as previously described. On sampling days 0, 1, 2, 4 and 8 five fish (3 female and 2 male) were sacrificed by cervical scission. The liver, gonads and carcass of the fish were analyzed separately for ^{14}C content. Frozen tissue was minced and homogenized in 5 volumes of 0.02 M Tris (pH 7.4) for 1 min using a Virtis Model 45 macro homogenizer. Tissue was treated with pronase (77.5 U/mg in 10 ml buffer; 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 250 mM NaCl) by adding 1 ml of enzyme/500 mg tissue and incubating for 2 h at 38°C. Two ml of the digest was dispersed in 10 mls scintillation cocktail and analyzed for ^{14}C content by liquid scintillation spectroscopy.

BaP Exposure Experiment I.

Fish were maintained at 25-26 °C in 30 L glass aquaria with a photoperiod of 16 h light and 8 h dark. Three static tanks each contained 70 fish; the female to male ratio was 3:2. Fish were fed both flake food (stress Flakes) and freshly hatched brine shrimp daily. One third of the water volume was changed every 48 h by siphoning; the contaminated water was disposed of through a charcoal filter, and fresh water saturated with BaP was added to replace the water removed.

One aquaria contained 10 μg BaP/L and 1% Tween 80 (BaP tank; BaP was added to the water from a concentrated stock solution with 40μg/ml BaP in 1% Tween 80. The second aquaria was a carrier control, and contained only 1% Tween 80 (Tween tank). The third

tank was a clean control (control tank). Ten fish (6 females and 4 males) were sampled from each tank on days 1, 2, 4, 8, and 16. A day 0 sample was also taken from the control tank. Before fish were removed from the tanks, eggs were harvested from females. Water changes on sampling days were made after sample fish were removed.

Fish were sacrificed by cervical scission and the livers, gills, and gonads were removed for analysis. The livers were weighed and placed into small Eppendorf tubes; the tubes were immediately dropped into liquid nitrogen for subsequent DNA alkaline unwinding analysis using a miniprep procedure adopted from Shugart (1988). A pooled sample of twenty mg of tissue was homogenized in a 1.5 ml Eppendorf microfuge tube in 100 μl of 1 N NH_4OH/ 0.2% Triton X-100 followed by addition of 500 μl of G-50 buffer (150 mM NaCl, 10 mM Tris-HCl, 25mM EDTA, 1 mM $MgCl_2$), pH 7.4. Proteins were removed by extracting the sample with 700 μl phenol/chloroform/isoamyl alcohol (CIP) followed by extraction with 700 μl of chloroform. The aqueous layer was removed after centrifugation and G-50 buffer in 1 M NaCl was added to a final volume of 2 ml. The samples were then analyzed for DNA alkaline unwinding.

The ovaries were weighed and placed in L-15 culture medium. The carcass was placed in 1.5 ml Eppendorf tubes and stored in liquid nitrogen. Ovarian tissue was examined for oocyte size, fecundity (daily clutch size), numbers of vitellogenic oocytes, and oocyte atresia (Greeley, et al., 1987; Lin, et al., 1989).

Fertilized eggs were placed into hatching solution immediately after being removed from the females (medaka hatching solution: 0.017M NaCl, 0.40mM KCl, 0.27mM $CaCl_2$ $2H_2O$. 0.66M $MgSO_4$ $7H_2O$, 1 mg/L methylene blue). The eggs were separated with fine forceps under a dissection microscope, and each individual clutch was divided into equal numbers and placed into petri dishes (60 x 15 mm). Replicate dishes held between 9-25 eggs. The width of the perivitelline space was determined by subtracting the outer sphere diameter from the inner sphere diameter by measurement (in mm) under a dissection microscope for each individual sample. Eggs were then incubated at room temperature and allowed to develop. These were observed daily for embryonic death and developmental staging (Kirchen and West, 1976, Carolina Biological Supply, Inc.). Death of the embryo was determined by uptake of the methylene blue dye. Time to hatch and hatching success were also recorded. Fry which hatched were moved to small culture dishes and fed from cultures of mixed paramecium and mixed nematodes for 8 d. They were then fed a regular diet of brine shrimp and Stress Flakes.

BaP Exposure Experiment II.

Adult medaka (male and female) were divided into four treatment groups with 5 fish per tank: A) 15 fish, untreated control; B) 15 fish treated with verapamil (1 µg/L); C) 15 fish treated with BaP (4 µg/L); and D) 15 fish treated with verapamil (Kurelec and Pivcevic, 1989), which inhibits the p-170 glycoprotein pump and subsequent removal of xenobiotic compounds from cells, 1)

μg/L) and BaP (4 μg/L). Medaka from each group were maintained in three aquaria containing 8 L of water. BaP-saturated water was prepared by passage through a Generator column containing glass beads coated with BaP (Shiu et al., 1988). No additional BaP water was added to the Bap and the BaP + verapamil tanks during the experiment. Tanks which contained verapamil were given a daily dose of verapamil dissolved in methanol; the doses were sufficient to deliver a final concentration of 1 μg/L of the drug. After 7 d of exposure, the medaka were sacrificed by spinal scission.The DNA from individual whole fish was extracted and analyzed using the alkaline unwinding assay as described by Shugart, 1988.

Bap Exposure Experiment III.

Adult medaka (male and female) were exposed for 42 h to BaP and BaP + verapamil in a small scale follow-up mini-exposure experiment. Three treatment groups were A) 6 fish, untreated control; B) 6 fish, treated with BaP (4 μg/L); C) 6 fish treated with Verapamil (1 μg/L) plus BaP (4 μg/L). The fish were maintained in small aquaria, each of which contained 5 L of water. BaP concentrations were maintained at a constant level by continuous saturation with a BaP generator. Verapamil, dissolved in methanol was added daily to a concentration of 1 μg/L.

After 42 h exposure, the fish were sacrificed by spinal scission and DNA was purified from whole individual fish by a modified procedure adopted from Maniatis et al., (1982). Whole fish were homogenized in a 10 ml glass homogenization tube with ten

strokes of a teflon pestle in 3 ml of extraction buffer (10 mM Tris-HCl, pH 7.4; 1 mM EDTA and 250 mM NaCl). To the homogenate was added 250 µl of 10% Sarkosyl and 50 µl of RNAase (2 mg/ml stock, DNAase-free RNAase). The mixture was incubated at 50°C for 30 min to digest RNA. Incubation continued for an additional 60 min after 6 µl of proteinase K solution (10 mg/ml stock solution) was added. The contents of the tubes were gently shaken during incubation to ensure complete digestion of RNA and protein.

After incubation, the digested samples were transferred to 6 ml SST serum separation vacutainer tubes. An equal volume of CIP (phenol-chloroform-isoamyl alcohol; 25:24:1) was added and the tubes mixed by inversion for 5 min. They were then centrifuged at 3000 x g for 20 min at room temperature and the aqueous phase re-extracted in chloroform to remove traces of phenol. The aqueous phase was collected, and the DNA in this phase was precipitated by adding 2 volumes of cold 100% ethanol. DNA was pelleted by centrifugation at 3000 x g for 20 min before being briefly dried in a vacuum and resuspended in 3.5 ml of alkaline unwinding buffer (10 mM Tris-HCl, pH 7.4; 1 mM EDTA and 250 mM NaCl). Alkaline unwinding analysis was performed according to the procedure described by Shugart (1988).

Results

Uptake and Accumulation of Radiolabeled BaP.

BaP in the water during the 8 d experiment declined following each water renewal event and radiolabeled [^{14}C]- BaP accumulated in the medaka (Figures 1 and 2).The initial rate of[^{14}C]-BaP accumulation into whole body tissues of the fish was high; during the remainder of the exposure time, it was lower (Figure 2,. BaP appeared to concentrate more extensively in the tissues of female medaka than in the tissues of the males (Figure 3).

Bap Exposure Experiment I.

The reproductive parameters (Table 1; days 1 and 16 of exposure) indicates that the fecundity of BaP-treated fish increased by approximately 250% during the 16 d experiment. In fish treated with Tween 80 only, fecundity actually decreased by 70%. The number of immature vitellogenic oocytes in the ovaries of fish treated with BaP increased by about 130% during the 16 d experiment, whereas the number of vitellogenic oocytes in ovaries of fish treated with Tween 80 did not change. The incidence of oocyte atresia did not appear different between the BaP or Tween 80 treated fish.

The results from the developmental studies are summarized in Table 2. No significant difference (t-Test) was found in the overall size of the eggs, the inner diameters or the difference between the two. These measurements were made under a dissection microscope within 6 h of fertilization and the data from each set

of replicate plates were pooled for this analysis. Exposure to BaP and Tween 80 had no obvious effect on the morphology of fertilized eggs. The average number of days over which hatching occurred was obtained by monitoring replicate plates within each treatment group. For example, on day 0, there were three replicate plates of embryos which hatched over a four and a half day period. In contrast, controls on day 16 had two replicate plates and the eggs on these plates required an average of 8 days from the first hatch to the last hatch. No significant difference (t-Test) was found between the groups when analyzed this way. All replicate plates were pooled for determination of the time of fertilization to the last hatch. No significant differences (t-Test) were seen between groups. The percent survival of successfully hatched fry in each group is give up to April 12 1990 (about a 30 d period). Since all fry from individual petri dishes were maintained in the same culture it was impossible to separate deaths that occurred as a result of transfer of fry with glass pipettes from any which might have been caused by exposure to BaP or Tween 80.

Alkaline unwinding analysis was performed on liver DNA from the medaka using the mini-prep method. The alkaline unwinding F values (% of the DNA in double stranded form) are shown at days 0, 1, 2, 4, 8 and 16 (Figure 4). The means of the alkaline unwinding F values within the three treatment groups were quite variable and are not significantly different on day 16 of exposure (ANOVA: $F= 0.774$; $F_{0.05,2,6}= 5.14$, $n= 3$, $N= 9$, $k= 3$; $p> 0.25$).

BaP Exposure Experiment II.

The alkaline unwinding F values from the second BaP experiment are shown in Figure 5. The F values for these four treatment groups were essentially the same except that DNA from fish treated with both verapamil and BaP had higher F values at 38°C (ANOVA: $F= 152.3$, $F_{0.05,3,32}= 2.92$, $n= 9$, $N= 36$, $k= 4$; $p< 0.001$). Medaka DNA from all four exposure groups was quite resistant to alkaline unwinding even at a temperature of 50°C.

Figure 6 shows a comparison of the extent of alkaline unwinding (measured by the decrease in fluorescence of DNA + Hoechsh dye # 33258) of BaP exposed medaka DNA with increasing temperature. Medaka DNA was resistant to unwinding between 23°C and 50°C, but began to unwind at higher temperatures.

Medaka DNA from the three exposed groups (BaP, verapamil, and BaP + verapamil had an increased resistance to alkaline unwinding (about a 25% decrease in fluorescence) in contrast to control and fresh, salt extracted DNA (about a 45% decrease in fluorescence) after 60 minutes of incubation as shown in Figure 7 (ANOVA: $F= 39.25$, $F_{0.25,4,20}= 2.87$, $n= 9$, $N= 25$, $k= 5$; $p< 0.001$). The freshly extracted medaka DNA showed an increased rate of unwinding in the first 30 minutes of incubation which was quite different from control and treated DNAs.

BaP Exposure Experiment III.

Figure 8 shows the alkaline unwinding F values for medaka DNA from the third experiment. The yield of high molecular weight DNA

was considerably higher than NH_4OH/ Triton X-100 extraction, possibly due to more efficient removal of protein which reduces loss of DNA to the aqueous-CIP interface during phenol extraction. The increased yield of DNA should make possible multiple DNA analyses from a single fish. There was no difference between the alkaline unwinding F values for DNA from control, BaP and BaP + verapamil treated fish at alkaline unwinding incubation temperatures of 4°C 38°C and 52°C (ANOVA: $F= 0.0123$, $p> 0.25$ at 38°C: $F=1.88$, $p> 0.10$ at 52°C; $F^{0.05}_{2,12}= 3.89$; $n= 5$, $N= 15$, $k= 3$). In addition, there was considerable unwinding of the DNA at lower temperatures in contrast to DNA extracted by the procedure of Shugart, 1988 in the previous exposure experiments.

Discussion

Japanese Medaka were exposed to water in which BaP dispersal was facilitated by the presence of a detergent (Tween 80). Since the maximal solubility of BaP in water without detergent is about 4 µg/L, the presence of Tween 80 may promote temporarily higher concentrations of BaP in water, but this BaP may not have been available to the fish. The rapid decline in BaP levels seen in Figure 1 could be attributed to either uptake into the medaka, binding to particulate organic matter or absorption to glass and plastic in the aquaria which is characteristic of hydrophobic polyaromatic hydrocarbons.

During the time that BaP levels decreased during the exposure, there was a rise in BaP levels in the tissues of the medaka. The increase is more rapid during the first day of exposure and changes to a slower but steady rate of accumulation on susequent days (Figure 2). The total amount of BaP accumulation in the 25 fish was less than 150 ng as compared with a decrease in BaP concentrations in water of greater than 30 μg; thus most of the BaP may have been absorbed to other materials in the aquaria or subjected to photodegradation. Female medaka also appeared to accumulate BaP at a higher level in their tissues than male fish. However, we do not yet know whether this is a result of metabolic or tissue specific differences between male and female medaka.

Medaka eggs were exposed to BaP only briefly (1 h after spawning). This exposure did not result in significant changes in the numbers of developmental abnormalities or in the morphology of fertilized eggs. The study of fecundity and ovarian oocyte atresia for BaP treated fish did not indicate overt ovotoxicity. In medaka, BaP may have actually stimulated oocyte development and egg production possibly via an estrogenic effect on the ovaries. BaP may inhibit ovarian function at higher effective concentrations. The lack of ovotoxicity in experiment I may have been caused by the medaka detoxifying and excreting BaP at the levels present in the water, or perhaps BaP was not biologically available to reproductive tissues in concentrations sufficient to greatly effect reproduction and development. Tween 80 alone may have exhibited some deleterious effect on fecundity, although this effect was not

seen in combination with BaP. The use of Tween 80 as a vehicle for BaP may have to be reconsidered.

The high variability in survival of embryos derived from BaP-exposed parents may have resulted from problems with glass pipette transfer techniques. Improved handling techniques for embryo transfer have been developed which should minimize damage to developing fish. Future studies will additionally focus on genotoxic and teratogenic effects of BaP exposure in medaka. The dominant lethal assay was developed as a measure of genotoxicity in mammals (mice) exposed to a large number of xenobiotic compounds (Bateman and Epstein, 1971). Previous studies using intraperitoneal injection of mitomycin C and ethyl methanesulfonate into medaka have demonstrated a considerable dominant lethal effect as measured by hatchability of fertilized eggs (Shimada and Egami, 1984). The medaka is currently being used for studies of teratogenesis by exposing developing fertile eggs to xenobiotic compounds (Cameron et al., 1985; Shi and Faustman, 1989). Fertilized medaka eggs can be easily monitored through a compound microscope during development. Future studies incorporating the dominant lethal assay, teratogenic analysis, and reproductive studies could help identify more specific genotoxic and cytotoxic effects. These studies would complement experiments described in this report in which parental medaka were exposed to BaP and the reproductive and developmental effects analysed and is more characteristic of environmental exposure to toxicants.

Alkaline unwinding analyses of liver DNA indicated that BaP may not have caused DNA strand breaks in medaka (Figure 4). Considerable variability of alkaline unwinding F values within treatment groups, though may make it necessary to modify the procedure to improve both the yield and integrity of DNA extracted. Flow cytometric analyses of liver and gill cells (which provides an estimate of the DNA content of cell nuclei) failed to detect an effect of BaP on nuclear DNA (data not shown).

Tween 80 was not used as a vehicle for dispersing BaP in the water in experiments II and III. The water in the second exposure was saturated with BaP at day 0 and was not renewed thereafter. In experiment II the concentration of BaP was initially high but rapidly declined to undetectable levels in the water by d 7 (Shugart et al., 1987). Verapamil, which inhibits the p-170 glycoprotein xenobiotic pump (Kurelec and Pivcevic, 1989); this drug was renewed daily in the verapamil and BaP + verapamil treatments.

The alkaline unwinding rates obtained from DNA extracted from whole medaka carcasses (Figures 5 and 6) showed that medaka DNA was very resistant to unwinding at incubation temperatures successfully used to measure DNA strand breaks in BaP exposed Bluegill sunfish and in other animal species. We found no significant difference in the DNA strand breakage of fish treated with BaP alone or those treated with verapamil. Also, resistance of medaka DNA to alkaline unwinding was greater for fish exposed to BaP, verapamil, and BaP + verapamil than it was for unexposed control fish (Figure 7).

Thus, high concentrations of salt (>250mM NaCl) and/or proteinase K - RNAase digestion during DNA extraction may have removed an unidentified unwinding inhibitor that, 1) associates with medaka DNA and 2) may be induced by exposure of the fish to xenobiotic compounds.

In summary, we found no evidence of BaP or verapamil induced genotoxicity as measured by direct DNA damage (strand breaks) in the medaka. In addition, little evidence was found for physiological effects of these two chemicals upon reproductive or developmental parameters. The low solubility of BaP in water may necessitate longer exposure times to overcome the natural xenobiotic defense mechanisms in medaka.

Several hypotheses for the resistance of medaka to DNA damage from xenobiotic compounds can be formulated: 1) these fish have efficient metabolism and excretion of BaP from the gills; 2) medaka may be able to induce or increase the activity of a xenobiotic pump (the use of higher concentrations of verapamil or vanadate may effectively inhibit the p-170 glycoprotein pump and permit toxicants to enter the cells at a higher rate); and 3) an as yet unidentified factor naturally associated with medaka DNA and induced to higher levels by exposure to xenobiotics may protect DNA from damage or facilitate its repair.

A continuation of these studies on BaP exposure in medaka will focus on the nature of the salt-extractable factor which apparently associates with the DNA. Proteins and polyamines (Bachrach, 1973) are potential candidates for such factors, though protein may be

the more likely candidate, for protease treatment greatly facilitates alkaline unwinding of DNA. This phenomenon may in itself be a marker of DNA damage because DNA-associating protein (rec A) is induced in E. coli after DNA damage has occurred (Hanawalt et al., 1979). The possibility of protein-protein or protein-DNA crosslinking cannot be excluded.

The dominant lethal assay and teratogenesis analysis will be used as an additional measure of genetic damage in the medaka after exposure to xenobiotics (Shimada and Egami, 1984). Agarose gel electrophoresis would be another means to determine the amount of double-stranded DNA breaks (Maniatis et al., 1982. Two other assays to estimate DNA damage are 1) the quantitation of BaP adducts (Shugart et al., 1987) and 2) ^{5}m-dCty (Shugart, 1990) levels in the DNA of exposed organisms. Other areas of analysis would employ flow cytometry, DNA repair assays, stress protein synthesis and detoxification enzyme activities.

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TABLE 1

MEDAKA REPRODUCTION

DAY	TREATMENT	female GSI[1]	Fecundity[2]	Atresia[3]	Vitellogenic Oocytes[4]
1	Control	5.5 +/- 0.4[5]	6.1+/- 1.1	0.3+/- 0.2	68.2+/- 7.6
	Tween 80	4.5+/- 0.9	8.5+/- 2.8	0.7+/- 0.7	68.8+/- 18.5
	BaP	5.3+/- 0.3	5.5+/- 1.7	6.5+/- 5.5	85.8+/- 19.4
16	Control	5.7+/- 0.5	11.3+/- 2.4	1.3+/- 0.8	94.5+/- 16.1
	Tween 80	4.4+/- 0.5	5.8+/- 1.1	1.5+/- 0.6	70.2+/- 6.5
	BaP	6.0+/- 0.3	13.3+/- 1.4	0.8+/- 0.5	113.5+/- 10.8

1. GSI: Gonadal Somatic Index; (ovary weight/body wt) X 100.

2. Fecundity: The number of mature eggs released per ovary or daily clutch size.

3. Atresia: The number of dead or damaged oocytes per ovary.

4. Vitellogenic Oocytes: The number of yolk-containing oocytes per ovary.

5. Values represented as means +/- SEM; n= 6 in each case.

TABLE 2

MEDAKA DEVELOPMENT

PARAMETER	CONTROL n= 21	TWEEN 80 n= 15	BaP[1] n= 36
Outer Diam. of Fertilized Eggs[2] (mm)	1.25 +/- 0.06[3]	1.20 +/- 0.06	1.25+/- 0.05
Inner Diam. of Fertilized Eggs[2] (mm)	1.10 +/- 0.06	1.10 +/- 0.06	1.10+/- 0.06
Size of the Perivitelline Space in Fertilized Eggs[2] (mm)	0.15 +/- 0.06	0.15 +/- 0.05	0.15 +/- 0.05
Number of Days Over Which Hatching Occurred[2]	8.00 +/- 1.41	4.50 +/- 3.53	7.33 +/- 0.58
Days from fertilization to last hatch[2]	12.75 +/- 2.43	12.73 +/- 3.02	15.24 +/- 2.61
Fry survival	83.3%	90.9%	67.3%

1. 10 ppb BaP in 1% Tween 80

2. Day 16 of exposure; mean +/- SEM

3. +/- SE.

BaP Concentrations in Water
During Exposure of Medaka

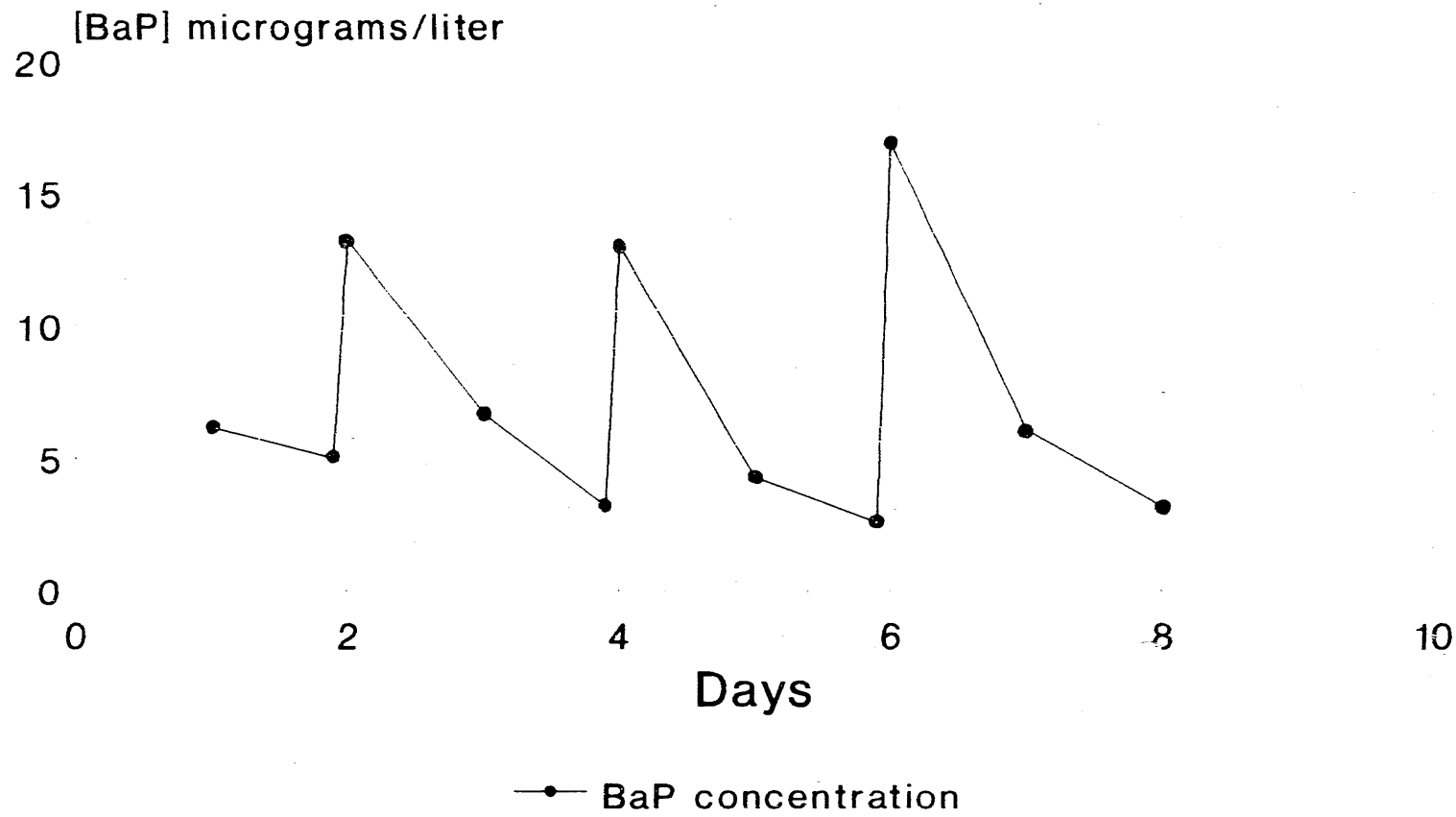

FIGURE 1. Concentration of BaP in water during an 8 d exposure of Medaka.

BaP Accumulation in Medaka

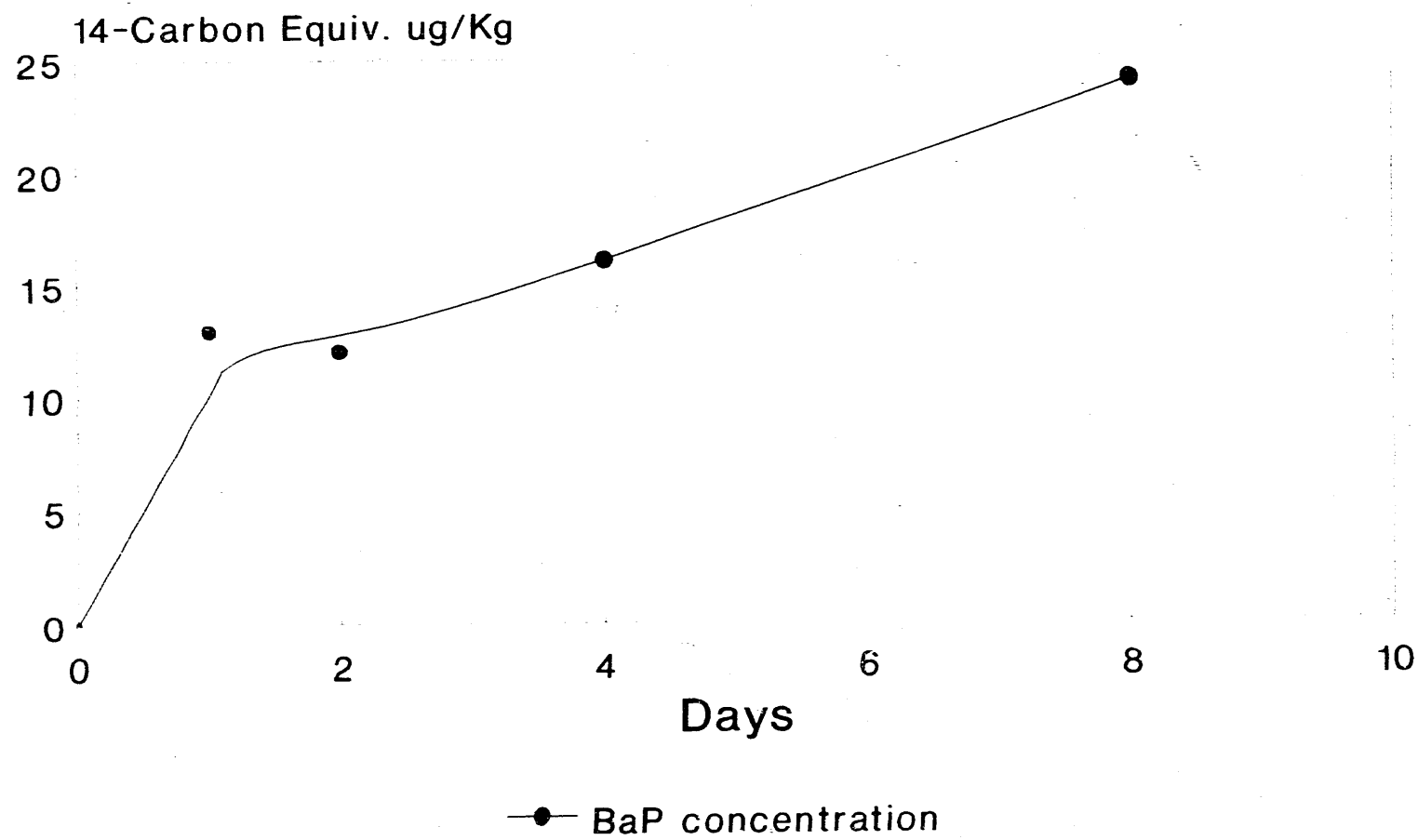

FIGURE 2. Accumulation of BaP in whole body tissues of adult Medaka over time, N= 15 female and 10 male fish

BaP Accumulation in Medaka
Males vs. Females

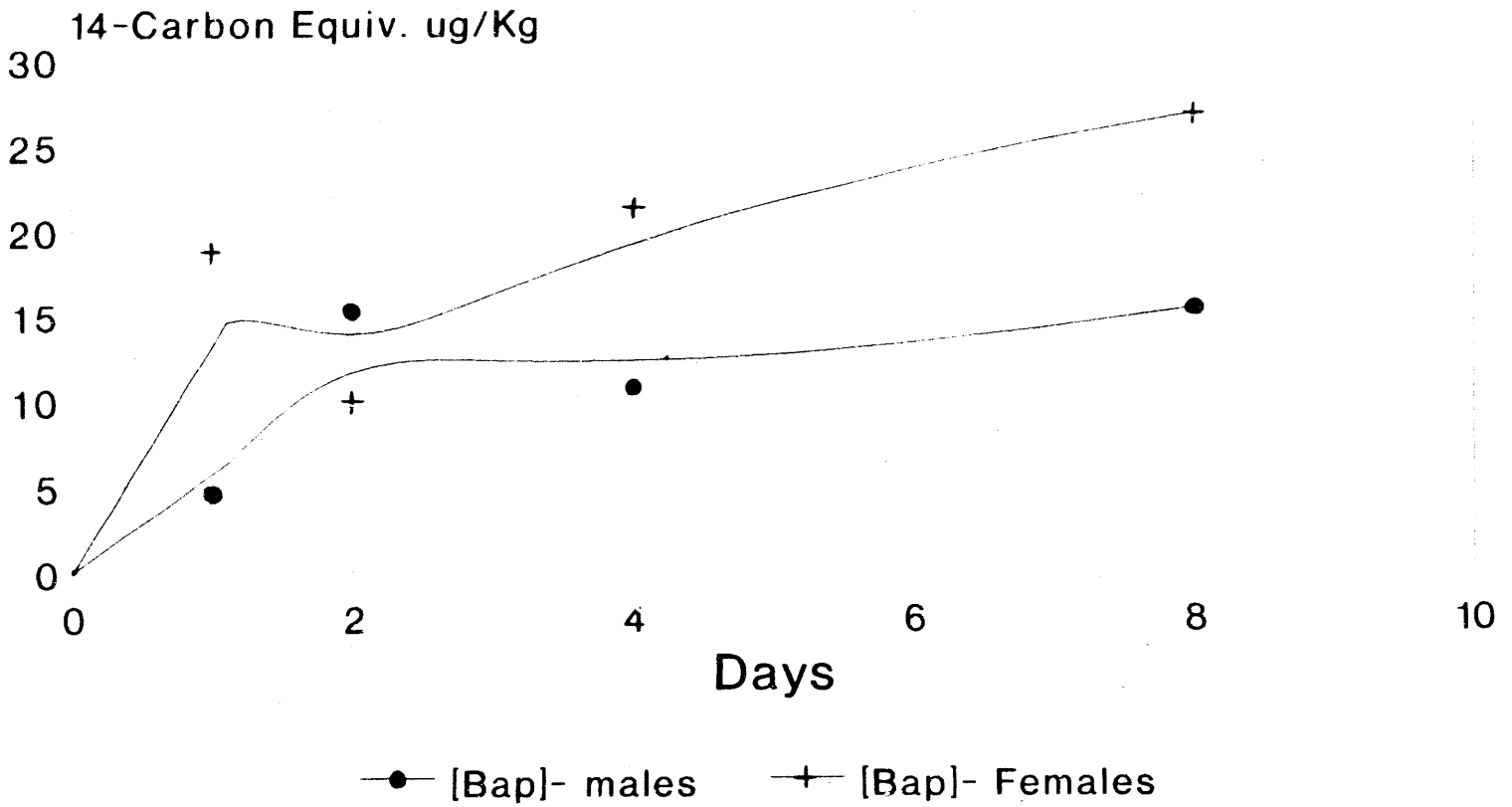

FIGURE 3. Accumulation of BaP in whole body tissues of male and female fish
N= 15 females; N= 10 males

F Values for DNA of Medaka
From Exposure I

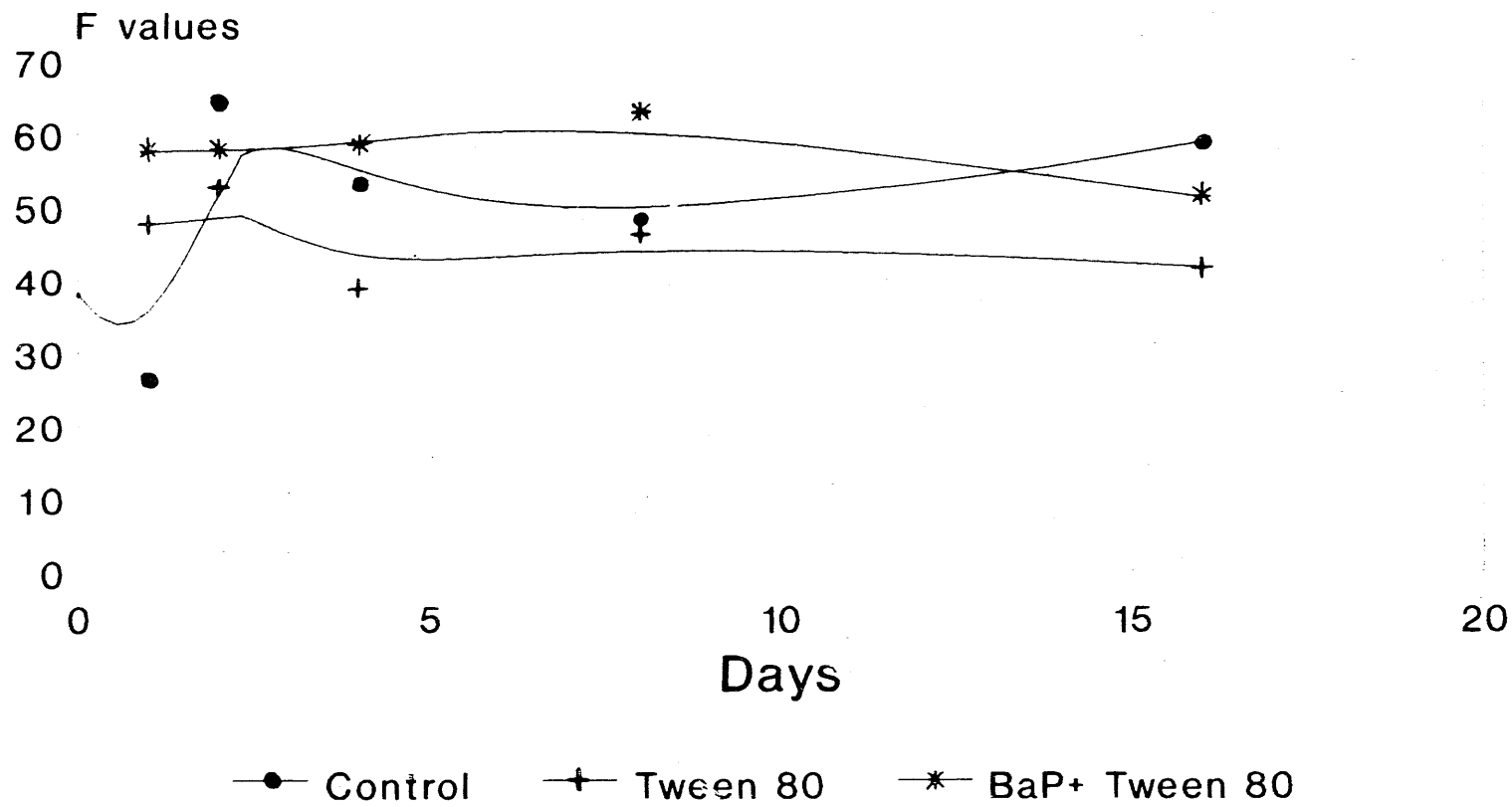

FIGURE 4. F values (% DNA in double stranded form) from livers of adult Medaka from BaP exp. I overtime.

F VALUES OF EXPOSURE II
ON MEDAKA DNA

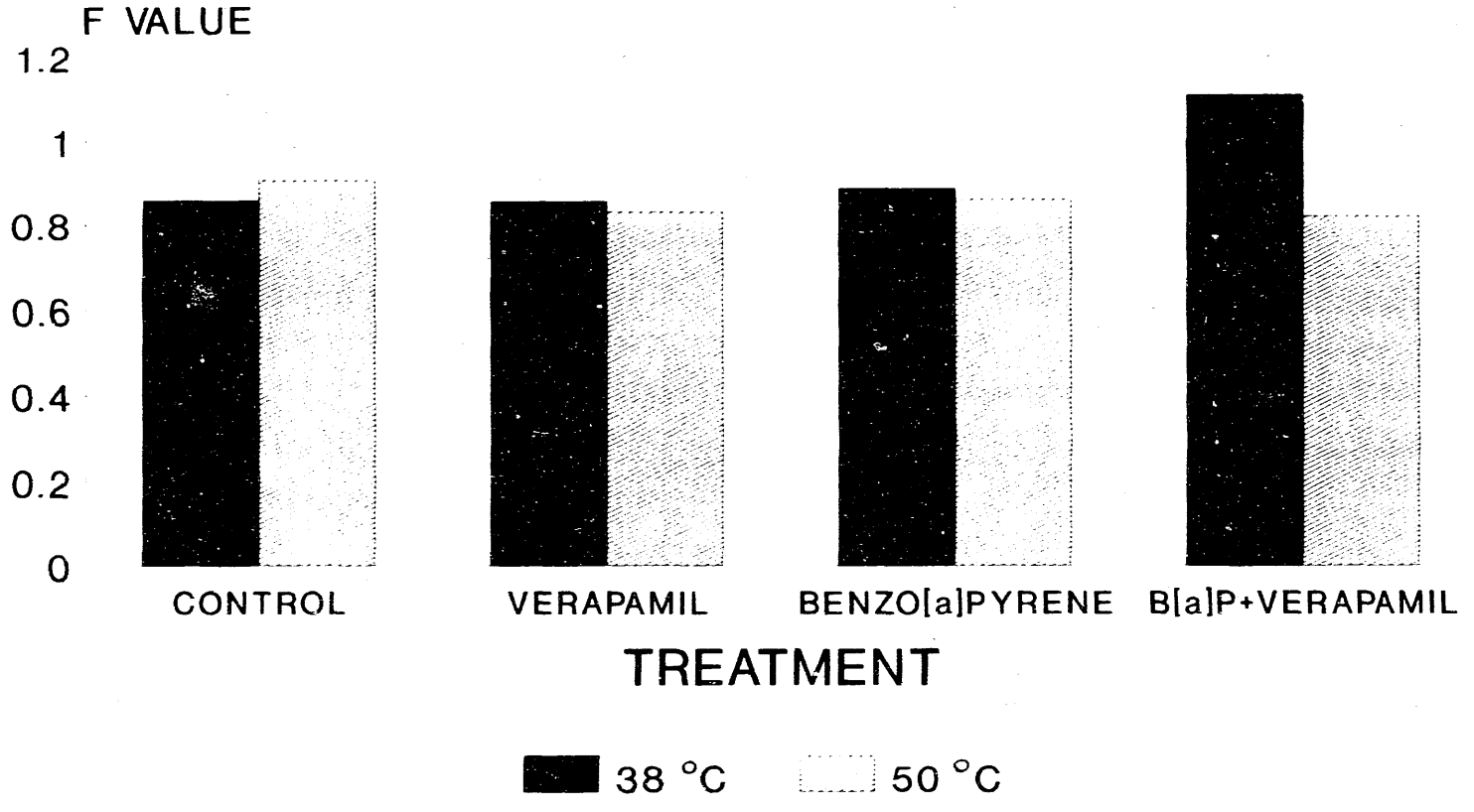

FIGURE 5. F values (% DNA in double-stranded form) for Medaka DNA from exposure II (7 days exposure).

THERMAL DENATURATION
OF BaP TREATED MEDAKA DNA
UNDER ALKALINE CONDITIONS

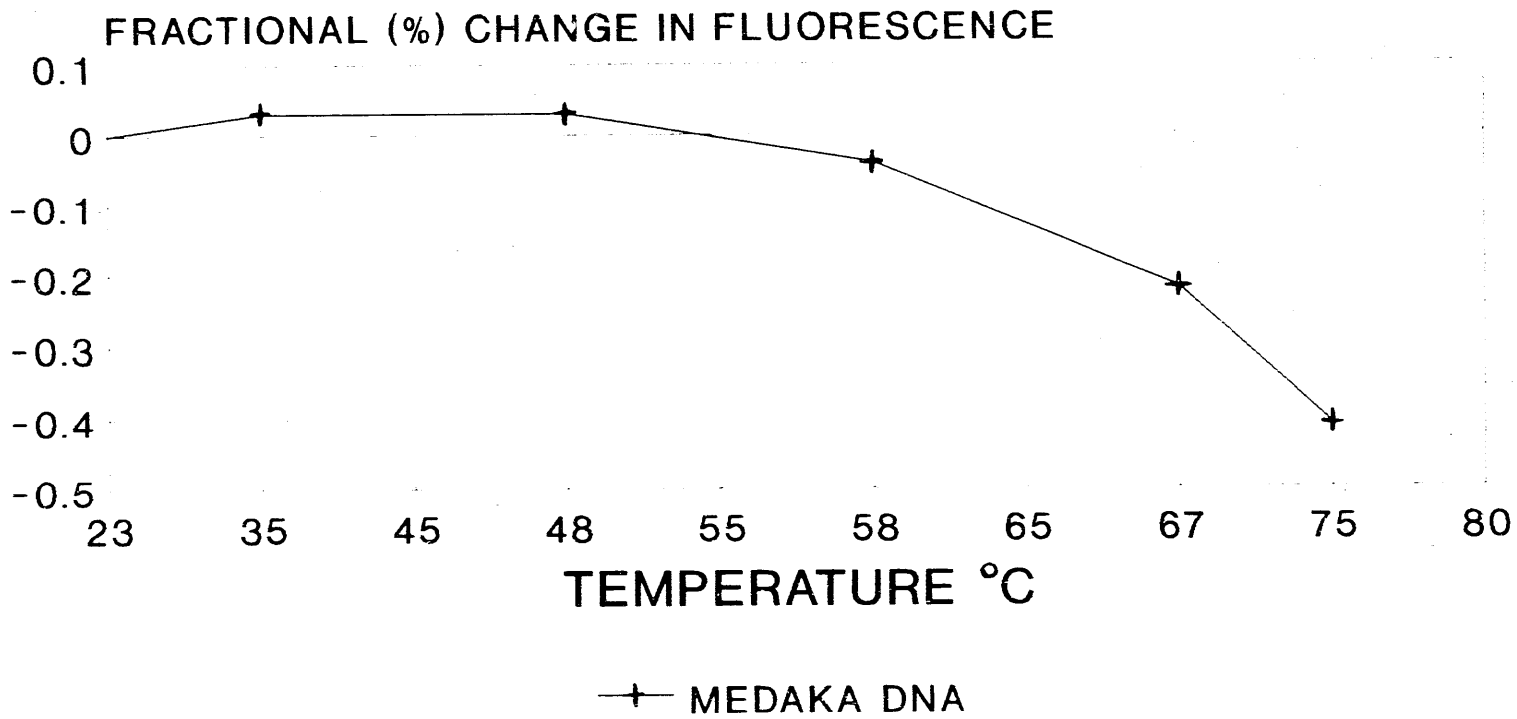

FIGURE 6. Alkaline unwinding of medaka DNA at various temperatures; exposed to BaP for 10 d.

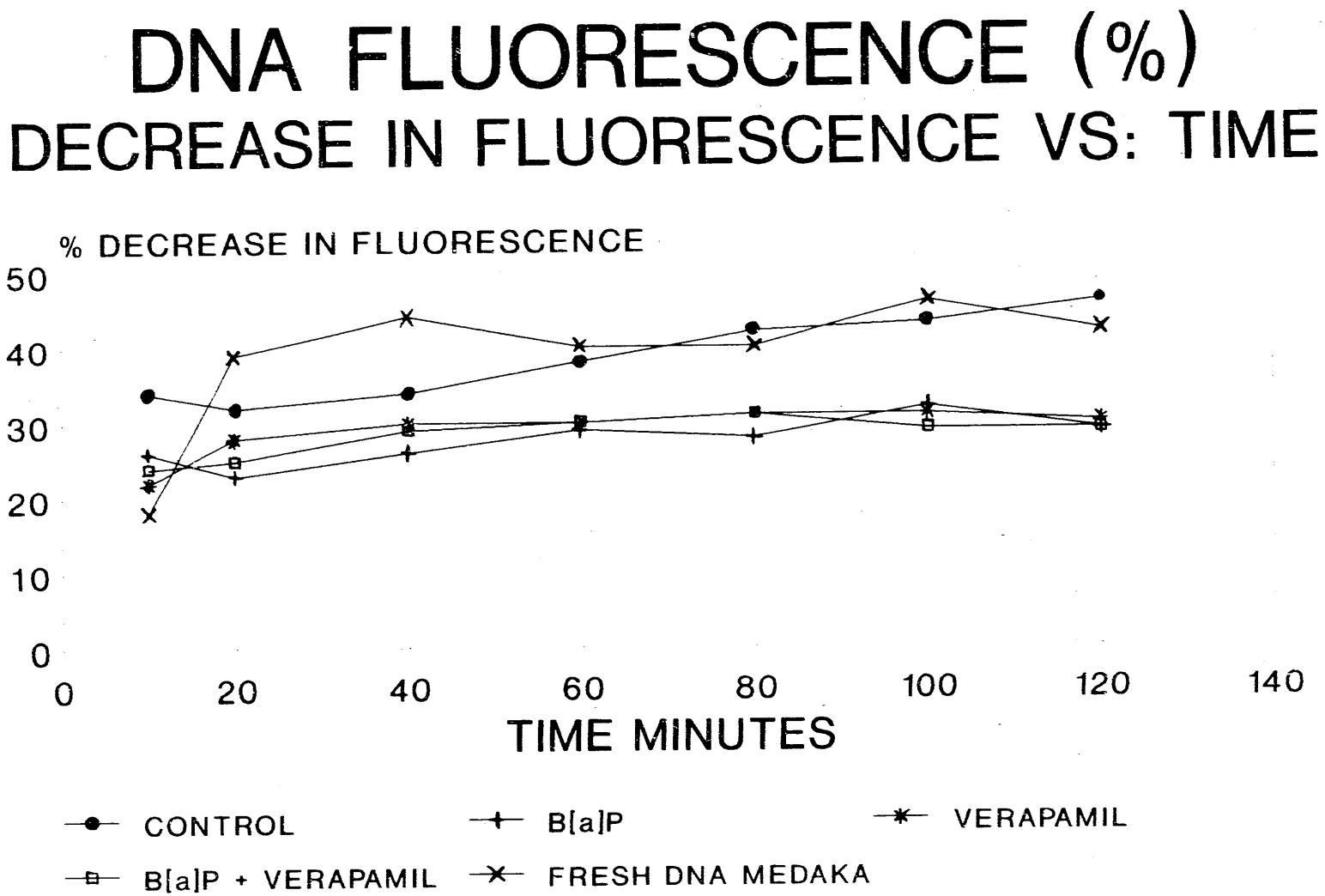

FIGURE 7. Fluorescence change between control, BaP and verapamil exposed Medaka (incubation at 60 C).

F VALUES OF EXPOSURE III
ON MEDAKA DNA

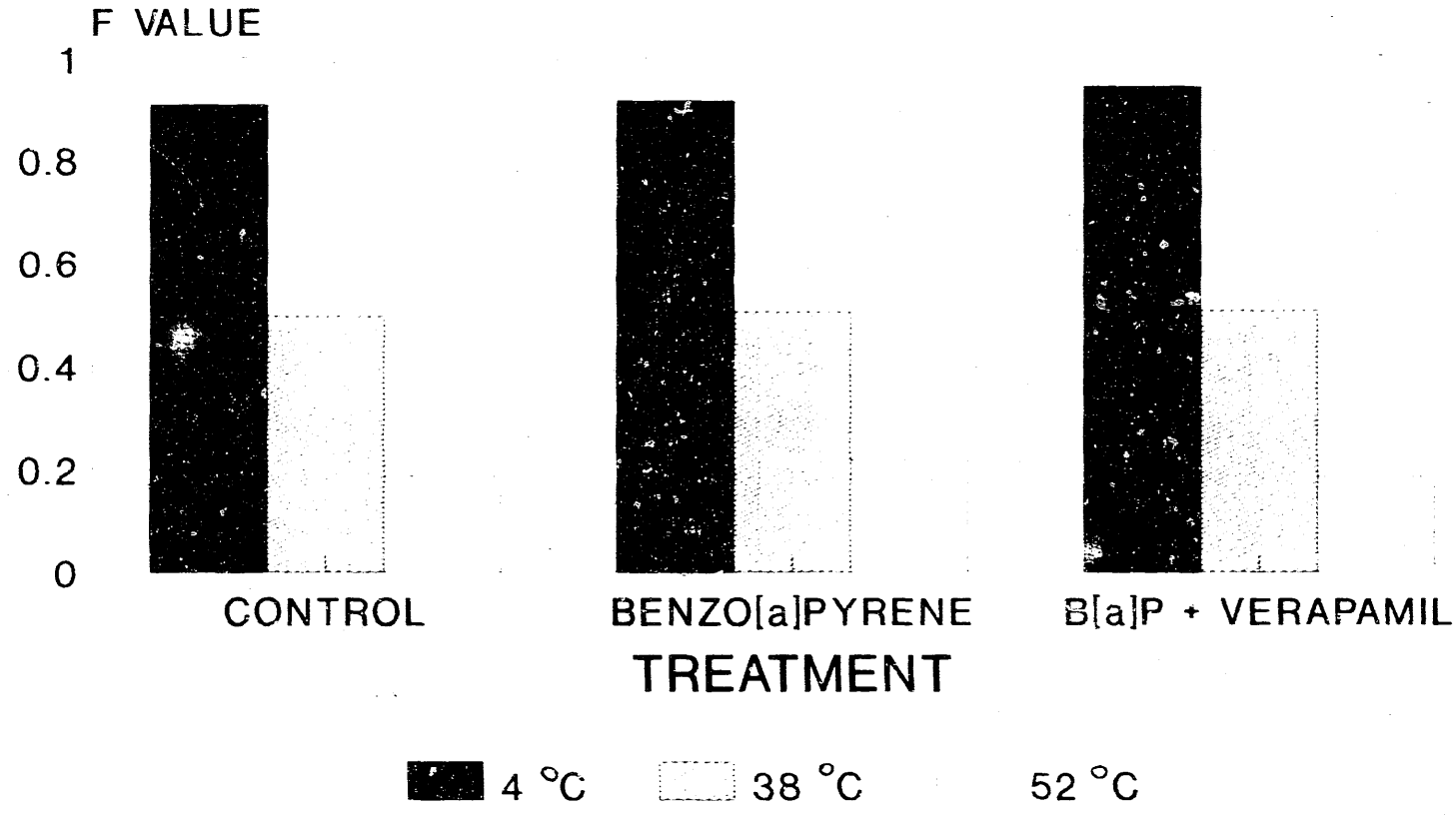

FIGURE 8. Comparison of F values (% DNA in ds form) of DNA from control and 42 hour exposed Medaka.

END

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