1974

Sensitivity of Vertebrate Embryos to Heavy Metals as a Criterion of Water Quality, Phase I

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Research Report No. 71

SENSITIVITY OF VERTEBRATE EMBRYOS TO HEAVY METALS AS A CRITERION OF WATER QUALITY PHASE I

by

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Project Number B-028-KY (Completion Report)
Agreement Number 14-31-0001-3890 (FY 1973)
Period of Project: July 1, 1972-June 30, 1973

University of Kentucky Water Resources Institute
Lexington, Kentucky

The results from continuing work on this project will be presented in the completion report for Project B-039-KY, scheduled for completion on June 30, 1974.

ABSTRACT

Avian, amphibian and fish embryos were given continuous treatment with inorganic mercury, methyl mercury, cadmium and lead, to determine the sensitivity of embryogenesis to metallic poisoning. All metals produced substantial degrees of lethality and/or gross anatomical anomalies at 10 ppb or less. Treatment with inorganic mercury at 10 ppb produced 100% kill of frog embryos. Chick and rainbow trout embryos suffered 10-20% lethality when exposed to 1 ppb of either inorganic or methyl mercury. Lead and cadmium at 1 ppb produced 24-32% lethality in chick embryos. No significant differences were observed in the embryopathic effects of inorganic or methyl mercury.

Concerning the toxic effects of mercury, cadmium and lead, the "embryonic stage" appears to constitute the critical "sensitive link" in the vertebrate life cycle. The reproductive potential of vertebrate populations may be severely restricted (e.g., embryonic mortality) by such pollutants at trace levels which may not prove hazardous to adult animals, and environmental standards based on tolerance levels for adults may not provide adequate protection for sensitive developmental stages.

Mercurial sensitivity of the goldfish, channel catfish and trout embryos increased in respective order, correlating with differences in egg size and hatching time of these species. A 50-fold difference in threshold levels was observed between goldfish and more sensitive trout embryos. This positive correlation suggests that fish with larger eggs and/or longer periods of embryological development are more susceptible targets of mercurial poisoning.

Results indicate that vertebrate embryos are particularly suitable bioassay and bioindicator organisms which may be used to determine protective limits for trace contaminants or to monitor the quality of water resources.

Keywords: water quality control, heavy metals, vertebrate embryos, bioindicator

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CHAPTER I

INTRODUCTION

Considering the extent to which mercury, cadmium, lead and certain other metals are being released into the environment, there is an urgent need to 1) identify the more critical or sensitive "target sites" of metallic poisoning in animals communities, 2) quantify the effects of such toxicants upon survival within animal populations, and 3) develop "sensitive" bioassay or bioindicator systems to assist in monitoring actual levels of environmental pollution and to aid in the evaluation of possible hazards of potential pollutants which may emerge as future industrial products or by-products.

It is our contention that environmental standards for metallic pollutants should be established at levels which will insure "safe limits" for the most susceptible stage(s) in the life history of organisms. With respect to vertebrate animals, our initial investigations indicate that the embryonic stage represents the critical "sensitive link" relative to the toxic effects of mercury, and certain other metallic poisons (33). It should be acknowledged that the National Technical Advisory Committee on Water Quality Criteria (1) and numerous other investigators (2-5) have underscored the importance of identifying the more susceptible stages within the life cycles of organisms in order to properly evaluate the inherent dangers of environmental pollution. Similarly, a number of sources have stressed the need for bioassay systems and bioindicators to assist in properly detecting and quantifying the lethal and/or toxic actions of environmental pollutants (1, 5-9). In particular, the Presidents Council on Environmental Quality (7)

has pointed out that there are insufficient indicators or bioassay systems with which to accurately monitor environmental changes and that the lack of such tools prevents an adequate assessment of the status of major environmental problems. The Council further indicates that the lack of an early warning system delayed the detection of the mercury pollution problem until it had reached critical proportions in certain areas.

We propose to study the sensitivity of vertebrate embryos to certain metals which are of serious consequence in environmental pollution (arsenic, cadmium, mercury, lead, zinc). In the course of this investigation, representative "species" of embryos will be exposed to various concentrations of metals, under controlled laboratory conditions, in order to 1) determine the range of concentrations at which individual metals kill or seriously impair fish, amphibian and avian embryos, 2) identify periods in embryogenesis which exhibit greatest susceptibility to metallic poisoning, and 3) construct a sensitive "bioassay system" in which vertebrate embryos may be used to assess toxic effects of actual or potential environmental pollutants.

Resulting data will be used to establish threshold values and toxicity indices to frequencies of embryonic lethality and/or teratogenicity produced by specific concentrations of test metals, and environmental standards for metallic pollutants will be critiqued with emphasis upon establishing "safe" or tolerable limits necessary to safeguard embryonic development and reproductive potential in vertebrate organisms.

CHAPTER II

RESEARCH PROCEDURES

Selection of animals. In this investigation we propose to establish a reliable indéx to toxicity thresholds for embryos of several representative Classes of vertebrates. Such comparative studies are essential in evaluating the hazards of metallic pollutants to different patterns of vertebrate reproduction and they afford the best means of identifying sensitive embryonic stages (or species) which may serve as practical bioassay or bioindicator systems.

Our choice of animals will include three species of freshwater fish--rainbow trout (Salmo gairdneri), channel catfish (Ictalurus punctatus), and the goldfish (Carassius auratus). In addition, the leopard frog (Rana pipiens) and the chick embryo (Gallus domesticus) will be used for test purposes. This selection includes species from 3 major Classes of vertebrates (Osteicthyes, Amphibia, Aves), representing aquatic, amphibian and terrestrial forms respectively. These species also represent two principal categories of embryonic development--holoblastic cleavage (Rana pipiens) in which the entire egg undergoes division to form the embryo, and meroblastic cleavage (fish, chick) in which the embryo forms from only a small portion (blastodisc) of the egg. There are substantial morphogenetic differences between these two patterns of embryonic development, and consequently they may respond differently to metallic poisoning. In addition, this selection includes forms with and without intermediate larval stages, embryonic periods of short to moderately long duration, as well as representa-

tives of poikilothermal (fish, leopard frog) and homeothermal (chicken) vertebrates. Thus, it will be possible to compare the effects of metals upon these different, representative patterns of vertebrate development.

Selection of metals for test purposes. In this study we shall use the heavy metals cadmium (Cd), lead (Pb), mercury (Hg), and zinc (Zn), and the transitional metal arsenic (As). All five metals have been identified as important water pollutants, and this selection should provide a reasonably broad spectrum of the toxic effects of such metals on vertebrate embryos. The heavy metals (Cd, Pb, Hg, Zn) will be used primarily as chlorides. Organo-mercurials (methyl mercuric chloride, dimethyl mercury) will be used in addition to mercuric chloride in order to compare relative toxicity levels. Arsenic will be used as a trioxide or as sodium arsenite.

Treatment of eggs and embryos. Metallic test solutions used in this study will be expressed as parts per million (ppm) or parts per billion (ppb), based on actual metal content. Initially, each species of embryos will be treated with high concentrations of metallic toxicants in order to exceed threshold tolerances. Once the lethal dosage is established for a specific metal, successively lower dilutions will be used until a "safe" range is determined for the most sensitive embryonic stage. Embryos will be given continuous exposure to test metals throughout development, except in specific instances where it may be desirable to work individually with a developmental stage which gives high sensitivity.

Fish and amphibian eggs, as well as larvae and fry, will be cultured in glass distilled water (conductivity of less than 1

micromho/centimeter), or in "natural" water obtained from specified collection sites in Kentucky. In the former, the concentration of salts will vary from the standard Holtfreter's solution (10) in that the proportion of sodium bicarbonate will be reduced by 50%. Eggs of aquatic species will be collected from spawning substrates (9, 11) or the adults will be artificially spawned, following guidelines described by Wharton (12) and Leitritz (13).

During incubation of fish embryos, the culture water will be regulated in a flow system, and turnover rate will be adjusted to the level which provides optimum control survival. Temperature and pH will be maintained constant, within the prefered range for each species (10, 13). Aquatic culture media will be monitored periodically for free oxygen, pH, temperature, and metal content, and eggs and larvae (or fry) will be inspected daily. The analysis of metals will be accomplished primarily by means of flameless atomic absorption spectrophotometry (14-20). Organomercurials will be analyzed by gas-chromatographic detection, generally after the methods of Westoo (21-24) and Tatton and Wagstaffe (25).

Particular attention will be given to trout embryos, especially during the "green" stage. They will be maintained in a constant temperature room at 56° F with a pH of 7.6-8.0 (13). Using continuous aeration, the oxygen level will be held near saturation for all amphibian and fish species (9, 13, 26, 27). Caution will be exercised in preventing over-crowding. Harmful exposure to artificial light will be precluded where necessary (28, 29). Otherwise, normal light-dark periodicity will be maintained (9).

Chick embryos will be maintained in a forced-draft incubator at 100° F, under conditions previously described by Birge (30, 31).

Test metals will be injected into the yolk sac, prior to incubation, in sufficient amounts to dilute the yolk to the desired levels of concentration (32). Yolk sac injections will be made with a hypodermic syringe, after cleaning the appropriate area of the shell surface with an alcohol swab. Immediately after each injection, the point of entry through the egg shell will be sealed with paraffin.

The conventional yolk sac injection procedure used in most toxicity experiments with chick embryos consists of depositing the test compound within the central area of the yolk mass (32). During our initial studies, we performed several experiments to determine the suitability of this procedure for the administration of toxic metals. Injections were made in which ^{203}Hg was deposited in the central yolk area of unincubated chicken eggs. Following 20 days of incubation, chick embryos were removed and analyzed for ^{203}Hg.. On an average, less than 2% of the mercury was detected in embryonic tissues. Photographs were then taken of similar ^{203}Hg-treated eggs with an Anger Scintillation Camera. The results showed poor distribution of the injected mercury. We then proceeded to develop a new method of administration in which a 0.1 ml aliquot of a metal-containing test solution was deposited in a needle track extending across the diameter of the egg yolk mass. This provided much greater dispersion of injected metals throughout the yolks of treated eggs, and was used in the experiments summarized below.

Control eggs will receive identical treatment as given the experimentals, except the metallic additive will be withheld.

Embryonic stages to be considered. Although embryonic development normally is a continuous process, there are distinct develop-

mental stages or periods which may respond differentially to metallic toxicants (34). The following stages and organ systems will be given particular scrutiny concerning responses to metallic poisoning:

1. Eggs, sperm and fertilization--fertilizability, sperm motility.
2. Cleavage--mitotic division of the egg to form the blastula.
3. Gastrulation--formation of the three basic germ layers.
4. Neurulation--initial development of the central nervous system.
5. Further organogenesis--formation of other basic organ systems.
   a. Sensory organs and further development of nervous system
   b. Heart and vascular system
   c. Gastro-intestinal system
   d. Urogenital system
   e. Limbs and gross body form
6. Hatching and yolk sac reabsorption.
7. Early post-hatching period.

Use of embryos for bioassay procedures. In judging the suitability of vertebrate embryos as possible bioassay systems, consideration will be given to 1) the level of sensitivity to metallic pollutants, 2) consistency and reliability of response to metal-induced toxicity, 3) degree of difficulty in interpreting test responses, and 4) ease and economy of operation.

The above procedures have been observed in collecting data under Phase I of this study, and also will be used in the continuing investigation (Phase II).

CHAPTER III

RESULTS

Frog embryos. All developmental stages of frog embryos have been treated with mercuric chloride, ranging in mercury concentrations from 10 parts per million (ppm) to 0.1 part per billion (ppb). A concentration of 0.1 ppm kills 100% of treated embryos through cleavage, blastula, gastrula and neurula stages. At 10 ppb there is 100% kill for cleavage and blastula stages, with approximately a 15% death rate for gastrula stages, as compared to controls. At 1 ppb initial studies still indicate a possible 9 to 10% kill of gastrula and neurula stages. These data are summarized in Table 1, where survival is based on observations of 100 embryos for each experimental and control sample.

Adult frogs and tadpoles (larvae) were treated with concentrations of mercuric ion varying from 50 to 0.5 ppm (Table 2). Treatment with 5 ppm mercury killed 100% of the frog larvae on the first day of exposure, while adult populations were unaffected for 10 days. The highest concentration of mercury at which larvae survived for 10 days was 0.5 ppm. Larvae treated with 2.5 ppm survived for 2 days, but developed severe eye abnormalities, resulting in apparent blindness.

While 100% of the treated larvae survived 1 ppm mercury for 4 days, a concentration of 0.01 ppm killed 100% of early frog embryos during the same period of exposure (Table 1, 10 ppb). These data show that larvae are up to 10 times more sensitive to direct exposure to mercury than are adult frogs, and that frog embryos are at least 100 and 1000 times more sensitive to mercury than are larvae and adults, respectively.

Chick embryos. To date, chick embryos have been treated with cadmium, methyl mercury, inorganic mercury, and lead. The test metals were administered by a new injection procedure in which the metallic additives were deposited in a "needle track" extending through the diameter of the egg yolk. Survival curves are presented in Figures 1-4.

Cadmium killed approximately 90, 60, 42 and 32% of chick embryos treated with yolk concentrations of 1.0, 0.1, 0.01, and 0.001 ppm (Figure 1). As seen in Figure 2 methyl mercury also is highly toxic to chick embryos, killing 75, 48, 23, and 20% of embryos treated at concentrations of 1.0, 0.1, 0.01 and 0.001 ppm, respectively. Treatment with inorganic mercury produced quite similar results (Figure 3). Though developmental anomalies were observed among cadmium and mercury-treated survivors, their relative frequencies as yet have not been tabulated.

We previously reported (33) that treatment with lead killed 75, 31, 8 and 6% of chick embryos at concentrations of 10, 1.0, 0.1, and 0.01 ppm, respectively. Also, serious anomalies were found among survivors at a frequency of 4% at 0.01 ppm, 8% at 1.0 ppm, and 55% at 10 ppm. Thus, at 0.01 ppm (10 ppb) approximately 10% of treated embryos either died or developed serious anomalies. The more common anomalies included absent eyes, hydrocephaly, and other deficiencies in the brain and spinal cord. These data are summarized in Figure 4, taken from Research Report No. 61 (33). The treatments with lead have been repeated, using the new "needle track" yolk sac injection procedure described above (Research Procedures). The new method of administration gave substantially more lethal values of 49, 38, 28 and 24% at concentrations of 1.0, 0.1, 0.01 and 0.001 ppm. Similarly, the new values given above for cadmium and

mercury, determined with the new injection procedure, indicate significantly higher percentages of induced lethality than previously reported (33).

Fish embryos. Eggs and embryos of the goldfish (Carassius auratus) were treated with methyl and inorganic mercury at concentrations of 100 to 0.001 ppm. Percent survival, expressed as survival of experimentals/survival of controls, was determined for each test concentration by observations on a sample population of 250 eggs, following continuous treatment from fertilization to hatching (3 days). The two forms of mercury produced near identical effects upon goldfish embryos, killing essentially 100% of all test populations treated at concentrations of 100 to 0.5 ppm (Table 3; Figures 5, 6). At concentrations of 0.1, 0.05, and 0.01 ppm, methyl mercury treatment resulted in 47, 94, and 97% survival rates, respectively. By comparison, survival values for populations treated with inorganic mercury at these same concentrations were 47, 92 and 97%.

Embryos of the channel catfish (Ictalurus punctatus) were treated with cadmium and mercury, using test populations of 250 eggs for each metal concentration studied. In all cases, treatment was continuous over the entire span of development (8 days). Exposure to cadmium resulted in survival rates of 1, 41, 80 and 100% at concentrations of 1, 0.1, 0.01, and 0.001 ppm, respectively. Treatment of catfish eggs with methyl mercury gave survival rates of 0, 17, 24, 83 and 96% for concentrations of 1, 0.1, 0.05, 0.01, and 0.001 ppm (Table 3; Figure 5). Using inorganic mercury at these same concentrations, survival rates of 0, 30, 34, 83 and 97% were observed (Table 3; Figure 6). Cadmium and mercury appear to be near equally toxic to catfish embryos.

Trout eggs also have been treated with methyl mercury, inorganic mercury and cadmium. As in all other cases, concentrations were based upon content of metallic ions, and embryos were treated continuously from fertilization to hatching (24 days). Sample size again was set at 250 trout eggs for each experimental and control population studied, and percent survival of each experimental population was determined as the number of surviving experimentals/ number of survivors in the corresponding control population.

Cadmium produced complete lethality when trout embryos were treated with concentrations of 100 to 1.0 ppm. At 0.1, 0.05 and 0.01 ppm survival frequencies of 59, 91 and 98% were obtained for cadmium-treated trout embryos.

Inorganic and methyl mercury proved significantly more toxic to trout embryos than cadmium. Mercury treatment produced "complete" lethality at all concentrations tested down to 0.05 ppm, and only 33% of the trout embryos survived treatment at 0.01 ppm (Table 3; Figures 5, 6). Furthermore, exposure at concentrations below 0.01 ppm indicated extreme sensitivity of trout embryos to mercury. Treatment with methyl mercury at 0.005, 0.002 and 0.001 ppm gave survival rates of 57, 76 and 90%, respectively, and 5% lethality still was observed at 0.0007 ppm (0.7 ppb). Near identical results were obtained with inorganic mercury (Table 3).

A significant correlation was found between exposure time and percent survival for mercury-treated trout embryos, as shown in Tables 4 and 5. Concentrations of mercury at 0.5 ppm or more generally produced a 100% kill within one day or less, but at concentrations ranging from 0.1-0.001 ppm, the frequency of lethality

generally increased with duration of exposure. However, a higher rate of lethality always was observed during the first three days, indicating that early developmental stages are particularly sensitive to mercurial poisoning. It also should be noted that surviving alevins (fry), which hatched on day 24, suffered little or no lethality during 3 days of postembryonic exposure (Tables 4, 5). In the goldfish, where hatching required 3 days, a generally similar response was noted (Tables 4, 5).

Attention should be directed to the high incidence of gross developmental anomalies found among mercury-treated trout alevins. When trout embryos were treated with mercury at 0.025 ppm, 35-43% of the surviving alevins were anomalous. At 0.002 ppm, nearly 10% of the surviving alevins were teratogenic, having severely defective vertebral columns or exhibiting partial twinning (Plate 1). Thus, mercury produced lethality or severe anomalies in approximately 35 and 53% of trout embryos when treatment was maintained at 0.002 ppm (2 ppb) and 0.005 ppm (5 ppb), respectively (Table 3).

It should be noted that the studies reported above deal with the effects of individual metals upon trout development. In all of these toxicity experiments the trout embryos have been maintained under standardized conditions, in a uniform, defined culture medium, and where the only detectable variable has been the metallic additive.

TABLE 1

PERCENT SURVIVAL OF AMPHIBIAN EMBRYOS (RANA PIPIENS)

FOLLOWING FOUR DAYS OF CONTINUOUS EXPOSURE TO

INORGANIC MERCURY[1]

Stage at Initiation of Treatment	Concentration of Mercury						
	10 ppm	1 ppm	0.1 ppm	10 ppb	1 ppb	0.1 ppb	Control
Cleavage	0	0	0	0	93	94	95
Blastula	0	0	0	0	82	78	84
Gastrula	0	0	0	80	85	95	95
Neurula	0	0	0	93	88	96	100
Tail Bud	0	0	20	80	93	95	95

[1] Each observation based on a sample size of 100 embryos.

TABLE 2

COMPARISON OF PERCENT SURVIVAL OF LARVAE AND ADULT FROGS TREATED WITH MERCURY[1]

Length of Exposure (Days)	Survival (%)													
	Mercury Concentration (ppm)													
	50		25		10		7.5		5.0		2.5		1.0		0.5	
	L	A	L	A	L	A	L	A	L	A	L	A	L	A	L	A
1	0	0	0	0	0	70	0	80	0	100	100	100	100	100	100	100
2						60		80		100	100	100	100	100	100	100
3						30		80		100	0	100	100	100	100	100
4						30		80		100	0	100	100	100	100	100
5						20		80		100	0	100	50	100	100	100
6						20		60		100	0	100	50	100	100	100
7						10		60		100	0	100	50	100	100	100
8						0		60		100	0	100	50	100	100	100
9						0		60		100	0	100	50	100	100	100
10						0		60		100	0	100	50	100	100	100

[1] Each observation based on a sample size of 100 animals.

TABLE 3

COMPARISON OF PERCENT SURVIVAL OF TROUT, CATFISH AND GOLDFISH EMBRYOS TREATED CONTINUOUSLY THROUGHOUT DEVELOPMENT WITH MERCURY AT CONCENTRATIONS OF 0.001 TO 10.0 PPM [1,2]

Concentration (ppm)	Percent Survival					
	Trout [3,4]		Catfish		Goldfish	
	CH_3Hg^+	Hg^{++}	CH_3Hg^+	Hg^{++}	CH_3Hg^+	Hg^{++}
0.001	90(3)	90(2)	96	97	100	98
0.002	76(9)	72(8)	-	-	-	-
0.005	57(10)	59(12)	93	89	99	99
0.007	46(17)	45(19)	-	-	-	-
0.010	33(20)	33(22)	83	83	97	97
0.025	28(35)	24(43)	-	-	-	-
0.050	0	0	24	34	94	92
0.075	-	-	-	-	65	65
0.100	0	0	17	30	47	47
0.250	-	-	-	-	24	22
0.500	0	0	0	0	2	4
1.000	0	0	0	0	0	0
5.000	0	0	0	0	0	0
10.000	0	0	0	0	0	0

[1] Percent survival was determined for each test point as the frequency of hatching for 250 treated eggs/frequency of hatching for controls. Anomalous animals which successfully completed the hatching process were counted as survivors. Animals which died subsequent to hatching were not counted as embryonic mortalities.

[2] Treatment was initiated immediately following fertilization and maintained continuously for 3 days post-hatching. Duration of embryonic exposure corresponded to the hatching time for each species, which was 3, 8 and 24 days for the goldfish, catfish and trout, respectively.

[3] Percentages of surviving trout alevins (fry) possessing gross anomalies are given parenthetically. Levels of spontaneous anomalies within control populations ranged from 0-1%.

[4] The differential response of the three species of fish illustrates the advantage of comparative studies in which different animals are treated under identical laboratory conditions.

TABLE 4

COMPARISON OF PERCENT SURVIVAL OF GOLDFISH AND TROUT EMBRYOS WITH DURATION OF EXPOSURE DURING DEVELOPMENT TO ORGANIC MERCURY

CH_3Hg^+ in ppm	Exposure Time in Developmental Days[1]											
	Goldfish			Trout								
	1	2	3	3	6	9	12	15	18	21	24	27
0.001	100	100	100	95	92	91	91	90	90	90	90	90
0.005	100	100	99	91	82	74	69	63	60	58	57	57
0.010	100	100	97	75	66	54	48	42	39	34	33	33
0.050	100	97	94	59	43	12	6	0	0	0	0	0
0.100	70	61	47	13	0	0	0	0	0	0	0	0
0.500	40	6	2	0	0	0	0	0	0	0	0	0
1.000	28	3	0	0	0	0	0	0	0	0	0	0

[1] Hatching occurred on day 3 in the goldfish and on day 24 in the trout. Data given on day 27 for the trout represent percent survival at 3 days posthatching.

TABLE 5

COMPARISON OF PERCENT SURVIVAL OF GOLDFISH AND TROUT EMBRYOS WITH DURATION OF EXPOSURE DURING DEVELOPMENT TO INORGANIC MERCURY

Hg^{++} in ppm	Exposure Time in Developmental Days[1]											
	Goldfish			Trout								
	1	2	3	3	6	9	12	15	18	21	24	27
0.001	100	100	100	98	96	93	91	90	90	89	89	89
0.005	100	100	99	83	75	68	63	62	61	60	59	59
0.010	100	99	97	72	61	53	45	39	36	34	34	33
0.050	100	94	92	69	19	6	0	0	0	0	0	0
0.100	89	50	47	4	0	0	0	0	0	0	0	0
0.500	56	21	4	0	0	0	0	0	0	0	0	0
1.000	27	3	0	0	0	0	0	0	0	0	0	0

[1] Hatching occurred on day 3 in the goldfish and on day 24 in the trout. Data given on day 27 for the trout represent percent survival at 3 days posthatching.

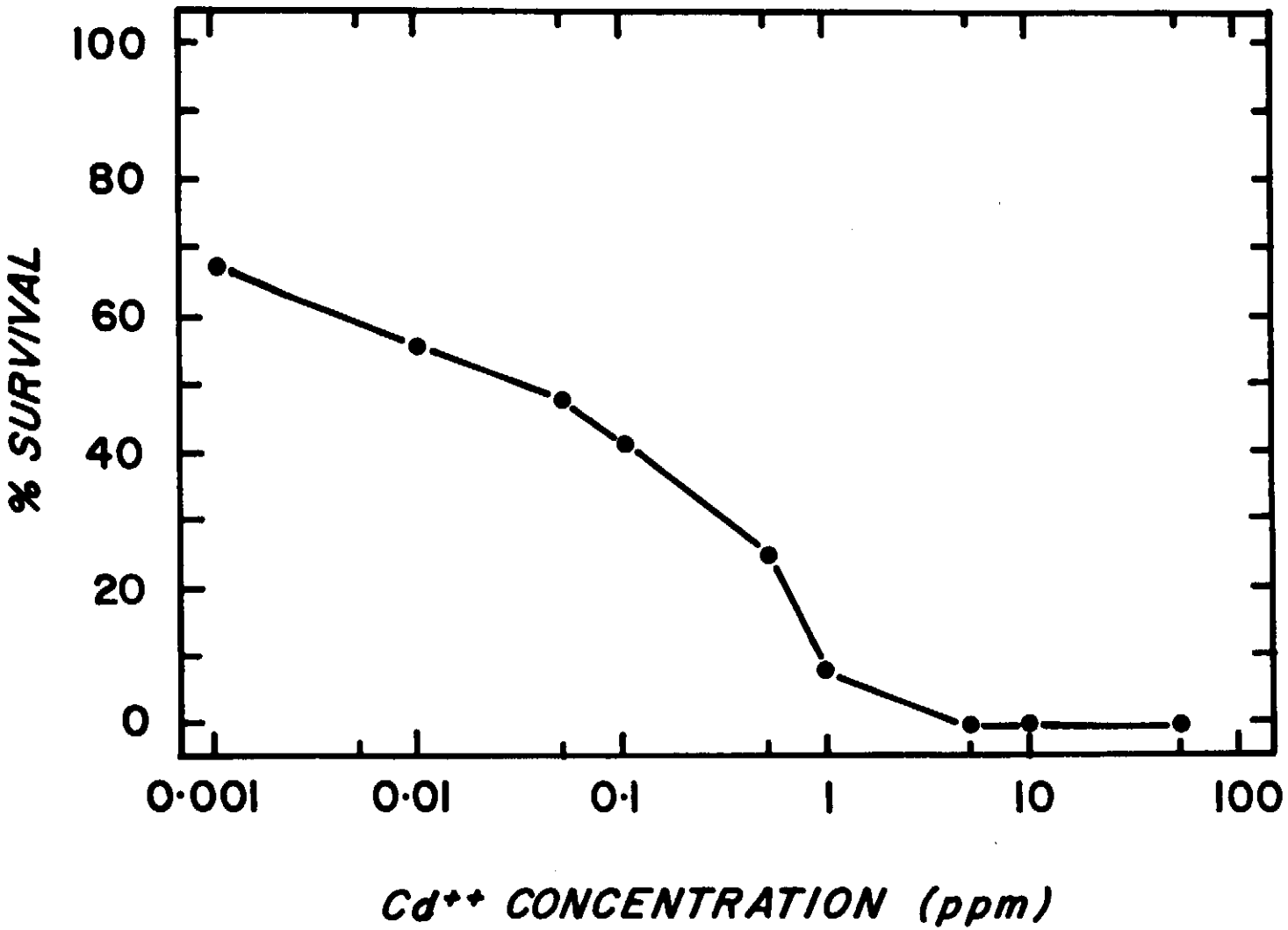

FIGURE 1. Sensitivity of chick embryos to cadmium administered by yolk sac injection at start of incubation. Experiment terminated one day prior to hatching (20 days). Each point represents frequency of survival for 100 experimental embryos/ 100 controls; plotted on a semi-logarithmic scale.

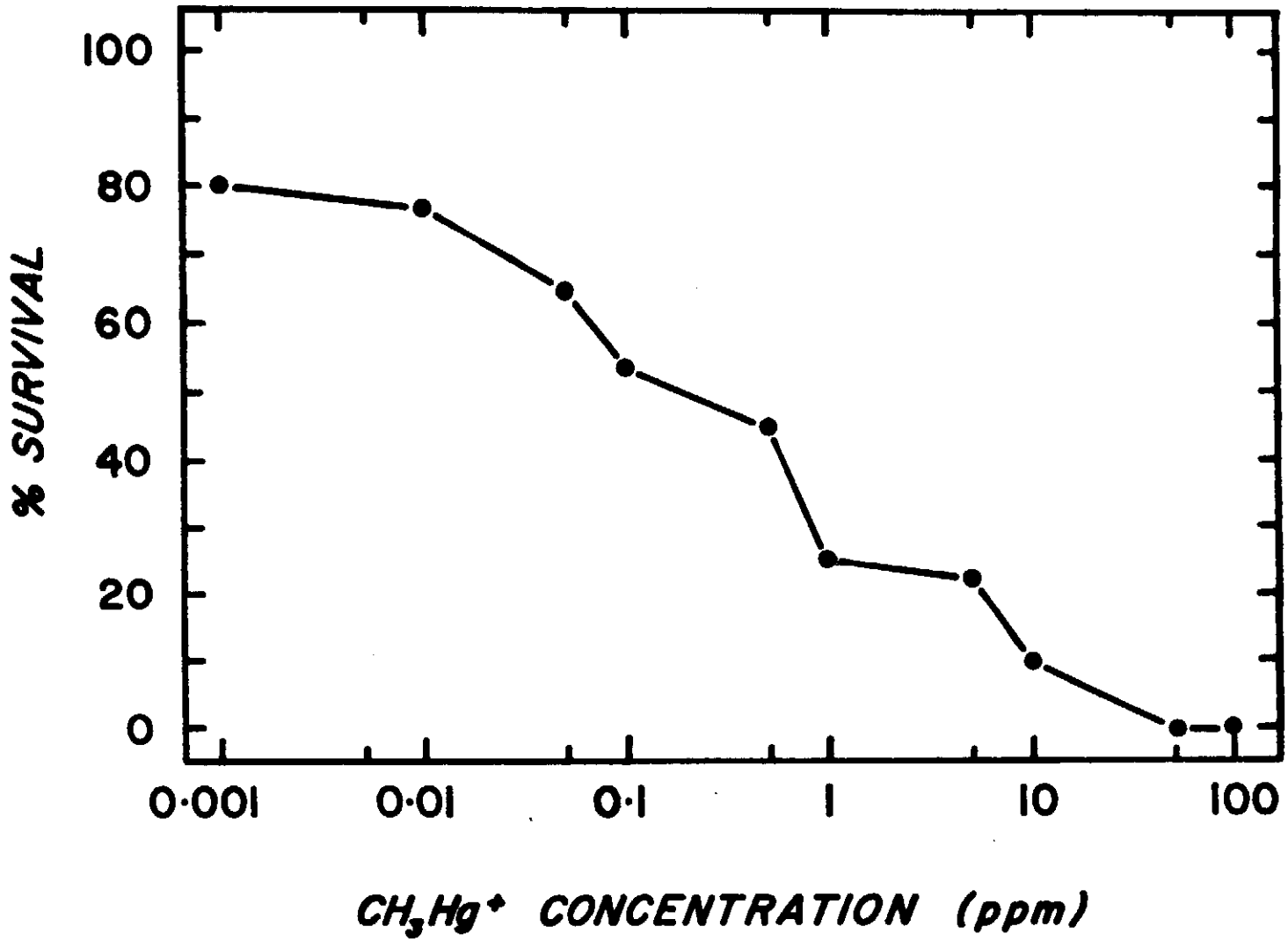

FIGURE 2. Sensitivity of chick embryos to methyl mercury administered by yolk sac injection at start of incubation. Experiment terminated one day prior to hatching (20 days). Each point represents frequency of survival for 100 experimental embryos/100 controls; plotted on semi-logarithmic scale.

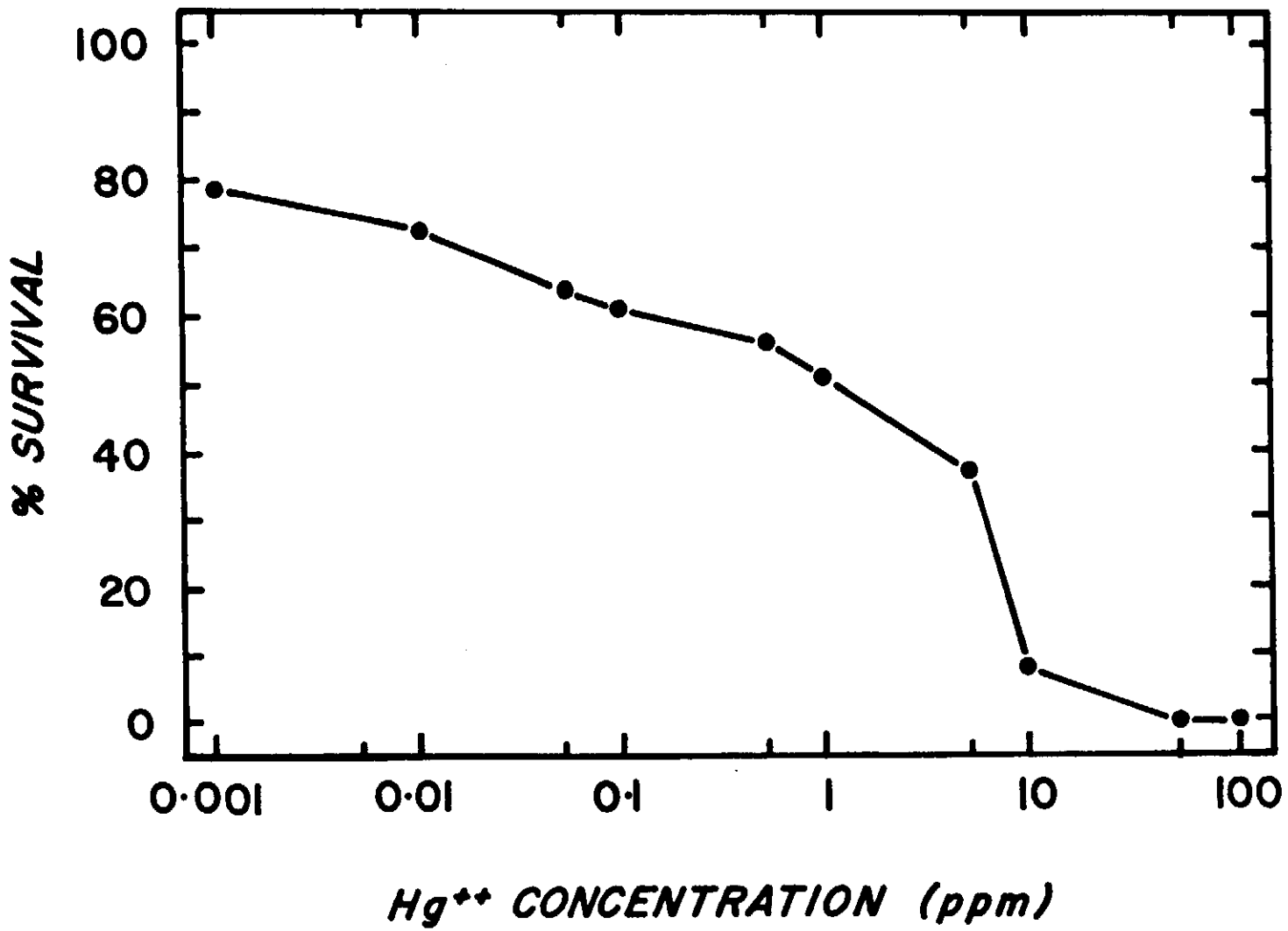

FIGURE 3. Sensitivity of chick embryos to inorganic mercury administered by yolk sac injection at start of incubation. Experiment terminated one day prior to hatching (20 days). Each point represents frequency of survival for 100 experimental embryos/ 100 controls; plotted on semi-logarithmic scale.

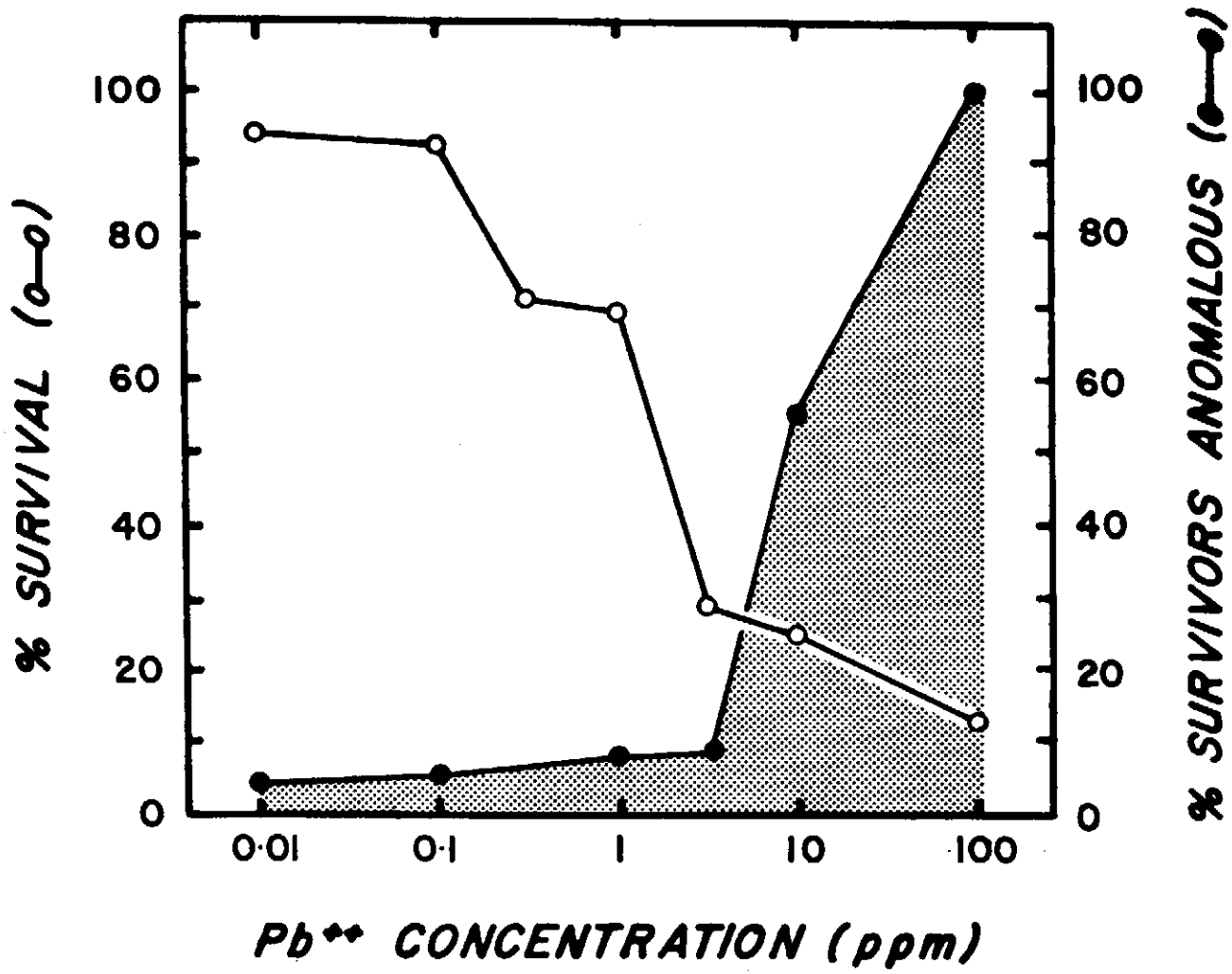

FIGURE 4. Sensitivity of chick embryos to lead administered by yolk sac injection at start of incubation. Experiment terminated one day prior to hatching. Each point represents survival of 100 experimental embryos/100 controls.

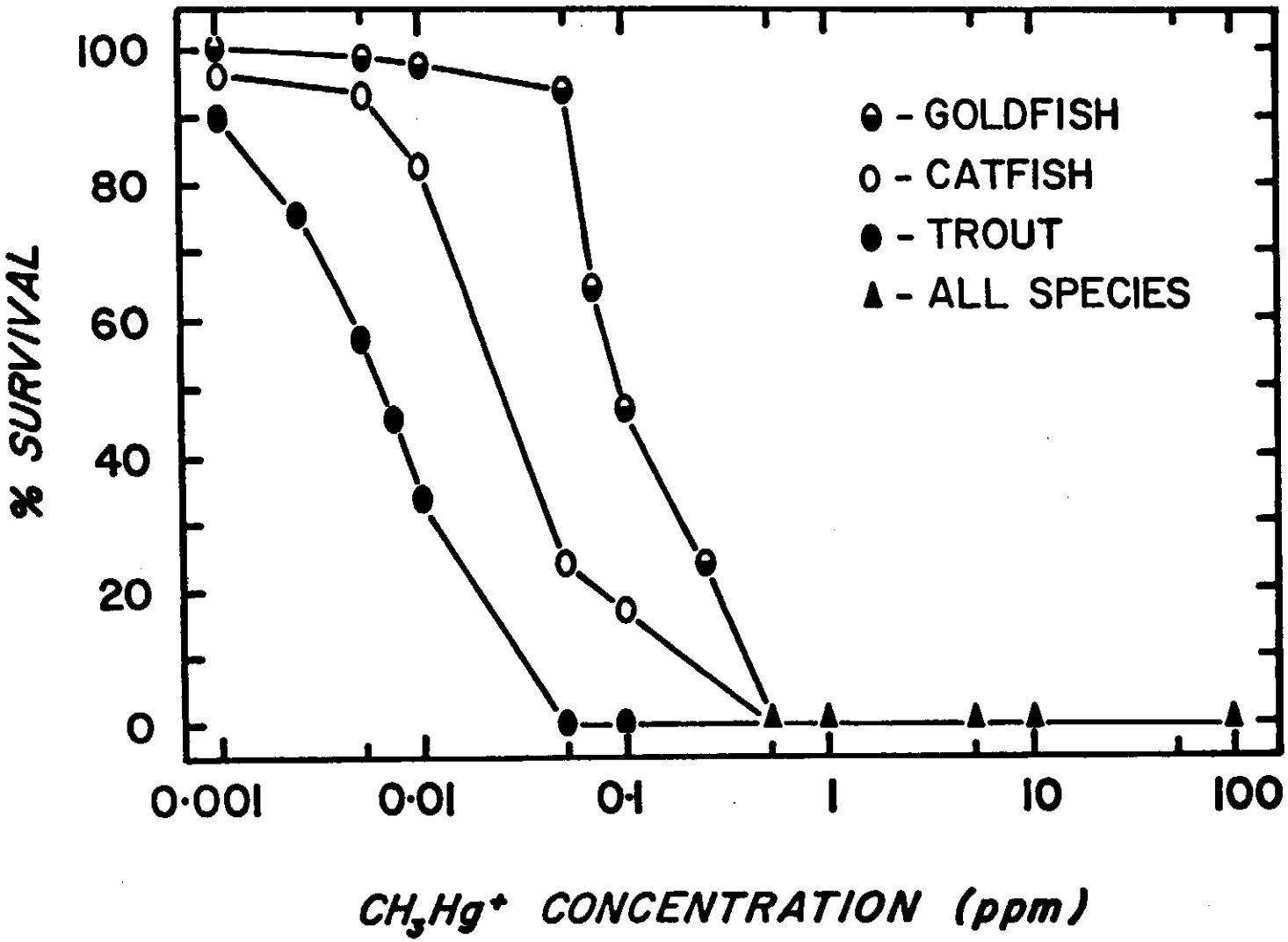

FIGURE 5. Comparison of toxic effects of methyl mercury on embryos of the goldfish, catfish and trout. Each point represents survival of 250 treated embryos/survival of 250 controls, plotted on a semi-logarithmic scale.

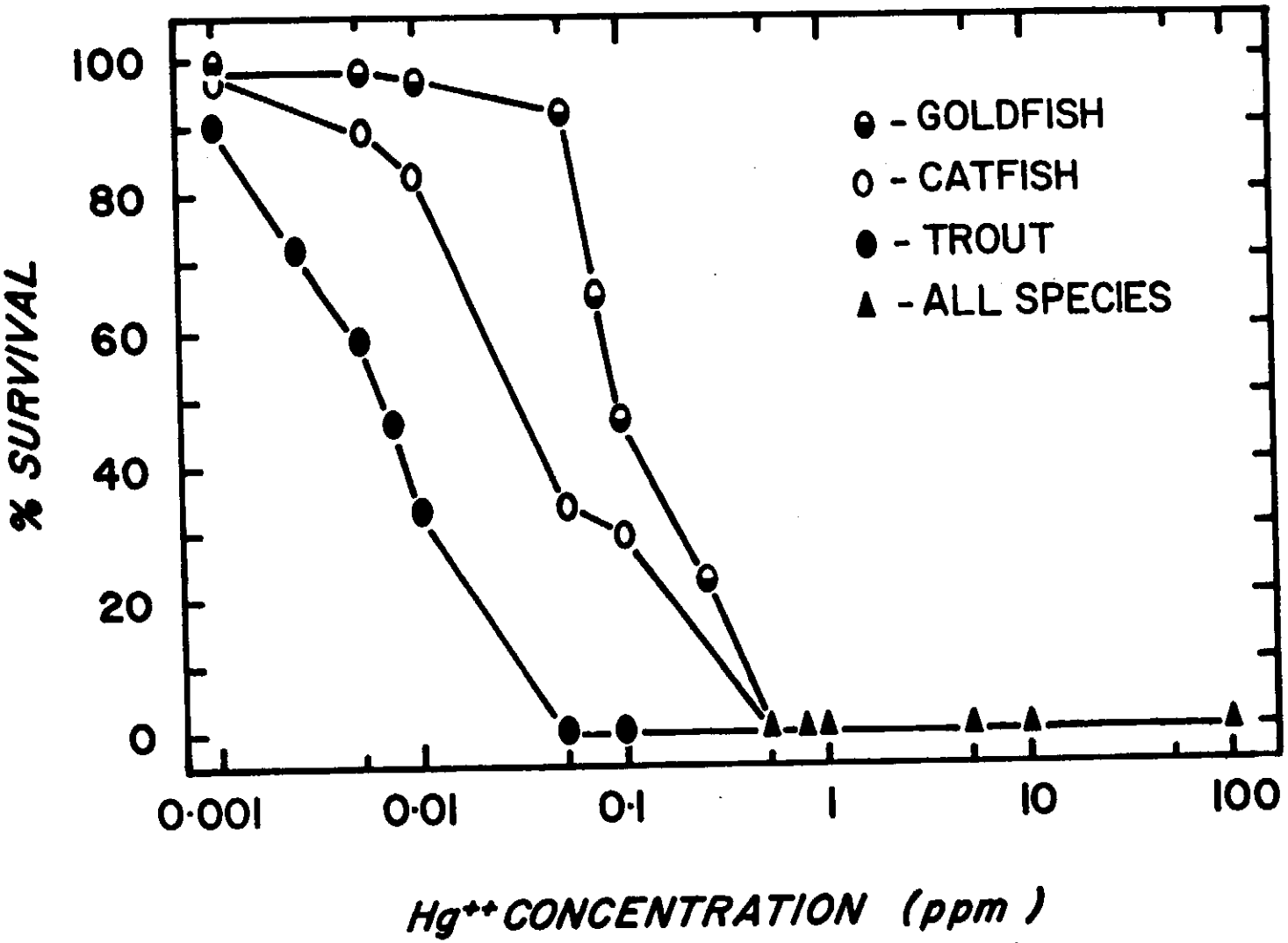

FIGURE 6. Comparison of toxic effects of inorganic mercury on embryos of the goldfish, catfish and trout. Each point represents frequency of survival for 250 treated embryos/ 250 controls; plotted on a semi-logarithmic scale.

PLATE I. Mercury-induced congenital deformities frequently observed in trout alevins. Photographs were taken two weeks subsequent to hatching at a magnification of 10X. The anomalies most commonly encountered included a) defects of vertebral column, b) absent or irregular fins, c) hydrocephalous brain development, and d) retarded yolk sac reabsorption.

1. Twinning of head and anterior trunk, ventral view.; Single yolk sac (arrow) possesses paired yolk stalks, one serving each partial twin.

2. Dorsal view of dicephalous alevin. Twinning extends to anterior border of dorsal fin (arrow).

3. Inflexible C-shaped curvature of tail, accompanied by hydrocephaly (arrow).

4. Mercury-induced skeletal anomalies including an acute lordotic spine and a defective, immovable lower jaw (arrow). Yolk sac reabsorption also is retarded (YS).

5. Acute flexion of spinal column, resulting from defective vertebral development. Retarded yolk sac reabsorption also is evident (YS).

6. Anomalous, immobile curvature of spinal column (arrows).

7. Defective dorsal fin and sharp angular flexion of spinal column.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

Judging from our results with mercury, cadmium and lead, it is clearly evident that vertebrate embryos are extremely sensitive to metallic pollutants. The frog embryo appears to be at least 1,000 times more sensitive to mercury than are adult frogs, and embryos of the chicken, goldfish, catfish and trout suffer high rates of mortality and/or congenital deformities when treated with mercury, cadmium and lead at concentrations which approach or fall within presently accepted "safe limits" for water resources (35, 36).

These data take on added significance in view of the USPHS standard of 5 ppb for mercury in drinking water set in 1970 (35, 37), the limiting human average daily intake (ADI) of 20-50 ppb set in 1966 by WHO (38), the interim guideline of 500 ppb set in 1970 by FDA (35), or the limit of 100 ppb for mercury in whole blood recommended in 1968 by the International Committee on Maximum Allowable Concentrations of Mercury Compounds (39). It becomes obvious that environmental standards for mercury have been based largely on human health requirements, and that most existing standards do not provide for the extreme sensitivity of vertebrate embryos. It seems apparent that many natural animal populations could be extinguished or reduced through a loss of reproductive potential (e.g., embryonic mortality, teratogenicity) produced by concentrations of mercury which may not be injurious to the health of adult organisms.

The commonly accepted public health standard of 5 ppb for mercury in water is essentially an LD_{50} value for trout embryos, as better than 50% either die prior to hatching or exhibit gross anatomical

defects when treated at this concentration. By comparison, lethal values for various mercurial compounds have been set as high as 580-1300 ppb and 2000-9200 ppb for adult channel catfish and rainbow trout, respectively (35, p. 60). Surveys indicate that certain metallic pollutants presently exist in various water resources in concentrations which we find lethal or damaging to vertebrate embryos (35, 36, 40).

It is becoming increasingly apparent that within the life history of vertebrates, the embryonic form is the critical "sensitive link" relative to the effects of such metallic pollutants as mercury and cadmium, and it seems obvious that the reproductive potential of natural vertebrate populations may be severely restricted by levels of contamination which may not seriously harm adult organisms. Therefore, it follows that tolerance levels for sensitive embryonic stages should be considered in establishing or revising protective environmental standards for such metals.

Attention should be drawn to the fact that embryos of different vertebrate species are differentially susceptible to the toxic effects of certain metals. As noted previously, cadmium is more toxic than mercury to domestic fowl embryos, while the reverse relationship is true for trout embryos. In addition, it is apparent that embryos of the trout and catfish, in respective order, are approximately 50 and 10 times more sensitive to methyl mercury than goldfish embryos. This estimate is based upon a comparison of the highest concentration at which there is 90% or better survival for these three species. These values are 0.05, 0.005 and 0.001 ppm for the goldfish, catfish and trout, respectively (Table 3). Egg size and hatching time for these species increase in the same

order as mercurial toxicity, and this correlation indicates that fish with larger eggs and/or longer periods of embryological development are more susceptible to mercurial poisoning. In the face of increasing levels of pollution, such data raise concern regarding 1) proper ecological balance in vertebrate communities, and 2) fate of commercially important vertebrate species which are subject to both economic and environmental stress.

A number of other investigations indicate that fish eggs and fry are highly sensitive to metallic poisoning. Most current studies in this area concern the toxic effects of zinc. Affléck (41) has reported high sensitivity of eggs and alevins (fry) of brown and rainbow trout to zinc poisoning, where concentrations as low as 0.01 ppm were found to be lethal or toxic. Similarly, Leitritz (13) has reported that zinc in a concentration of 0.04 ppm is lethal for young rainbow trout of two to four weeks of age. Pickering and Vigor (11) have shown an acute toxicity of zinc to eggs and fry of the fathead minnow. They found median tolerance limits (TL_m) of 3.92-3.98 milligrams/liter for 1-day old eggs. The TL_m values dropped to 2.47-2.63 after two days of development, and were 1.57-1.69 by completion of the 12th day. The newly hatched fry were still more sensitive to zinc sulfate (TL_m of 0.95 mg/L for 2 days exposure). Corresponding TL_m values for adults, under the conditions imposed in the study, were not given. However, other reports indicate lethal concentrations of zinc sulfate for adult minnows ranging from 10 mg/L for 48 hours in freshwater (42), to values as high as 400 mg/L for 200 minutes in distilled water (43). Skidmore (3, 44) has shown that zebrafish eggs also increase in sensitivity to zinc sulfate during the course of embryonic development. The later

embryonic stages (2-4 days) and newly hatched fry (4-13 days old) were highly susceptible to the toxic effects of zinc. Young fish up to 40-days of age were still found to be more sensitive to zinc than adults, as illustrated by their threshold concentration of 1.3 ppm (zinc sulfate) compared to a value of 10 ppm for adults.

Certain investigations with the fathead minnow indicate that fecundity (45) and early fry (11) are more sensitive to zinc than are eggs. Similarly, eggs of the king salmon (46) and brook trout (47) have been reported to be somewhat less sensitive to copper than newly hatched alevins. Though toxic at higher concentrations, zinc and copper are essential trace metals which are required for the normal development of vertebrate embryos (48-51). Accordingly, at trace levels, they appear to be less embryopathic to early developmental stages than certain non-essential heavy metals, such as mercury.

Initial investigations also indicate that vertebrate embryos are particularly suitable "test organisms" for use in bioassay or bioindicator systems. Embryos as indicators or bioassay organisms may be used experimentally by Federal or State agencies (or other investigative personnel) to determine protective limits for environmental contaminants and to monitor environmental quality of natural resources. In compliance with present or future Federal and State regulations, private industry may utilize "embryo indicators" as screening or pre-screening systems to evaluate toxicity of a wide range of commercial products and waste materials.

The majority of proposed monitoring systems are based on the use of adult "test" animals in 1) acute or chronic toxicity bioassay, generally expressed in TL_m values (9, 52), or 2) the assessment of

avoidance or other behavioral responses which are measurable in sublethal concentrations of toxicants (53). The sensitivity of such bioassay systems to metallic pollutants generally does not approach that which we find in vertebrate embryos. As a comparison, 1-2 ppb mercury produces lethality or gross congenital anomalies in appreciable percentages of treated trout embryos, whereas 3 ppb is the lowest concentration which induces measurable behavioral or learning responses in the goldfish (53).

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