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Project Summary

Usefulness of the Self-Fertilizing Cyprinodontid Fish, *Rivulus marmoratus* as an Experimental Animal in Studies Involving Carcinogenesis, Teratogenesis and Mutagenesis

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Rivulus marmoratus is a naturally self-fertilizing cyprinodontid fish inhabiting mangrove marshes throughout the Caribbean. As a result of internal self-fertilization, this oviparous species is composed of a number of isogenic, homozygous lines (clones), several of which have been identified by histocompatibility experiments and maintained in laboratory culture for over 30 years.

Simplified culture and handling methods are given and data are presented on the reproduction, growth and development of rivulus under laboratory culture as a prelude to the evaluation of its potential as a bioassay animal. Several types of bioassays were run and evaluated using rivulus: behavioral, carcinogenic, teratogenic, toxic, and mutagenic. Advantages and disadvantages of using rivulus for such bioassays are discussed. Behaviorally, rivulus is capable of detecting and avoiding water contaminated with H_2S. They respond (EC50 = 123.6 ppb H_2S) by leaping from the water and remaining emergent for various periods of time while respiring cutaneously. Hepatocellular carcinoma among other pathologic changes were observed in livers of rivulus a year after exposure of adults and larvae to diethylnitrosamine (45, 30, 15 ppm in water) for 5 weeks and 12 weeks, respectively. No pathologic changes were found in embryos exposed similarly. High rates of various skeletal malformations resulted in offspring of adults exposed to dibutyl phthalate (DBP) and 2,3,4,6-tetrachlorophenol (TECP) at concentrations of 20, 10 and 5% (DBP - 0.740.

This Project Summary was developed by EPA's Environmental Research Laboratory, Gulf Breeze, FL, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

Rivulus marmoratus (common name = rivulus) is a self-fertilizing cyprinodontid fish inhabiting mangrove marshes throughout the Caribbean. As a result of natural internal self-fertilization, this oviparous species produces isogenic, homozygous lines (clones). Several clones have been established for nearly 30 years by Dr. Robert W. Harrington,

Jr. from single wild-caught hermaphrodites taken in Florida. Rivulus possesses a number of attributes that make it attractive as an experimental animal. It is hardy, easy and inexpensive to maintain in the laboratory and has a wide salinity tolerance, short generation time and desirable reproductive and genetic aspects. Our main purpose was to investigate the potential of rivulus as an experimental animal useful in bioassay studies and particularly in the study of carcinogenicity, teratogenicity and mutagenicity.

Methods

Laboratory Culture of Rivulus marmoratus

Rivulus were held singly in stackable glass culture dishes in 14 o/oo synthetic seawater. Temperature is held at 25° ± 0.5°C and the photoperiod is set at 14 hr. Frozen brine shrimp (*Artemia*) are fed to adult rivulus and live brine shrimp nauplii are fed to the young. Reproducing adults are held singly in culture dishes outfitted with teflon bottom screens to prevent the fish from eating their eggs. Fish are fed and eggs are removed three times a week and water is changed about once a month. This system has the advantages of simplicity and economy. Also, chances of unwanted contamination of the culture water are minimal because the entire apparatus is made of teflon and glass.

Reproduction, Growth and Development of Rivulus marmoratus

Because internal self-fertilization is the natural mode of reproduction in rivulus, other unusual characteristics relating to reproduction result. Eggs may be emitted at various times during embryological development either singly or in clusters. The average intraparental incubation time is about 10 hr. A relatively large proportion (22.5%) of eggs are non-viable (assumed unfertilized). Healthy adult rivulus during reproductive periods produce about an egg per day on an average.

Growth experiments indicated that culture dishes 11.4 cm in diameter were optimum for rearing all life stages but the 5.1 cm culture dishes were convenient for early life stages. Also, food should be changed from live nauplii to frozen brine shrimp at about 2 months after hatching. The first eggs were laid about 6 months after hatching.

The embryonic development of rivulus (Table 1) is similar to that of other cyprinodontid fishes. Although hatching usually takes place in 12 to 15 days after fertilization, a delay in hatching may extend this up to 14 additional days. The control of hatching or the cause of delayed hatching in rivulus is not known.

There are several advantages and disadvantages to using rivulus embryos for research purposes. The main advantage, aside from genetic, is that under laboratory conditions eggs may be obtained throughout the year. However, because fertilization and the early stages of development (often through blastulation) are intraparental, observations must be restricted to the latter stages. Also, because eggs are not fertilized synchronously it is difficult to obtain a number of eggs in the same stage of development.

Behavioral Bioassay using Rivulus marmoratus

Rivulus has the capacity to leap from the water and remain emergent for extended periods of time while respiring cutaneously. This behavior has been observed in the laboratory in response to contamination of culture water by H_2S, ammonia, formalin and rotenone.

This study was designed to evaluate the use of rivulus as a test animal for early warning systems in biological monitoring.

Flow-through bioassays were carried out in covered beakers outfitted with

Table 1. *Summary of the Development Stages of* Rivulus marmoratus

Stage	*Time (hr)*	*Description*
1	*2.5*	*1 cell*
2	*3.5*	*2 cells*
3	*4*	*4 cells*
4	*4.5*	*8 cells*
5	*5.5*	*16 cells*
6	*6.5*	*32 cells*
7	*8*	*Early blastula*
8	*9.5*	*High blastula*
9	*10.5*	*Late or flat blastula*
10	*15*	*Gastrulation begins, expanding blastula*
11	*19*	*Epiblast covers 1/3 yolk*
12	*22*	*Epiblast covers 1/2 yolk, germ ring and embryonic shield are forming*
13	*24*	*Epiblast covers 2/3 yolk*
14	*25.5*	*Large yolk plug, embryonic shield enlarging*
15	*31*	*Epiboly complete*
16	*34.5*	*Head and tail regions recognizable*
17	*36*	*Optic vesicles appear, somite formation begins*
18	*43.5*	*Optocoeles are prominent, auditory vesicles form*
19	*53*	*Optic cup and lens formation*
20	*55.5*	*Heart beats, no circulation*
21	*58*	*Body movement*
22	*62*	*Circulation*
23	*71*	*Increased vitelline circulation*
24	*73*	*Urinary bladder and otoliths first appear, pigmentation on brain, trunk, and yolk near embryo*
25	*77*	*Pectoral fins appear, otoliths are prominent*
26	*90*	*Liver first appears, pigmentation on optic cup*
27	*105*	*Increased pigmentation and body movement*
28	*140*	*Pigmentation of the optic cup obscures the lens, circulation in pectoral fin and liver, caudal fin developing*
29	*180*	*Gas bladder and anal fin formation*
30	*211*	*Rays in caudal fin form, dorsal fin develops, the jaw appears*
31	*240*	*Pectoral fin movement, circulation in the caudal fin, a delay in hatching can occur*
32	*310*	*Hatching, rays in the dorsal and anal fin form*
33	*post-hatching*	*Fish is actively swimming*

shelves onto which the fish could jump. Each of five beakers were supplied by individual head tanks containing either test or control solutions. Dissolved oxygen concentration was held at 2 ppm ± 0.05 ppm in all test containers and one control container; the other control container was kept at air-saturation. Both position of beakers and assignment of fish were random. Randomly selected fish were allowed 1 hr acclimation which was followed by 1 hr of observation. Each fish was used only once. H_2S and O_2 tensions were monitored during all 11 runs.

Results, analyzed by probit analysis, indicate an EC50 (median effective concentration) of 123.59 ppb H_2S and 95% confidence limits were 63.68 and 181.97. Also, there was a positive correlation ($p < 0.05$; Spearman rank correlation) between H_2S concentration and length of time emergent.

Although more comprehensive testing is required, the data indicate rivulus shows promise in water quality management for the following reasons: 1) the behavior is easily quantified; 2) the response is rapid; 3) normal variation in environmental parameters such as salinity and temperature do not elicit the response; 4) by the use of fish from the same clone genetic variability can be eliminated; and 5) rivulus adapts quickly to laboratory conditions and large numbers can be held at low cost.

Carconogenesis Bioassay using Rivulus marmoratus

Liver cancer (hepatocellular carcinoma) has been induced in a number of fish species including rainbow trout and several species of small aquarium fish. Among the most active chemical agents which cause liver cancer are the aflatoxins and the nitrosamines. The purpose of this study was to establish the sensitivity of various life stages of rivulus to diethylnitrosamine (DEN) so that the potential of rivulus as a test animal for carcinogenic studies may be evaluated.

Adult rivulus (30 fish per concentration) were exposed to DEN in the culture water in concentrations of 45, 30, 15, and 0 mg/l for 5 weeks. Larvae (< 1 month old; 30 per concentration) were exposed to the same concentration levels for 12 weeks. Embryos (56 per concentration) were exposed to DEN in the culture water in concentrations of 1000, 100, 32, 10 and 0 mg/l for 1 week. All surviving rivulus were held in uncontaminated culture water for one year, then killed in 10% buffered formalin and prepared for histological examination. Slides of liver sections were examined and various pathologic changes were described by Dr. John C. Harshbarger (Registry of Tumors in Lower Animals, Smithsonian Institution).

Results indicate that DEN induced hepatocellular carcinoma, among other pathologic changes, in rivulus as has been shown for a number of other experimental animals. Histological analyses of the livers of adults and larvae are summarized in Tables 2 and 3. Figure 1 compares normal liver tissue and solid pattern hepatocellular carcinoma from a fish exposed to 30 ppm DEN for 12 weeks. No pathologic changes were seen in rivulus exposed to DEN as embryos. There were no obvious indications of tumors when whole livers were examined grossly

Advantages of using rivulus in studies involving induction of carcinoma include: 1) a variety of isogenic, homozygous strains may be used; 2) tissue transplants are possible within clones; 3) no aeration is necessary in the simple culture system, thus volatile chemicals such as DEN are not lost; and 4) embryo studies are possible throughout the year. It is not known whether clear determination of hepatocellular carcinoma could be made with an induction period shorter than one year.

Table 2. *Pathologic Changes in Livers of* Rivulus marmoratus *Adults Exposed to Diethylnitrosamine for 5 Weeks*

Nominal Water Concs. (mg/l)	*No. Fish (Start)*	*No. Fish[a] (End)*	*Fish with Neoplasms[b]*	*Incidence[c]*				
				Hepatocellular Carcinoma	*Incipient Neoplasms*	*Cholangioma*	*Adenofibrosis*	*Granuloma*
0	*30*	*23*	*0/23(0)[d]*	*0/23(0)*	*0/23(0)*	*0/23(0)*	*0/23(0)*	*0/23(0)*
15	*30*	*20*	*0/20(0)*	*0/20(0)*	*0/20(0)*	*0/20(0)*	*1/20(5.0)*	*0/20(0)*
30	*30*	*21*	*7/21(33.3)*	*6/21(28.6)*	*2/21(9.5)*	*1/21(4.8)*	*13/21(61 9)*	*5/21(23 8)*
45	*30*	*16*	*5/16(31 3)*	*5/16(31.3)*	*0/16(0)*	*0/16(0)*	*8/16(50.0)*	*2/16(12.5)*

[a]Fish were killed one year after end of exposure period.
[b]No of fish with neoplasms (hepatocellular carcinoma, cholangioma, or incipient neoplasms)/total no. of fish
[c]No. of fish with pathologic change/total no of fish.
[d]Nos. in parentheses are percents

Table 3. *Pathologic Changes in Livers of* Rivulus marmoratus *Larvae Exposed to Diethylnitrosamine for 12 Weeks*

Nominal Water Concs (mg/l)	*No Fish (Start)*	*No. Fish (End)[a]*	*Fish with Neoplasms[b]*	*Incidence[c]*				
				Hepatocellular Carcinoma	*Incipient Neoplasms*	*Cholangioma*	*Adenfibrosis*	*Granuloma*
0	*30*	*23*	*0/23(0)[d]*	*0/23(0)*	*0/23(0)*	*0/23(0)*	*0/23(0)*	*0/23(0)*
15	*30*	*18*	*17/18(94.4)*	*12/18(66.7)*	*7/18(38.9)*	*4/18(22 2)*	*16/18(88.9)*	*11/18(61.1)*
30	*30*	*14*	*12/14(85 7)*	*9/14(64.3)*	*4/14(28.6)*	*1/14(7.1)*	*6/14(42.9)*	*7/14(50.0)*
45	*30*	*17*	*16/17(94.1)*	*13/17(76.5)*	*4/17(23.5)*	*7/17(41 1)*	*14/17(82 4)*	*7/17(41.2)*

[a]Fish were killed one year after end of exposure period.
[b]No. of fish with neoplasms (hepatocellular carcinoma, cholangioma or incipient neoplasms)/total no. of fish.
[c]No. of fish with pathologic change/total no. fish.
[d]Nos in parentheses are percents

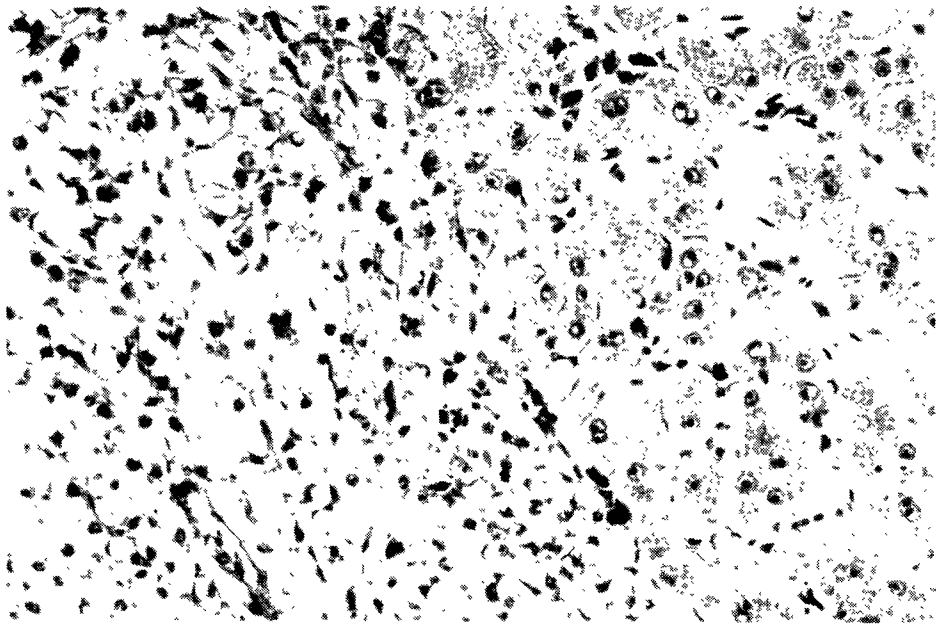

Figure 1. Liver section from rivulus exposed to 30 ppm DEN for 12 weeks. Border of hepatocellular carcinoma (dark) and normal tissue (light) is distinct. H & E x 400.

Teratogenicity Bioassays and Chronic Full Life Cycle Exposure of Rivulus marmoratus *to Sublethal Levels of Selected Toxicants*

Coastal and marine environments have become contaminated with a variety of organic and inorganic pollutants. Many of these pollutants are known teratogens, mutagens, and carcinogens but there is little or no information on the vast majority. Often, effects such as tumors and various abnormalities are seen in natural populations from contaminated areas; however, laboratory experiments on affected populations are often difficult or impossible because of problems associated with maintaining laboratory populations One way to gain insight into the mechanisms of action of the various pollutants is to expose species which are conducive to laboratory culture to suspect chemicals and observe responses throughout the full life cycle. This work was designed to evaluate the potential of rivulus for such studies. Five priority chemicals were selected for this study, among them the known teratogen, dibutyl phthalate (DBP). The other toxicants used were pentachlorophenol (PCP), 2,3,4,6-tetrachlorophenol (TECP), 2,3,5-trichlorophenol (TRCP) and bromoform.

Sublethal water concentrations for the full life cycle chronic study were chosen as fractions (20, 10 and 5%) of static 96-hr LC50 tests on newly hatched larvae Randomly chosen adults were exposed to the various concentrations for eight months during which time eggs were collected, incubated and reared. Eggs for each fish were randomly placed in either uncontaminated water or water contaminated with the same chemical and to the same degree as that of parental adults. This was done to distinguish between prehatching developmental effects and post-hatching chronic exposure effects. At the end of the exposure period all offspring were killed, examined grossly and group was cleared and stained for the determination of skeletal abnormalities.

The chronic exposure levels, based on larval LC50's, were as follows: PCP - 0 074, 0.037, 0.185; TECP - 0.220, 0.110, 0.055; TRCP - 0 360, 0.180, 0.090; DBP - 0.740, 0.370, 0.185; and bromoform - 8 40, 4 20, 2.10 mg/l.

Production of infertile eggs over all exposures and controls averaged 22.6%. The proportion of viable eggs that hatched varied from about 70 to 90% overall. The most common abnormality among embryos was an edematous pericardial cavity but this showed no clear relationship to chemical or concentration. Mortality rates among post-hatch offspring were variable but dose-related in some cases, such as in response to TECP exposure Gill and fin erosion was clearly the result of exposure to TECP and this undoubtedly contributed to the dose-related mortalities. Also, degree of damage to fins and gills was dose-related

Skeletal abnormalities observed in cleared and stained fish were divided into six categories: vertebral fusion, deformed centra, abnormal neural and/or hemal spines, abnormal dorsal fins, abnormal pectoral fins and abnormal pelvic fins. Rates of skeletal abnormality in controls were about 30% and this compares well with previous work done by Dr. R. W. Harrington, Jr on the same clones of rivulus. Preliminary work on wild-caught fish from Naples, Florida, indicate spontaneous rates of skeletal abnormality near zero.

A teratogenic response from the DBP exposures was evident in the rates of vertebral fusion and neural and hemal spine deformity A histogram of such effects in offspring reared in uncontaminated water shows a clear dose-response relationship (Figure 2). Evidence for a teratogenic response was also seen in offspring from TECP exposed fish. There was no clear evidence for teratogenic response in offspring of PCP, TRCP, or bromoform treated fish. However, offspring exposed to bromoform developed abnormal dorsal fins.

The data demonstrate not only the advantages of using rivulus in full life cycle exposures, but also the sensitivity of rivulus to chemically induced skeletal malformations. This latter finding is significant because to date there are no aquatic vertebrate test animals routinely used for determination of teratogenic potential of various pollutants. Because of the many attributes of rivulus including genetic uniformity of clones, it is anticipated that further development of this aquatic vertebrate assay would produce a powerful tool useful in water quality management

Conclusions

Mutagenesis Bioassay: Investigations with Rivulus marmoratus *and Other Selected Fish Species*

Objectives of this section include: 1) to characterize the genetic system of rivulus and to determine the long-term effects of known mutagens, and 2) to

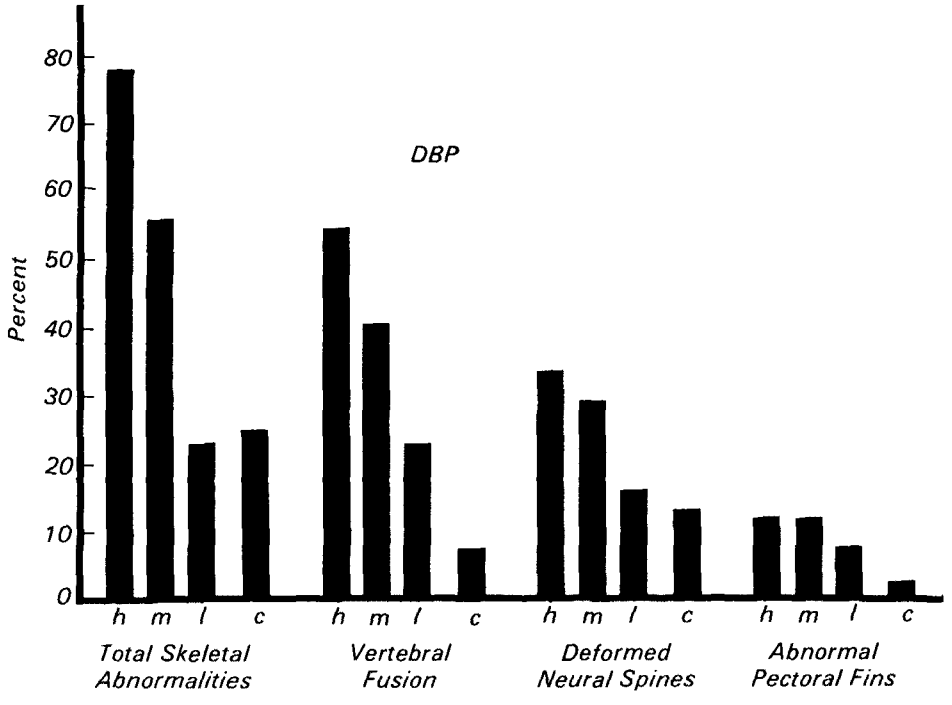

Figure 2. *Percentages of various skeletal abnormalities in offspring of DBP exposed rivulus.*

investigate possible short-term procedures for assessing mutagenic effects on the rivulus system. These objectives were subsequently amended to include other fish species when rivulus proved unsuitable for the experiments we had in mind.

The initial intent was to identify gene markers in rivulus by starch gel electrophoresis. Several fish from each clone and 15 to 30 wild-caught fish (caught from Naples, Florida) were screened for 14 enzyme systems representing an estimated 28 loci.

There were no electrophoretic differences found between clones and wild-caught fish. Thus, genetic distance between clones, occurrence of self-fertilization and segregation of alleles could not be verified. The finding of no electrophoretic difference supports the view that the Florida populations of rivulus were derived from a single small founder population.

As part of the second objective we attempted to establish a permanent line of cultured cells from rivulus to provide sufficient quantities of material to investigate the metabolism of mutagens and carcinogens and to evaluate sensitivity to induction of mutation, DNA repair and neoplastic transformation by these chemicals. The attempts at tissue culture of rivulus cells have not been successful. Alternatively, the culture of toadfish (*Opsanus tau*) cells up to fourth passage was successful.

The karyotype of rivulus was evaluated for cytogenetic analysis, specifically sister chromatid exchange (SCE) analysis, and found unsuitable. Other marine and freshwater species were surveyed and the toadfish was found most suitable, having relatively large chromosomes. Attention was therefore diverted from rivulus to the toadfish.

Culture of peripheral leukocytes, an effective method for preparing metaphase chromosomes, was successful with the toadfish. Leukocyte preparations were then used for sister chromatid exchange assays. Rates of sister chromatid exchange in mammalian cells show a dose-response relationship with concentration of mutagens and carcinogens. Techniques used to differentiate sister chromatids *in vitro*, when applied to toad fish leukocytes, were successful and an increased rate of SCE was obtained when cells were treated with the mutagen ethyl methanesulfonate. No increase in rate of SCE was found with bromoform exposure. This line of research is being pursued under another grant.

Another set of experiments involved characterization of the nature of the toadfish cytochrome P450 system. A series of comparative studies were done to determine the effects of hepatic microsomal enzyme preparations (S-9) from rats and toadfish pretreated with standard enzyme inducers (3-methylcholanthrene [MC] and Arochlor [AC]) and untreated on the metabolism of a polycyclic aromatic hydrocarbon (benzo (a) pyrene) and an aromatic amine (2-aminoanthracene) to *Salmonella* mutagens.

Neither benzo (a) pyrene (BP) nor 2-aminoanthracene (AA) were mutagenic in the absence of S-9 protein. All fish S-9 and S-9 from MC- and AC-pretreated rats resulted in little activation. The extent of activation of BP and AA under optimal conditions of S-9 type and concentrations for fish and rat were comparable. These results support the growing evidence of similarities between fish and mammal enzyme systems which metabolize pro-mutagenic xenobiotics.

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***W. P. Davis** is the EPA Project Officer (see below).*

The complete report, entitled "Usefulness of the Self-Fertilizing Cyprinodontid Fish, Rivulus marmoratus *as an Experimental Animal in Studies Involving Carcinogenesis, Teratogenesis and Mutagenesis," (Order No. PB 82-249 194; Cost: $13.50, subject to change) will be available only from:*

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