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

Wesley J. Birge
University of Kentucky

John J. Just
University of Kentucky

RESEARCH REPORT NO. 61

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By

WESLEY J. BIRGE
Principal Investigator

JOHN J. JUST
Co-Investigator

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UNIVERSITY OF KENTUCKY
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Dr. Wesley J. Birge
Principal Investigator

Dr. John J. Just
Co-Investigator

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The results from continuing work on this project will be presented in the completion reports for Project B-028-KY, scheduled for completion on June 30, 1973; and B-039-KY, which will be completed on June 30, 1974.

ABSTRACT

Embryonic and/or larval stages of the leopard frog (Rana pipiens), domestic fowl (Gallus domesticus) and the goldfish (Carassius auratus) were treated with cadmium chloride, mercuric chloride, lead chloride, zinc chloride and sodium arsenite. The principal objectives were (1) to determine the sensitivity of vertebrate embryos to certain metals which are of consequence in water pollution, and (2) to ascertain the suitablility of vertebrate embryos as bioassay organisms for monitoring metallic pollutants within water resources. Vertebrate embryos were found to be highly sensitive to the toxic effects of all the metals studied. Concentrations of mercury as low as 10 ppb, with continuous treatment, produced a 100% kill of frog embryos and a significant degree of lethality in chick embryos. Cadmium and lead also produced detectable levels of lethality and/or anomalous development when administered to chick embryos at concentrations of 10 ppb. At 0.5 ppm mercury and cadmium produced 100% lethality in populations of goldfish embryos treated for four days. Lead and zinc were less toxic to the latter, producing approximately 80% lethality under similar conditons.

These results indicate that vertebrate embryos are substantially more sensitive to metallic pollutants than are adult forms, and that they may constitute a valuable tool for monitoring the quality of water resources.

Keywords: water quality control*, heavy metals*, vertebrate embryos* and water pollution

CHAPTER I

INTRODUCTION

The principal objectives of this study were twofold. Initially, we attempted to (1) determine the sensitivity of vertebrate embryos to certain metals which are of consequence in water pollution (Arsenic, Cadmium, Mercury, Lead, Zinc); (2) to ascertain the lowest concentrations at which these metals either kill or seriously impair fish, amphibian and avian embryos, and (3) to identify the periods in embryogenesis which exhibit greatest susceptibility to these toxic substances.

In the second phase of this study, we have (1) examined the feasibility of using vertebrate embryos as "sensitive indicators" of water quality, and (2) tested the proposition that vertebrate embryos are significantly more susceptible to certain pollutants, particularly heavy metals, than are their adult counterparts.

CHAPTER II

RESEARCH PROCEDURES

Selection of animals. Our choice of animals included a species of freshwater fish, the goldfish (Carassius auratus), the leopard frog (Rana pipiens), and the chick embryo (Gallus domesticus, White Leghorn strain). This selection includes species from 3 major Classes of vertebrates (Osteicthyes, Amphibia, Aves), representing aquatic, amphibious and terrestrial forms respectively. These 3 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. In addition, this selection includes forms with and without intermediate larval stages, as well as embryonic periods of short to moderately long duration. This group of test animals also includes a representative of poikilothermal (fish, leopard frog) and homeothermal (chicken) vertebrates.

Adult fish in spawning condition were obtained from hatcheries in the Kentucky-Virginia area. Leopard frogs were purchased from Dr. George Nace of the U.S. Amphibian Facilities, University of Michigen. Fertile chicken eggs were obtained from the Poultry Department, University of Kentucky.

Selection of metals for test purposes. In this study, we have used 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) were used primarily as chlorides, and arsenic was used as sodium arsenite.

Treatment of animals. In the treatment of embryos, the concentrations of the aforementioned compounds were calculated to give parts per million (ppm) of the specific metals to be tested. Initially, individual metals were administered in high dosages, in an attempt to exceed threshold tolerances. Once a lethal dosage was established for a species, successively lower dilutions were tried until a "safe" range was determined for the most sensitive embryonic stage. Special attention was given to any significant reduction (drift) in the concentration level of the metallic additive during exposure.

Chick embryos were maintained in a forced-draft incubator at 100° F. Two alternative methods were used in administering the metals to the chick embryos: (1) in order to accomplish continuous exposure over the full range of development, the test metals were injected into the yolk sac (prior to incubation) in sufficient amounts to dilute the yolk to the desired levels of concentration; (2) in addition, metals were injected directly into the air space of the egg in amounts calculated to dilute egg volume to a specified concentration. This provided a second means of administering full-term exposures. Furthermore, it enabled us to compare the results of our studies with other published works, and to test the sensitivity and reliability of these two methods.

Air cell and yolk sac injections were made directly 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 was sealed with paraffin.

Amphibian eggs and larvae were reared in aquatic culture dishes (pyrex). Fish eggs also were maintained in aquatic culture under controlled conditions, using pyrex chambers. Amphibian and fish eggs were either collected from spawning substrates or the adults were artificially spawned, and the eggs were maintained in an excess volume of modified Holtfreter's solution, prepared in glass distilled water (conductivity of less than 1 micromho/centimeter). During incubation, the culture medium was changed at 12 hour intervals. Temperature and pH were maintained constant, within the optimum range for each species tested. Using continuous aeration, the oxygen level was held near saturation for all aquatic eggs tested, and caution was exercised in preventing overcrowding.

Culture media was monitored daily for pH and temperature, and at periodic intervals for metal content (atomic absorption spectrophotometry). Due to the tendency of mercury and other metals to adsorb on glass surfaces, special care was taken to use "chemically clean" Pyrex glassware. Developing eggs were inspected daily, and the embryos and larvae of each species considered were given continuous exposure over their entire span of development.

CHAPTER III

DATA AND RESULTS

During the course of this study, facilities have been established for handling, treating and maintaining the embryonic forms of the three species of vertebrates under consideration. Numerous pilot studies have been performed in order to develop technical facility. With this initial background, we have proceeded to treat a substantial number of fish, amphibian and avian embryos, as well as amphibian adults and tadpoles (larvae), with a number of heavy metals. The results of these experiments are summarized below.

All developmental stages of frog embryos have been treated with mercuric chloride, ranging in concentration 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 a 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 5 to 10% kill of gastrula stages. These data are summarized in Table I, where survival is based on observations of 100 embryos for each experimental and control sample. The sensitivity of frog embryos to mercuric ions is most interesting in view of the commonly accepted fresh water standard which allows 5 ppb of mercury.

Adult frogs and tadpoles (larvae) were treated with concentrations of mercuric ion varying from 100 ppm to 0.1 ppm. The data presented in Table II show that the adults can tolerate concentrations of 7.5 ppm

or less for at least 20 days, while the tadpoles are able to tolerate only 1 ppm or less during a ten day period. Treatment with 5 ppm, a concentration in which 80% of the adults survived 20 days, proved lethal to larvae in only one day. Tadpoles treated with 2.5 ppm could survive 3 days but developed severe eye abnormalities, resulting in apparent blindness. Of those tadpoles treated at 1 ppm, however, 50% survived for at least 12 days with no apparent abnormalities. These data show that the tadpoles are 7.5 to 10 times more sensitive to the Hg ion than are the adult frogs, and that early embryos are at least 100 times more sensitive than tadpoles (Table I).

The sensitivity of adult frogs and tadpoles has also been compared for cadmium and arsenic. As seen in Table III, 50% of the adults treated with 50 ppm of cadmium survived at least 20 days, while the same concentration killed tadpoles in less than one day. Cadmium appears to be 5 to 7.5 times more toxic to tadpoles than adults. Concerning arsenic, tadpoles again appear to be substantially more sensitive than adult frogs (Table IV).

Our studies on the effects of cadmium, lead, zinc, and arsenic upon embryonic stages of the frog are not as yet complete. However, preliminary indications suggest that early embryos are many times more sensitive to metals than are tadpoles.

Though not selected as a test metal in this study, lithium has long been known as an acute toxin and teratogenic agent to many invertebrate and vertebrate embryos. For comparative purposes, we have treated adult frogs and tadpoles with lithium in concentrations of 10 to 1000

ppm (Table V). It is interesting that this classic teratogenic agent is significantly less toxic to adult frogs and tadpoles than the other metals which we have studied. The comparative toxicity of these metals to frog tadpoles is summarized in Figure 1. The order of toxicity from highest to lowest includes mercury, cadmium, arsenic and lithium.

To date, chick embryos have been treated with inorganic mercury, cadmium and lead, by the yolk sac injection method. Survival curves are presented in Figures 2, 3 and 4. Cadmium has been found to be most toxic, followed by lead and mercury.

Cadmium kills approximately 90%, 50%, 20% and 6% of chick embryos treated with concentrations of 1.0, 0.1, 0.01, and 0.001 ppm (Figure 3). Embryos surviving treatment of 0.01 ppm or more exhibited a substantial number of serious anatomical anomalies. As we are still analyzing these results, complete data are not available at this time.

Concerning treatment with lead, 75%, 31%, 8% and 6% of the embryos are killed at concentrations of 10, 1.0, 0.1, and 0.01 ppm, respectively (Figure 4). Also, serious anomalies are 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 the treated embryos either die or develop serious anomalies. The more common defects include hydrocephaly, absent eyes and deficiencies in the brain and spinal cord. As seen in Figure 2, inorganic mercury produces about the same degree of lethality in chick embryos as does lead.

In our preliminary studies with fish, we have treated eggs of the goldfish (Carassius auratus) with mercury, cadmium, lead and zinc, at

concentrations ranging from 100 ppm to 0.5 ppm. The percentage survival was determined for each test concentration by observations on a minimum of 75 eggs. Higher concentrations were extremely toxic. At 10 ppm, both mercury and cadmium produced a 100% kill in 1 to 2 days, while lead and zinc treatment produced 100% lethality in 4 days. Only 10-12% of the embryos survived treatment with mercury and cadmium at 1 ppm for three days, and there was no survival at four days. With a concentration of 0.5 ppm, these same metals killed all but 14% (Hg) and 20% (Cd) of the embryos within 3 days and there were no survivors at 4 days. Lead and zinc were found to be somewhat less toxic. Using these metals at 0.5 ppm, there was approximately 50% survival for three days and 16-20% by four days. The goldfish was chosen as a substitute for the bluegill in this investigation as the latter was not available in spawning condition throught a sufficient period to permit adequate study.

Recently we have established extensive facilities for maintaining fish embryology in a constant temperature room, and we are now studying the effects of metals on trout eggs. Though initial data have yet to be compiled, it appears that trout eggs are more sensitive to inorganic mercury than those of the goldfish.

The duration of this study was not sufficient to permit the treatment of each embryo species with each of the five metals considered. As a consequence, it remains to study the effects of cadmium, lead, zinc and arsenic upon the frog embryo, the effects of zinc and arsenic upon the chick embryo, and the effect of arsenic on goldfish embryos. These studies are being continued under support of a matching grant from O.W.R.R. (Project no. B-028-KY).

TABLE I

PERCENT OF RANA PIPIENS SURVIVING FOUR DAYS OF CONTINUOUS MERCURY TREATMENT

Stage at initiation of treatment	Concentration of Mercury (Hg) ion						
	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

TABLE II

COMPARISON OF PERCENT SURVIVAL OF LARVAE AND ADULT FROGS WITH VARIOUS CONCENTRATIONS OF Hg^{++}.[a]

Length of Exposure (Days)	Survival (%) Mercury Concentration (ppm)															
	50		25		10		7.5		5.0		2.5		1.0		0.5		0	
	L	A[b]	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			100	100	100		100	100
5						20		80		100			50	100	100		100	100
6						10		60		100			50	100	100		100	100
7						0		60		100			50	100	100		100	100
8								60		100			50	100	100		100	90
9								60		100			50	100	100		100	90
10								60		100			50	100	100		100	90
11								60		80			50	100	90		100	90
12								60		80			50	100	90		100	90
13								60		80			50	100			100	90
14								40		80			50	100				90
15								40		80			50	100				90
16								40		80			50	100				90
17								40		80			50	100				90
18								40		80			50	100				90
19								40		80			50	100				90
20								20		80			50	100				90
21								0		80			50	100				90

a. Rana pipiens and R. catesbeiana were used in this study.

b. L = Larvae; A = Adult. Each datum point represents observations on a minimum of 25 animals.

TABLE III

COMPARISON OF PERCENT SURVIVAL OF LARVAE AND ADULT FROGS WITH VARIOUS CONCENTRATIONS OF Cd^{++}.[a]

Length of Exposure (Days)	Survival (%) Cadmium Concentration (ppm)															
	1000		500		250		100		75		50		25		10		0	
	L	A[b]	L	A	L	A	L	A	L	A	L	A	L	A	L	A	L	A
1	0	0	0	60	0	80	0	100	0	100	0	100	81	100	96	100	100	100
2				0		0		90		100		90	24	100	92	100	100	100
3								20		40		90	19	100	88	100	100	100
4								10		40		90	5	100	81	100	100	100
5								10		40		90	5	100	81	100	100	100
6								10		20		70	5	100	77	100	100	100
7								10		20		70	5	100	77	100	100	100
8								10		20		70	5	100	73	100	100	87
9								10		20		50	5	100	65	100	100	87
10								10		20		50	5	100	54	100	100	87
11								10		20		50	0	100	50	100	90	87
12								10		20		50		100		100	80	87
13								10		20		50		100		100	80	87
14										20		50		100		100	80	87
15										20		50		100		100	80	87
16										20		50		100		100	80	87
17										20		50		100		100		87
18										0		50		100		100		87
19												50		100		100		87
20												50		100		100		87
21												50		100		100		87
22												50		100		100		87
23												50		100		100		87
24												50		100		100		87
25												40		100		100		87

a. Rana pipiens and R. catesbeiana were used in this study.

b. L = Larvae; A = Adult. Each datum point represents observations on a minimum of 25 animals.

TABLE IV

COMPARISON OF PERCENT SURVIVAL OF LARVAE AND ADULT FROGS WITH VARIOUS CONCENTRATIONS OF As^{++}.[a]

Length of Exposure (Days)	Survival (%) Arsenic Concentration (ppm)													
	500		100		50		25		10		5		0	
	L[b]	A	L	A	L	A	L	A	L	A	L	A	L	A
1	0	0	69	100	94	100	100	100	94	100	100	100	100	100
2			0	23	44	100	80	100	88	90	92	100	100	100
3				8	6	90	80	100	88	90	92	100	92	100
4				8	0	70	50	100	81	90	92	100	88	100
5				8		50	40	100	81	90	92	100	85	100
6				0		30	30	100	75	90	92	100	81	100
7						30	10	100	38	90	85	100	81	100
8						30	10	100	31	90	85	100	74	100
9						30	10	100	31	90	85	100	74	100
10						20	10	100	31	90	76	100	74	100
11						20	10	100	31	90	76	100	64	100
12						20	0	100	31	90	76	100	64	100
13						20		100	25	90	76	100	64	100
14						20		100	25	90	76	100	64	100
15						20		100	19	90	76	100	64	100
16						20		100	19	90	76	100	64	100
17						20		100	19	90	76	100	64	100
18						20		100	13	90	76	100	64	100
19						20		100	13	90	76	100	64	100
20						20		100	13	90	76	100	64	100
21						20		100	13	90	76	100	64	100
22						20		100	13	90	76	100	64	100
23						20		100		90	76	100	64	95
24						20		100		90		100		95
25						20		100		90		100		95

a. Rana pipiens and R. catesbeiana were used in this study.

b. L = Larvae; A = Adult. Each datum point represents observations on a minimum of 25 animals.

TABLE V

COMPARISON OF PERCENT SURVIVAL OF LARVAE AND ADULT FROGS WITH VARIOUS CONCENTRATIONS OF Li^+.[a]

Length of Exposure (Days)	Survival (%) Lithium Concentration (ppm)													
	1000		500		250		100		50		10		0	
	L	A[b]	L	A	L	A	L	A	L	A	L	A	L	A
1	0	40	33	100		100	100	100	93.7	100	100	100	100	100
2		0	0	60		100	81.3	90	93.7	100	93.7	100	100	100
3				40		100	62.5	90	93.7	100	93.7	100	100	100
4				0		20	56.3	90	93.7	100	93.7	100	93.3	100
5						0	56.3	90	93.7	100	93.7	100	93.3	100
6							18.7	90	81.3	100	93.7	100	93.3	100
7							12.5	70	68.7	90	87.5	100	93.3	100
8							12.5	70	68.7	80	81.3	100	93.3	100
9							12.5	60	68.7	70	76.1	100	93.3	100
10							6.3	10	68.7	40	61.5	100	93.3	100
11							6.3	0	53.3	10	53.8	100	86.6	100
12							0		50.0		53.8	100	80	100
13									18.7		46.1	100	80	100
14											40	100	80	100
15											40	100	80	100
16											40	80	80	90
17												60		90
18												60		90
19												60		90
20												60		90
21												60		90
22												60		90
23												60		90
24												60		90
25												60		90

a. Rana pipiens and R. catesbeiana were used in this study.

b. L = Larvae; A = Adult. Each datum point represents observations on a minimum of 25 animals.

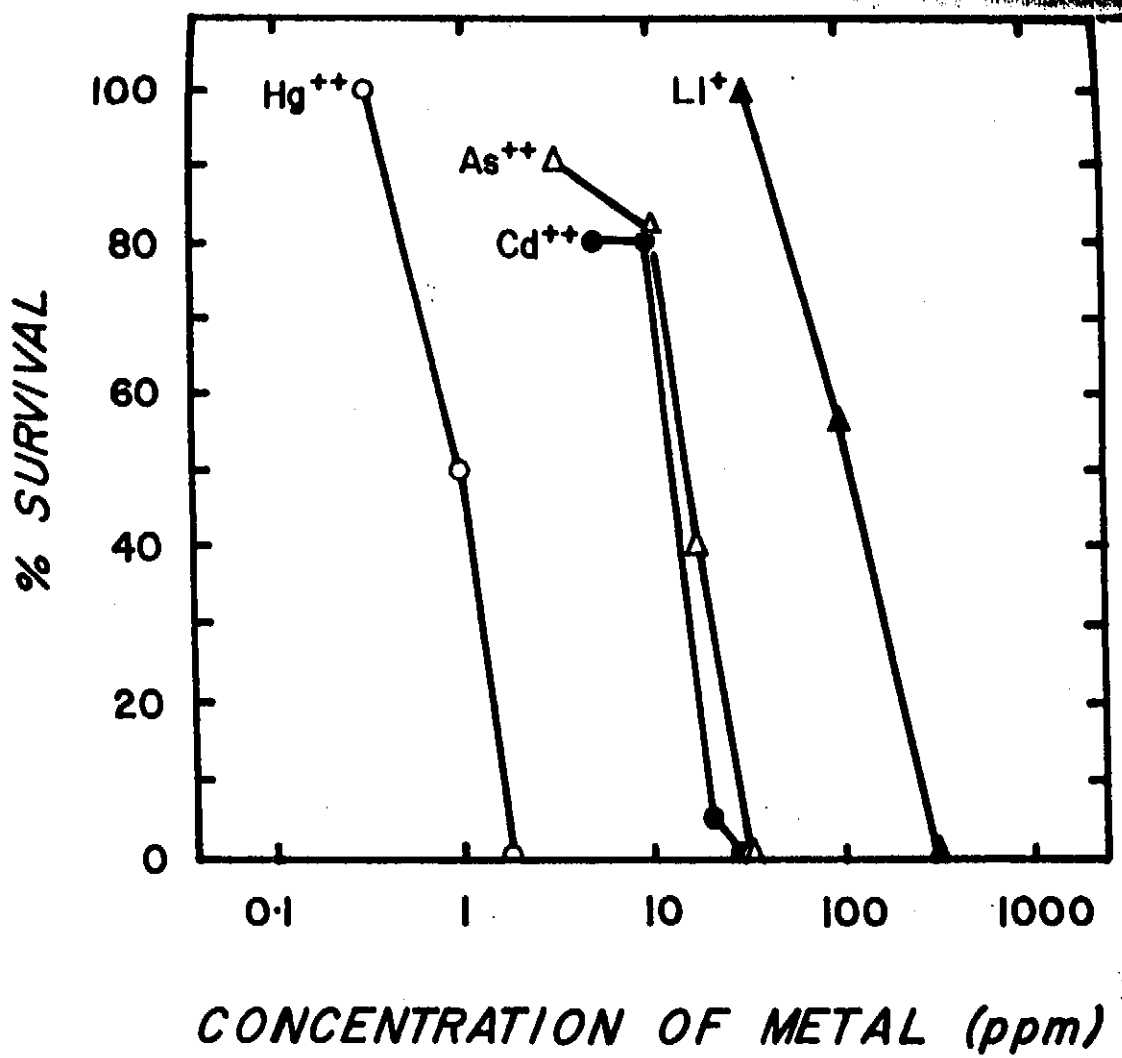

FIGURE 1. Comparison of toxic effects of mercury, cadmium, arsenic and lithium upon tadpoles treated for 5 days.

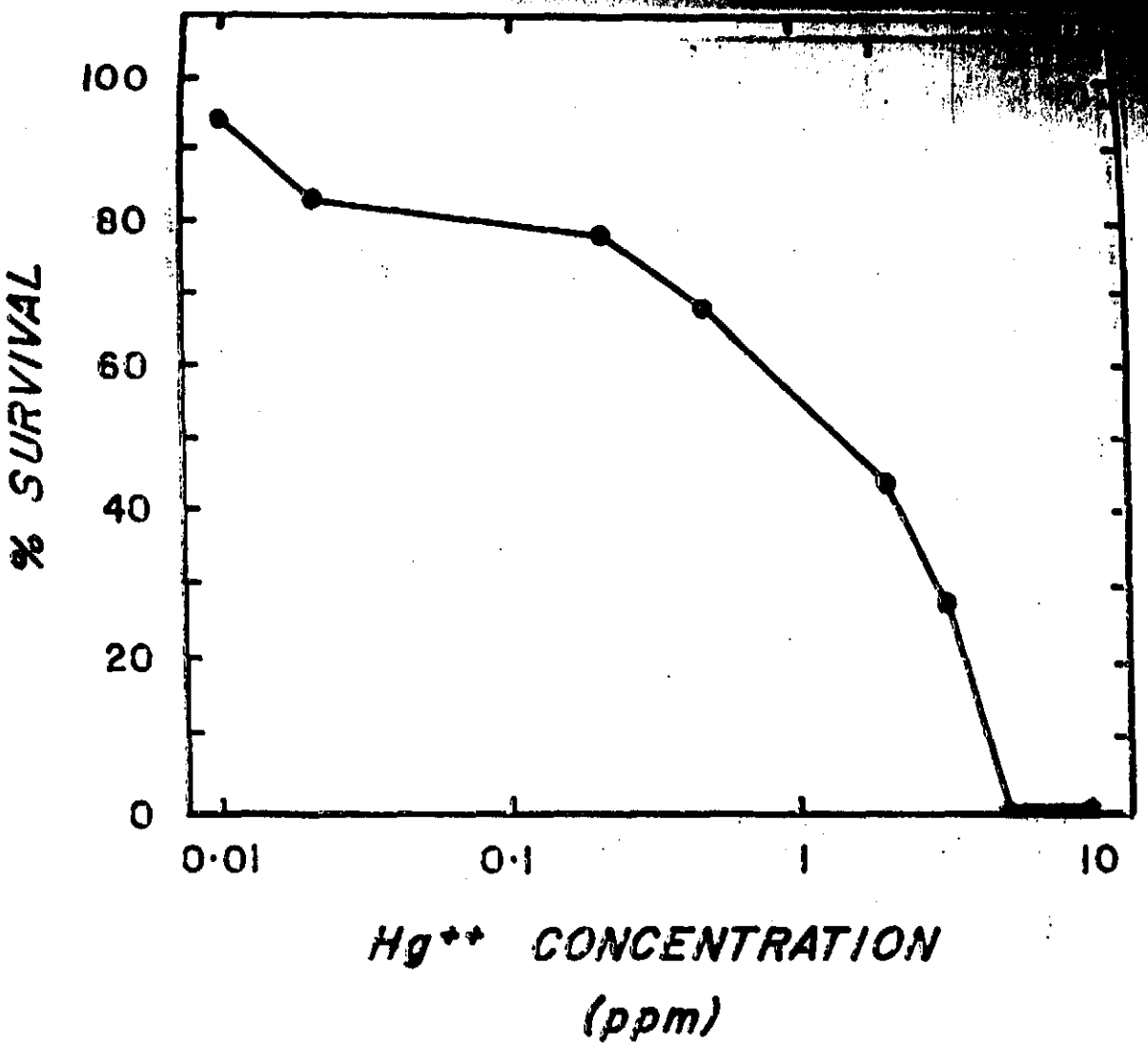

FIGURE 2. Sensitivity of chick embryos to inorganic mercury administered by yolk sac injection at start of incubation. Experiment terminated one day prior to hatching. Each point represents survival of 100 experimental embryos/100 controls.

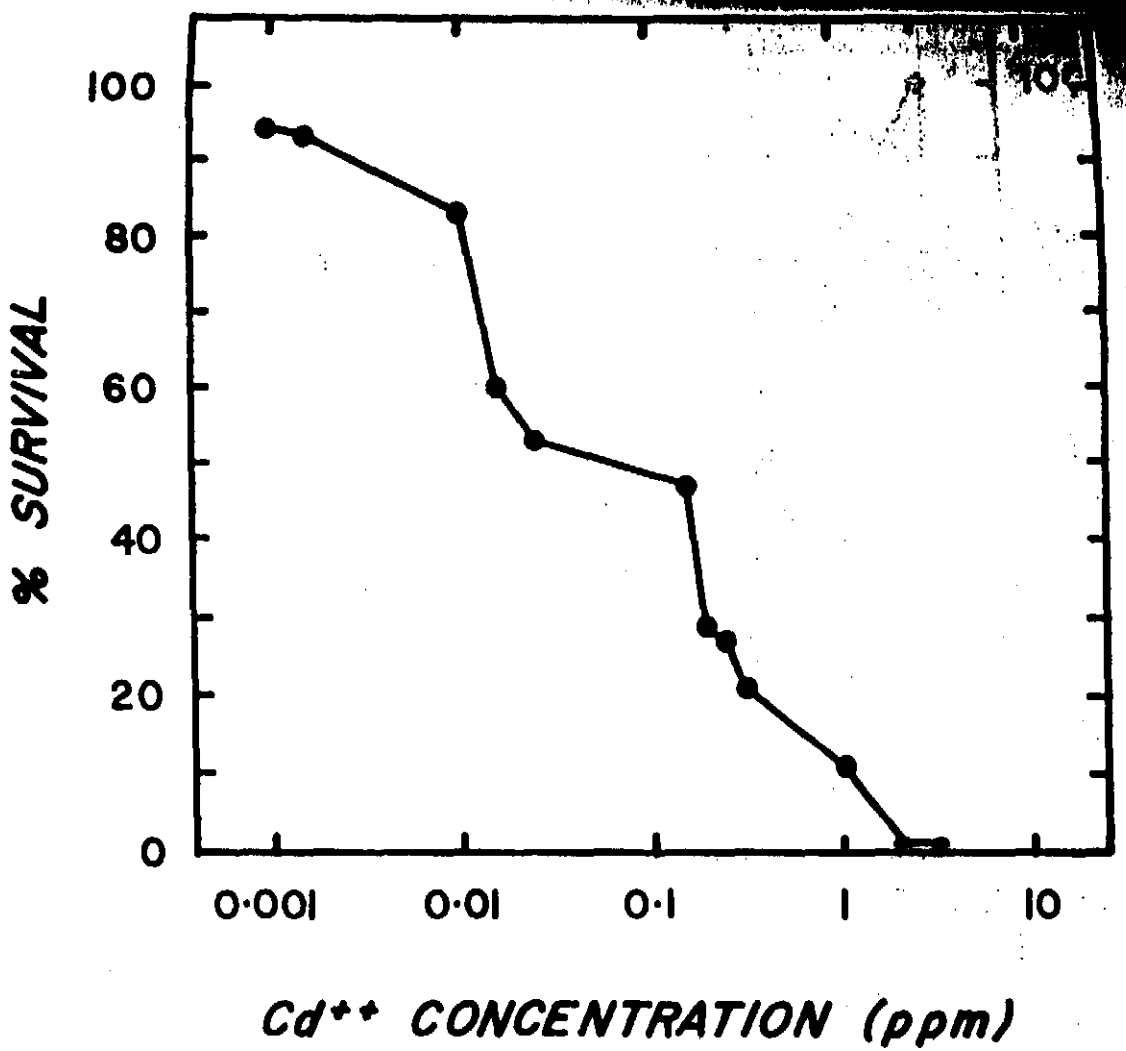

FIGURE 3. Sensitivity of chick embryos to cadmium administered by yolk sac injection at start of incubation. Experiment terminated one day prior to hatching. Each point represents survival of 100 experimental embryos/100 controls.

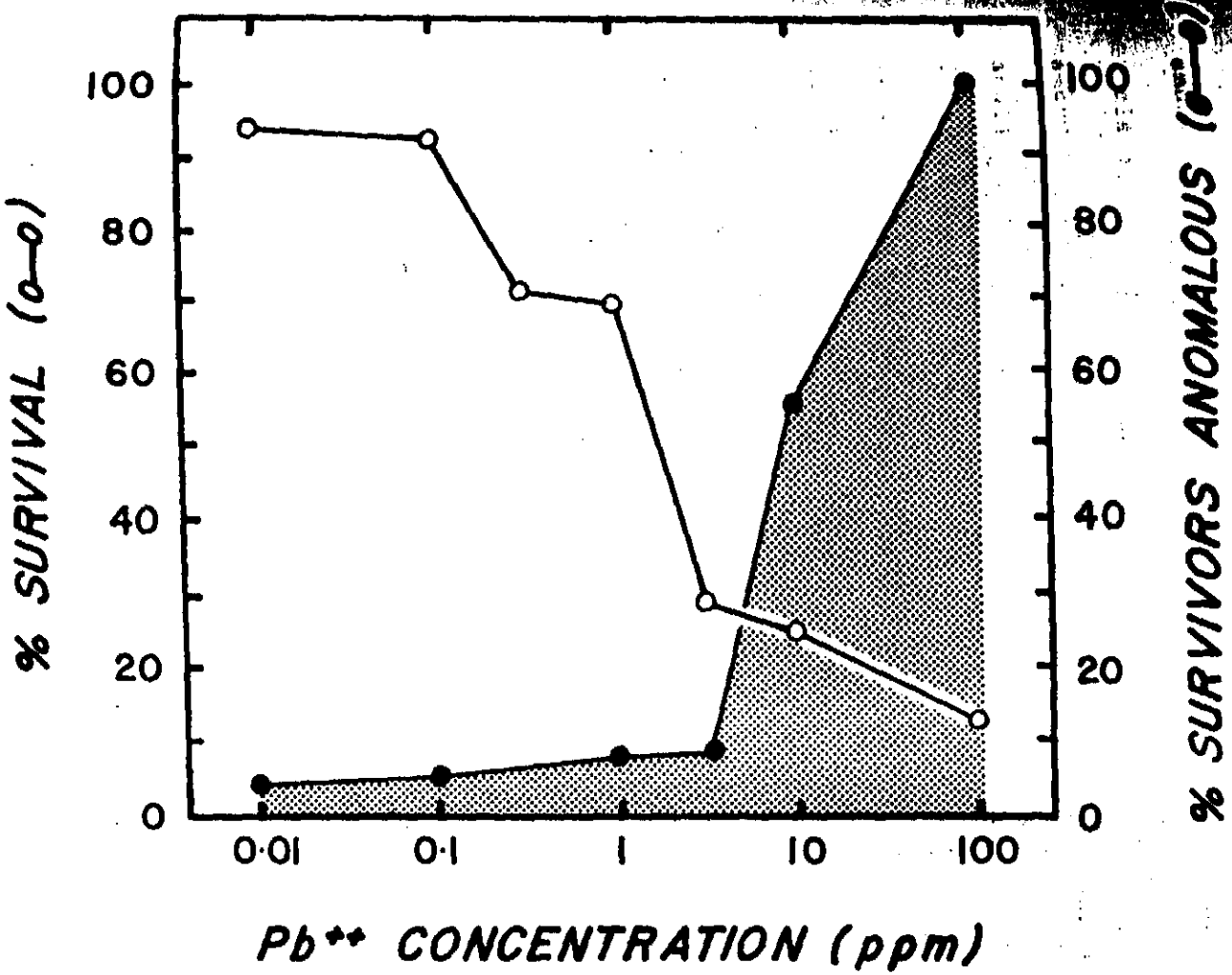

FIGURE 4. Sensitivity of chick embryos to lead administered by yolk sac injection at start of incubation. Experiment terminated one day prior to hatching. Each point represents survival of 100 experimental embryos/100 controls.

CHAPTER IV

CONCLUSIONS

We feel these initial results verify our contention that vertebrate embryos constitute extremely heavy-metal-sensitive stages in the life cycle of vertebrate animals. These data strongly indicate that embryonic stages are much more sensitive to metallic poisoning than are adult vertebrates. The more sensitive stages of the frog embryo appear to be at least one thousand times more susceptible to poisoning by inorganic mercury than are adult frogs. Concentrations of mercury, cadmium, and lead as low as 10 ppb (or less) are distinctly harmful to vertebrate embryos.

Also, it is apparent that the level of sensitivity for a particular metal may vary significantly among embryos of the different species of vertebrates. For example, while mercury is more harmful to amphibian embryos than cadmium or lead, the reverse relationship is found for avian embryos. These data suggest that species may vary substantially in their ability to reproduce in a polluted environment, and this underscores the need for a comparative index to the toxic effects of metals upon embryos of different vertebrate species.

It is our contention that water quality standards must be properly assessed with regard to dangerous pollutants to permit "safe limits" for the most sensitive or susceptible stage in the life history of an organism. With respect to vertebrate species, such "safe limits" should allow for the extreme sensitivity of vertebrate embryos to metallic pollutants. In this respect, the foregoing data on the

toxicity of metals should aid in the evaluation of adequate water quality standards suitable for maintaining animal life and reproduction.

In addition to the above results, our initial experiences with vertebrate embryos are encouraging in terms of developing a sensitive bioassay system for the detection of metallic pollutants occurring within water resources. These embryonic forms are sufficiently sensitive for such test purposes, and they may be cultured under conditions which will permit bioassay determinations.

PUBLICATIONS

Wesley J. Birge and John J. Just. Lethal and teratogenic effects of heavy metals upon embryonic development in the domestic fowl. In preparation.

John J. Just and Wesley J. Birge. Effects of inorganic mercury upon embryonic and larval development in the frog. In preparation.