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CONTAMINANTS PROGRAM

DISAPPEARANCE OF BOREAL TOADS IN COLORADO: A CONTAMINANT INVESTIGATION

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INTRODUCTION

Population sizes of many, but not all, amphibian species have experienced declines on all six continents on which they occur (Phillips 1990, Vial and Saylor 1993). In some cases, complete extinctions of populations have occurred (Roberts 1981, Corn and Fogleman 1984, Heyer et al. 1988, Beebee et al. 1990, Bradford 1991, Koonz 1992, Carey 1993, Sherman and Morton 1993, Pounds and Crump, Laurance et al. 1996, Drost and Fellers 1996, Lips 1998). While sporadic die-offs of amphibians were observed before 1970, major declines were documented in temperate areas beginning in the 1970's (Roberts 1981, Corn and Fogleman 1984, Heyer et al. 1988, Bradford 1991, Koonz, 1992, Carey 1993, Sherman and Morton, 1993); amphibian disappearances in the tropics have been observed more recently (Pounds and Crump 1994, Laurance et al. 1996, Lips 1998). The global scope and the rapidity with which these declines have occurred have led to the conclusion that anthropogenic causes must be largely at fault (Phillips 1990). While habitat destruction, introduction of predators, and exposure to toxicants certainly have played important roles in causing some amphibian declines (Phillips 1990), a perplexing number of die-offs have occurred in relatively pristine areas, such as national parks and forests of the western United States (Corn and Fogleman 1984, Bradford

1991, Carey 1993, Sherman and Morton 1993, Drost and Fellers 1996), and montane rainforests of Central America and Australia (Pounds and Crump 1994, Laurance et al. 1996, Lips 1998). These die-offs in relatively pristine areas share most or all of these characteristics: declines spread rapidly (within 1-2 yrs) over broad geographical areas, populations experience 50-100% mortality, declines are more pronounced at higher altitudes, declines occur in only some of the total number of species in a locality, mortality is principally among adult and metamorphosed individuals, and disease appears to be the proximal cause of death (Scott 1993, Laurance et al. 1996). Mass mortalities of salamanders in the genus *Ambystoma* differ from this pattern in that die-offs are geographically more restricted and mortality is highest among larvae just at metamorphosis (Worthylake and Hovingh 1989, Berna 1990, Jancovich et al. 1998, Carey et al. unpubl. data). While disease is the direct, or proximal cause of death in a number of these instances, no proof exists concerning the ultimate, or indirect causes of death. While disease has been a factor in the population biology of all living species since early in evolution (Anderson and May 1982), the recent number and severity of disease outbreaks among amphibians suggest that man-made environmental changes might be causing increased incidences of disease. At

least four hypotheses exist concerning why amphibians have recently succumbed to disease:

1) human activities might be spreading virulent pathogens from one habitat to another, thus exposing amphibians to disease vectors to which they have had no previous exposure,

2) environmental change(s) might cause the mix of soil and water microorganisms to change in a way that exposes amphibians to higher numbers of virulent pathogens,

3) environmental changes might cause the virulence of the pathogens to increase,

4) environmental changes that stress amphibians might cause immunosuppression and increased vulnerability to disease.

Boreal toads *(Bufo boreas boreas)* and northern leopard frogs *(Rana pipiens)* were once common in the Rocky Mountains of Colorado. During the mid-1970's to early 1980's many populations of these animals became extinct in western Colorado (Corn et al. 1989, Carey 1993). Mass mortalities were sufficiently wide-spread that the toads disappeared from entire drainages or mountain ranges, with the result that the boreal toad was designated as "endangered" by the Colorado Division of Wildlife. The few remaining populations appear to be stable, but they are neither increasing in size nor are the few remaining populations recolonizing historically occupied habitat.

While montane populations of leopard frogs also became extinct during those years (Carey 1993), the presence of populations at lower altitude in Colorado precluded a similar designation for this species. In both these species, population extinction occurred within a few years, despite the fact that these organisms are probably quite long lived as adults, because mortality occurred in adults and metamorphosed juvenile age classes (Carey 1993).

The direct cause of death of montane populations of these toads and frogs was disease (Carey 1993). However, the indirect, or ultimate environmental causes have never been determined. It is known that the pH of bulk precipitation in the Colorado Front Range declined significantly during the years from 1972-1978 (Carey 1993). While Corn et al. (1989) and Corn and Vertucci (1992) have ruled out pH as directly lethal to amphibians, such pH changes could have contributed to disease by either dramatically affecting the population mix of soil microorganisms and thereby the exposure of amphibians to different levels of virulent pathogens, or by stressing the amphibians sufficiently to cause immunosuppression or excessive immunostimulation (Carey et al. 1996). Insufficient funds have been available to pursue these possibilities or the possibility that pH

changes acted synergistically with other environmental factors to cause disease in these animals.

Montane populations of tiger salamanders in Colorado have experienced a different pattern of die-offs. A number of mass mortalities of larval salamanders at metamorphosis and newly metamorphosed individuals have been noted over the past 7 years (Carey et al. unpubl. data). These die-offs are also due to disease (Carey et al. unpubl. data). However, due to the facts that the die-offs only involve the year-class of young for that year and also that these extinctions are local, occurring in just one pond at a time, salamanders have recolonized ponds in which die-offs have occurred and no obvious populational disruptions have been evident. However, because these die-offs need to be taken seriously as potential indicators of even greater mortality in the future, the indirect cause(s) need to be identified.

A wide variety of metals and other contaminants are known to be immunosuppressive and are known to affect soil and water microorganism populations (Sharma and Reddy, 1987; van Elsas et al. 1992). The questions addressed by this study are: 1) what levels of metals and organochlorines exist in sediment and amphibian tissues in breeding areas of montane amphibians in

Colorado, and 2) what is the effect of metal exposure on immune function in leopard frogs?

STUDY AREA AND METHODS

Concentrations of Metals and Organochlorines in Sediment and Tissues in Breeding Areas of Boreal Toads in Colorado

Sediment samples were taken during summer, 1994 to 1996, with a stainless steel spoon from the top 20 mm of sediment in breeding ponds of extant populations of boreal toads and from ponds in which breeding was known to have taken place historically (Table 1 and Fig. 1). To cover a large geographical area, only one sediment sample was taken per site. Possible bias in the data exist because a large number of samples from extinct sites were collected within the same drainage, namely the East River drainage in the West Elk Mountains. Samples were taken to a depth of 20 mm because sediment is thought to accumulate at 1 mm/yr in lakes in the Colorado Rockies (W. M. Lewis, pers. comm.). Therefore, if abnormal contaminant levels existed at the time of the mass extinctions in the mid-1970's, they should show up in these samples.

In the first year of sediment and tissue sampling (1994), one boreal toad tadpole sample was collected from

Table 1. Sites and dates of sediment collection from breeding ponds of boreal toads (one composite sample/site/time).

Location	Date	Latitude	Longitude	County
Sites where boreal toads are extinct				
Mill Crk	10/01/94	38°42'	107°6'	Gunnison
Caribou	08/15/94	39°58'	105°35'	Boulder
Mt. Bellvw	07/12/96	39°00'	107°01'	Gunnison
Got. Nat. Ar.	07/12/96	38°59'	107°01'	Gunnison
Mt. Gothic	07/12/96	38°57'	106°59'	Gunnison
Rustlr Glch	07/12/96	38°59'	107°01'	Gunnison
Trl 401	07/12/96	38°58'	106°59'	Gunnison
Copper Cr	07/15/95	38°58'	106°56'	Gunnison
Love Lk	08/08/96	37°40'	107°02'	Mineral
Sites where boreal toads are still breeding				
Grgtown	08/01/94	39°41'	105°42'	Clear Creek
TriPass	07/10/94	39°00'	106°56'	Gunnison
DennCrk	07/31/94	38°50'	106°20'	Chaffee
Lost Lk	10/15/94	39°57'	105°37'	Boulder
Brown's Crk	06/24/95	38°39'	106°12'	Chaffee
Hart Lk	06/24/95	38°50'	106°20'	Chaffee
2 Pond	07/24/95	39°46'	105°51'	Clear Creek
Lost Lk	07/03/96	39°57'	105°36'	Boulder
Cottnwd Lk	07/13/96	38°46'	106°21'	Chaffee
Cottnwd Crk	06/12/96	39°48'	106°19'	Chaffee
Herman Glch	06/24/96	39°42'	105°51'	Clear Creek
HESBO	06/27/96	39°46'	105°48'	Clear Creek
Mt. Bethel	06/24/96	39°42'	105°52'	Clear Creek
Kettle Trn	06/20/96	40°30'	105°32'	Larimer
Fern Crk	06/12/96	37°42'	107°07'	Mineral
Cucumber	06/13/96	39°29'	106°03'	Summit
Lowr. 10 mi	06/13/96	39°34'	106°08'	Summit
Montezuma	06/13/96	39°35'	105°52'	Summit

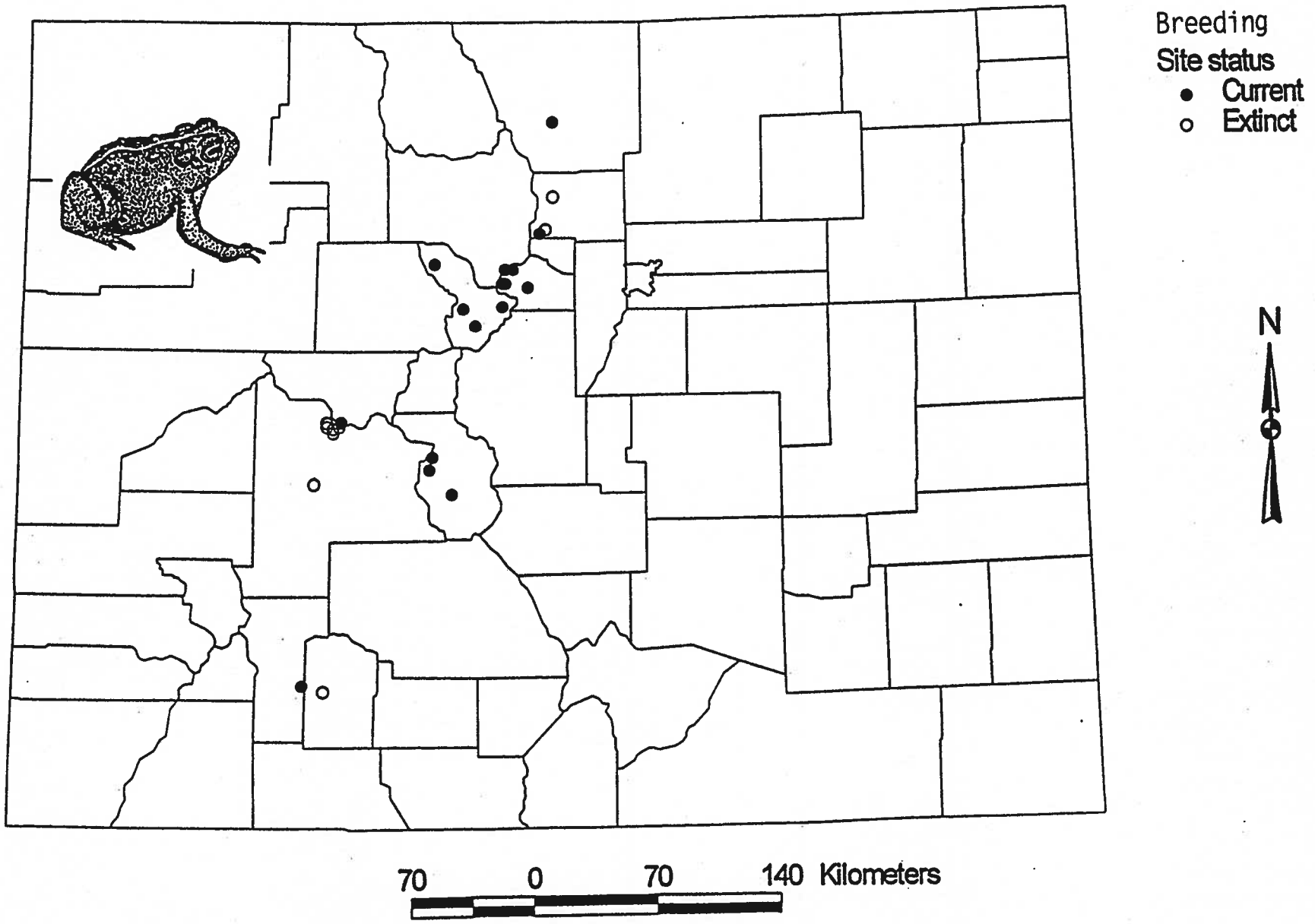


Figure 1. Map of sediment collecting localities for boreal toad breeding areas.

each of the following populations; Georgetown, I-70 (Herman Gulch), Triangle Pass (Whiterock), and Denny Creek and analyzed for metal concentrations. Boreal toad tadpoles collected in 1994 at Denny Creek and Herman Gulch were analyzed for organochlorines. In 1995, one boreal toad tadpole sample was collected from the Mt. Bethel population; the gastrointestinal tract was removed and analyzed for metals separately from the rest of the tissue. In both 1995 and 1996, three boreal toad tadpole samples were collected from the Kronke site; the gastrointestinal tracts were removed from two of these samples and analyzed for metals separately from the rest of the tissue. Metal concentrations in tadpole samples with gastrointestinal tracts removed were then compared to metal concentrations in the whole body tadpole sample. At sites where boreal toads were not available (eg. Chatfield, Mag.Pond, Pueblo), other amphibian species were collected to assess metal concentrations in amphibian tissue. To get sufficient tissue samples for metal analysis, at least 8 g (i.e. 20-50 tadpoles) of tadpole larvae had to be analyzed for each site. At sites where boreal toads were not available, other amphibian species were collected to determine metal concentrations in amphibian tissue. Therefore, values reflect the average of the homogenized mass, rather than for a single individual tadpole.

Sediment and tissue samples were frozen in glass jars. All sediment and tissue samples were sent to contract laboratories through the U.S. Fish and Wildlife Service Patuxant Analytical Control Facility (PACF) in Patuxant, Maryland. Quality-control procedures for both inorganic and organic analyses done by the laboratories included sample spikes, duplicates, and blanks. All analytical data were reviewed by the PACF.

Analyses for chlorinated hydrocarbons were performed by Mississippi State Chemical Laboratory. Following extractions using acetone and ether, samples were analyzed for chlorinated hydrocarbons by using electron capture chromatography. Because all sediment and tissue samples contained concentrations of chlorinated hydrocarbons which were below detection limits, samples collected during 1995 and 1996 were analyzed only for metals.

Analyses for trace elements were done at Environmental Trace Substances Research Center in Columbia, Missouri. Samples were analyzed for selected trace elements using inductively coupled argon-plasma atomic-absorption spectrometry after complete digestion of the sample in strong acids. Analyses for arsenic and selenium were done using hydride-generation atomic absorption, and

analyses for mercury were done by flameless cold-vapor atomic absorption.

Statistical comparisons of metal concentrations were done by Dr. Howard Ramsdell of the Dept. of Environmental Health, Colorado State University. Means were compared between extant and extinct sites for boreal toads with Student's t-statistic. Data were log transformed to adjust for lack of normality in the distribution of values. Calculations were performed using SAS. Correlation analyses were performed using the method of least-squares regression using Microsoft Excel 97.

Concentrations of Metals in Water in Breeding Areas of Boreal toads in Colorado.

Unfiltered water samples were obtained during 1995 only from sites with extant toad populations. Water samples were collected in Nalgene 60 ml high density polyethylene bottles and immediately preserved with Ultrex triple distilled nitric acid to pH <2.0. Manganese, iron and zinc concentrations were determined using an Instrumentation Laboratory Video 22 (Allied Analytical Systems, Franklin, MA) atomic absorption spectrometer with air-acetylene flame. Detection limits for manganese, iron and zinc were 20, 50, and 10 ug/L respectively. Cadmium, copper and lead were determined

using a Thermo Jarrell Ash SH4000 with CTF 188 controlled temperature graphite furnace with detection limits of 0.1, 1.0 and 1.0 ug/L respectively. Ammonium phosphate monobasic (0.1%) was used as a matrix modifier for all furnace analyses. Both systems used Smith-Hieftje background correction. Instruments were calibrated before each use and accuracy evaluated using external quality assurance standards (High-Purity Standards, Charleston SC). These analytical methods provide metal concentration results considered to be acid soluble (or acid leachable).

Concentrations of Metals in Sediment and Tissues in Breeding Areas of Tiger Salamanders in Colorado

Sediment samples from breeding ponds of tiger salamanders were collected and analyzed as described earlier for boreal toad breeding areas. One sediment sample was taken from each of three ponds that have not recently experienced a mass-die off of salamanders and one sediment sample was taken from each of four ponds in which die-offs have occurred since 1994 (Table 2). Because of the large size of the larvae (40-60 g), 1-2 larva(e) per site was (were) analyzed for metal concentrations. Therefore, bias may exist in the data because of the lack of the ability to obtain an average

concentration from many carcasses per composite sample per site.

Table 2. Sites for collection of sediment from breeding ponds of salamanders.

Location	Latitude	Longitude	County
Sites where salamander die-offs have occurred			
MudLk	39°58'	105°31'	Boulder
Guffy Pond	38°37'	105°22'	Fremont
Keblr Pass	38°54'	107°11'	Gunnison
Cunn. Res.	38°41'	107°02'	Gunnison
Sites where salamanders have not experienced mass mortality since 1994.			
Castle Cr.	38°47'	105°56'	Chaffee
ZirkB-5	40°35'	106°41'	Routt
ZirkB-6	40°36'	107°43'	Routt

Metal Exposure Affects on Amphibian Immune Function

The complete results from the statistical analysis of sediment and tissue samples of boreal toads were not available until May 23, 1998. Since laboratory tests had to be done in the summer of 1997, we had to guess what metals might be most important in terms of potential causes of boreal toad diseases. Furthermore, no studies of which we are aware test metal effects on adult

amphibians. Therefore, lethal levels are unknown. However, because mortality occurred in adult and metamorphic juvenile age classes during the mass die-offs in the 1970's, experiments involving exposures of adults must be top priority. Tests on immune function are expensive to run because of costs of animal purchase, care, daily water changes, radioactive tracers, and sterile test tubes, etc. for cell culture. Remaining funds, after analyses of contaminants in sediment and amphibian tissue, could support only a few immunological tests.

Immunological Methods

Blood Sampling. Blood was collected by heart puncture with a 1 ml syringe using a 20 ga needle. For differential blood count and mitogen studies, the syringes were heparinized with sterile heparin. Syringes were not heparinized for blood collection for complement analysis. Due to the fact that sampling with two different syringes was needed from the same animal, each animal experienced two heart punctures on the day it was sampled. No toads died in the two weeks following cardiac punctures.

Responsiveness of lymphocytes to a mitogen. About 0.5 to 0.8 ml blood was placed in sterile nutrient broth

(RPMI [Sigma] containing 5% fetal calf serum). Lymphocytes were separated by centrifugation for 30 min at 1200 rpm on a Percoll density gradient. After three washes in sterile amphibian phosphate buffered saline, cells were counted and the viability of the cells was checked by trypan-blue exclusion. The cells were then reconstituted in a volume of RPMI to reach a final concentration of 5×10^4 cells/ 100 µl. Cells were pipetted into 96-well flat-bottomed plates and were incubated in the presence or absence of 1.25 µg/ml PHA (phytohemagglutinin, a T-cell mitogen) added at the initiation of the culture in 100 µl in complete RPMI. This concentration of PHA was found in preliminary tests to cause the highest amount of stimulation of toad lymphocytes. Six control and six mitogen wells are run per animal. After incubation for 72 hr at 29 C in a 5% CO_2/95% air environment, the cells were pulsed with ^3H-thymidine (0.5 µci/well) for 18 hr and then harvested onto a glass fiber filter using a Harvard cell harvester. ^3H-thymidine absorbed by the cells was quantified by liquid-scintillation spectrometry. The average counts per minute of cells stimulated by PHA were averaged for each animal and then divided by the average value of unstimulated control cells for that animal. These averages, comprising the "stimulation index" for each animal, were then averaged to arrive at the mean for each test group.

Complement (CH_{50}) levels. Approximately 0.2-0.4 ml of blood per animal were placed in 1.7 ml Eppendorf centrifuge tubes and allowed to clot for at least 1 hr on ice. Plasma was removed by centrifugation and stored at -70C. Sheep erythrocytes were standardized to a standard optical density and then sensitized by coating with rabbit anti-sheep erythrocyte antibody (Sigma) at 18C (Hudson and Hay 1980). Varying dilutions of toad plasma were added to the erythrocytes and the mixture was incubated at 29 C for 1 hr. Tubes were then centrifuged and the OD of the supernatant determined at 541 nm. The CH_{50} (amount of complement necessary to lyse 50% of the erythrocytes) titer was calculated as specified in Green and Cohen (1977).

Experiment 1. Do current concentrations of metals in water cause immunosuppression in leopard frogs?

Data on metal concentrations in water in various extant boreal toad breeding areas were used to determine if current exposure levels in these ponds can induce immunosuppression (Steve Brinkman, CDOW, per.com.,1998). Zinc and manganese were chosen because of their relatively high concentrations in water at HESBO and 2 Pond.

The control group of 10 frogs was maintained in Holtfreter's solution (artificial pond water. Three groups of 10 adult leopard frogs were exposed to 0.1, 1 and 10 mg/l manganese chloride; another three groups were exposed to 0.01, 0.1 and 1 ug/l zinc chloride for three weeks. Each frog was individually maintained in 20 cm x 10 cm x 7 cm plastic containers with lids. Five hundred ml of the control or test metal water was placed in the container each morning and then replaced again 12 hours later. Frogs were held under these exposure conditions for 3 weeks. At the termination of the 3 week period, blood samples were taken as described above.

Experiment 2. Does concurrent exposure to cold, low pH and metals cause immunosuppression in leopard frogs?

Boreal toads largely died out during the winter, or to be exact, didn't reappear in the spring following the hibernation season. They may have died in early fall prior to hibernation or at any time during the winter, or during snowmelt in the spring. Due to the low pH of snow in the 1970's when toads and frogs were dying in the Colorado Rockies, the possibility exists that a combination of cold, low pH, and metals caused immunosuppression and increased susceptibility to disease. Cold is a potent immunosuppressor in leopard frogs (Maniero and Carey 1996).

Seventy-five frogs (not used in any previous metal experiment) were divided into groups. Each frog was held individually in its own plastic container and exposed to 500 ml of water. The control group of 12 frogs was exposed to 5 C and pH 7.0 in Holtfreter's solution for 6 weeks. A second group of frogs was exposed to 5 C and pH 4.0 in Holtfreter's solution for 6 weeks. A third group was exposed to 5 C, 5 ug/l CuCl2.2H20, pH 4.0 in Holtfreter's solution for 6 weeks, and a fourth group to 5C, 10 ug/L CdCl2, and pH 4.0 in Holtfreter's solution for 6 weeks. A final group of frogs were held for 6 weeks at 5 C, pH 4.0 in Holtfreter's solution, and 5 ug/L CuCL2.2H20 and 10 ug/L CdCl2. Water was changed 3 times daily to ensure that exposure to low pH and metals was consistent at all times of each 24 hr period. Blood samples were taken from each frog at the end of the 6 week period and tested for mitogenic responses of lymphocytes and complement.

RESULTS AND DISCUSSION

Concentrations of Metals and Organochlorines in Sediment and Tissues in Breeding Areas of Boreal Toads in Colorado

Sediment Samples

Trace element concentrations in sediment samples collected at boreal toad sites are listed in Table 3.

Table 3. Trace element concentrations (µg/g dry weight) in sediment samples collected from boreal toad breeding ponds.

Site	Collection Date	% Moisture	Al	As	B	Ba	Be	Cd	Cr	Cu	Fe	Hg	Mg	Mn	Mo	Ni	Pb	Se	Sr	V	Zn
Tri. Pass	10\1\94	67.8	15900	1.20	5.4	110.0	0.60	0.44	8.20	5.8	13400	0.013	3090	261.0	<.8	4.8	10.0	0.86	58.4	30.0	45.1
401 Ponds	7\10\94	56.7	18900	3.83	4.1	157.0	1.00	4.00	17.00	19.0	12400	0.020	5260	107.0	0.9	13.0	32.0	1.10	18.9	31.0	261.0
Herman Gich	8\1\94	76.3	31600	0.70	2.0	331.0	1.20	0.40	28.00	42.0	22500	0.049	5620	154.0	3.6	17.0	40.0	0.77	52.2	37.0	107.0
Grgtown	8\1\94	54.6	21700	2.80	2.5	293.0	1.10	2.30	44.00	49.0	36200	0.459	7920	641.0	2.0	25.0	300.0	0.87	57.2	50.0	419.0
Lost Lk	10\15\94	39.4	3730	1.40	<.8	60.0	0.10	0.30	7.20	17.0	10900	0.018	1450	72.3	1.0	3.2	10.0	0.42	16.9	18.0	26.0
Denny Crk	7\31\94	86.1	16900	0.77	4.2	208.0	0.58	0.88	14.00	18.0	18700	0.071	4620	321.0	2.9	7.9	32.0	0.97	41.8	32.0	95.9
Mud Lk.	10\15\94	27.0	2650	0.60	<.8	46.3	0.15	0.40	7.70	4.0	5460	0.013	710	28.8	<.8	4.4	6.0	0.21	5.9	10.0	20.6
Caribou	8\15\94	78.0	27400	1.80	2.7	297.0	0.61	0.81	20.00	59.0	42900	0.130	6420	253.0	1.0	13.0	35.0	0.99	77.8	72.0	88.1
Mill Crk	10\1\94	58.0	21200	0.96	1.0	109.0	0.53	0.40	3.40	3.8	28700	0.009	4340	392.0	<.9	2.0	9.0	0.34	143.0	59.0	60.1
Hart Lk	6\24\95	77.0	15600	0.99	4.5	160.0	0.62	0.50	15.00	17.0	16800	0.064	3740	212.0	2.0	7.6	33.0	0.64	33.0	30.0	52.8
2 Pond	7\24\95	44.2	5840	2.20	<6	147.0	34.70	5.70	<5	20.0	5390	0.016	2670	101000.0	52.0	25.0	30.0	0.20	1530.0	<8	3420.0
Copper Cr	7\15\95	85.9	10500	2.30	6.7	155.0	0.41	0.85	8.80	12.0	4900	0.089	2450	59.7	1.9	7.8	33.0	0.68	49.5	16.0	47.5
Brown's Crk	6\24\95	57.0	10600	0.63	2.7	40.3	2.50	0.56	6.00	13.0	7230	0.024	2990	208.0	2.2	5.7	34.0	1.00	34.8	20.0	77.7
Lowr 10 ml	6\13\96	74.0	18200	2.20	6.5	214.0	1.00	0.40	103.00	38.0	35000	0.042	8930	762.0	1.4	26.0	32.0	3.10	32.9	66.0	90.5
Mt. Gothic	7\12\96	58.7	22500	6.90	9.3	199.0	1.00	1.30	24.00	13.0	19700	0.040	4130	153.0	3.7	13.0	32.0	2.30	77.9	59.0	102.0
Lost Lk	7\3\96	64.8	7880	6.60	2.0	144.0	0.51	0.50	13.00	146.0	23600	0.085	2810	334.0	2.6	7.0	46.0	0.89	52.1	39.0	62.9
Trl 401	7\12\96	64.9	23700	3.50	8.5	189.0	1.40	8.40	21.00	25.0	12400	0.024	5900	165.0	0.7	15.0	86.0	2.20	26.6	42.0	834.0
Rustlr Gich	7\12\96	50.1	12100	15.00	4.6	138.0	1.40	1.20	13.00	27.0	33500	0.043	2440	126.0	1.3	22.0	30.0	2.90	43.6	33.0	72.3
Cucumber	6\13\96	56.2	53000	2.80	4.5	266.0	1.70	<.2	21.00	30.0	32200	0.058	7040	227.0	5.1	22.0	65.0	0.48	78.7	70.0	115.0
Fern Crk	6\12\96	37.7	26900	1.40	3.2	172.0	0.78	<.2	11.00	9.2	32600	0.023	4100	661.0	<.4	6.4	21.0	0.15	79.4	108.0	107.0
Got. Nat. Ar	7\12\96	53.0	13100	15.00	2.0	68.7	1.20	1.60	18.00	28.0	26900	0.034	8180	317.0	3.9	35.0	44.0	2.40	27.0	43.0	163.0
Brown's Crk	7\22\96	78.3	12700	0.72	1.9	42.6	2.85	0.56	6.40	15.0	8580	0.024	3950	284.0	2.5	6.5	36.0	1.20	48.2	21.0	95.5
Cottonwd Crk	6\12\96	60.3	15400	2.00	3.1	103.0	0.65	0.20	12.00	72.4	20300	0.200	7520	275.0	6.7	8.9	24.0	2.20	77.6	55.0	95.1
Cottonwd Lk	7\13\96	69.8	16300	1.20	4.7	159.0	0.79	0.56	18.00	71.0	18900	0.053	6960	226.0	3.7	16.0	27.0	2.90	100.0	35.0	85.6
Kettle Trn	6\20\96	13.1	1370	0.20	<.6	15.9	0.08	<.1	2.20	<.6	1980	0.007	506	38.1	<.3	2.0	3.0	0.20	1.9	3.5	10.0
Love Lk	8\8\96	76.2	20700	1.80	4.9	199.0	0.92	<.2	10.00	12.0	14000	0.023	2910	287.0	0.6	6.1	20.0	0.44	64.4	42.0	52.6
Mt. Bethel	6\24\96	49.1	7030	0.71	1.0	80.2	0.32	<.2	13.00	13.0	13500	0.013	2220	176.0	2.5	8.8	15.0	0.40	10.6	20.0	45.6
Herman Gich.	6\24\96	49.3	27900	2.80	2.9	354.0	1.10	0.30	29.00	39.0	37400	0.052	5350	924.0	3.4	16.0	70.0	0.30	44.5	44.0	139.0
HESBO	6\27\96	45.4	7520	6.00	<6	128.0	22.00	4.90	<5	22.0	9460	0.017	3340	70300.0	75.0	20.0	100.0	<.2	1010.0	<7	1700.0
Montezuma	6\13\96	43.3	12400	6.30	<.6	227.0	0.77	2.80	20.00	34.0	19500	0.023	3660	543.0	1.2	14.0	250.0	0.46	13.0	27.0	788.0
Mt Bellvw	7\12\96	58.6	8900	15.00	2.8	60.5	1.30	0.98	11.00	27.0	24500	0.033	3240	330.0	4.6	26.0	33.0	11.00	29.2	28.0	83.3

Highest metal concentrations in sediments were found at three sites; the Georgetown site, the 2 Pond site, and the HESBO site. Of 31 total sediment samples, the sample collected at the Georgetown site contained highest concentrations of mercury (0.459 ug/g) and lead (300 ug/g). The 2 Pond and the HESBO sites in Clear Creek county contained relatively high concentrations of manganese, molybdenum, cadmium, strontium, and zinc. The Trl 401 site in Gunnison county contained relatively high concentrations of cadmium (8.4 ug/g) and zinc (834 ug/g). And the Montezuma site in Summit county contained relatively high concentrations of copper, lead, and zinc. Some background metal concentrations are listed in Table 4. Copper concentrations exceeded background in 21 out of 31 total sediment samples. Other metals in which some of the 31 sediment samples exceeded background concentrations include; lead (8), zinc (7), cadmium (6), and arsenic (3).

Table 4. Pre-mining metal concentrations in sediment samples collected from the upper Arkansas River drainage basin (Church *et al.* 1993)

Metal/Metalloid	Background[1] (ug/g DW)
Arsenic	<10
Cadmium	<2
Copper	14
Lead	39
Zinc	158

[1] Average values determined from floodplain cores

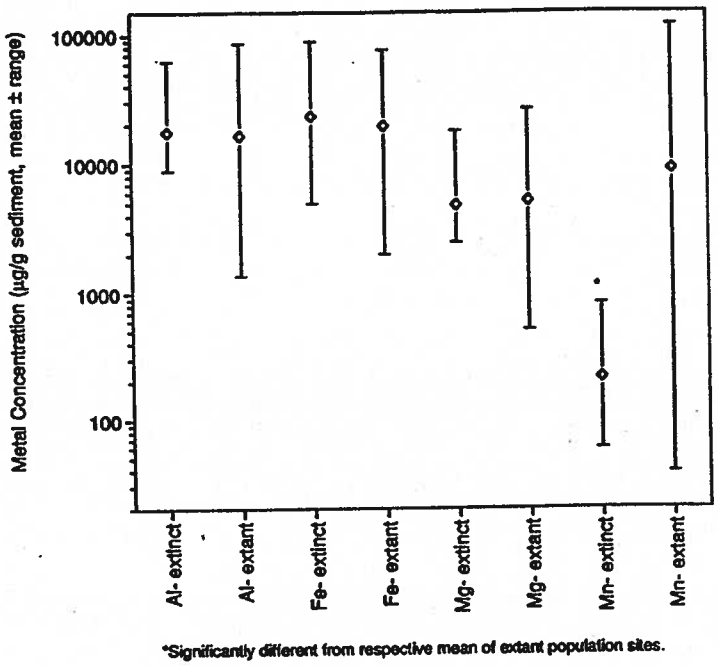
Average ± SD concentrations of metals for extant and extinct boreal toad breeding sites are shown in Table 5. Considerable variation in sediment concentrations existed among sites and considerable overlap in concentrations exists among sites (Fig. 2). Statistical comparisons of concentrations for each metal were done on log-transformed data because the values were not normally distributed. Although differences between means were small, concentrations of cadmium, arsenic and selenium were found to be significantly higher, whereas levels of manganese are significantly lower, in ponds in which boreal toads are now extinct compared to extant sites (Fig. 2). Cadmium, arsenic and selenium all have recognized toxic effects in mammals, but their toxicity to eggs, larvae or metamorphosed amphibians is as yet unclear. The concentrations of these three metals in the sediments would not be expected to be associated with water concentrations high enough to cause acute lethality, at least based on the current literature (Ramsdell, pers. comm). This observation, however, does not rule out the possibility of subacute effects such as immunosuppression.

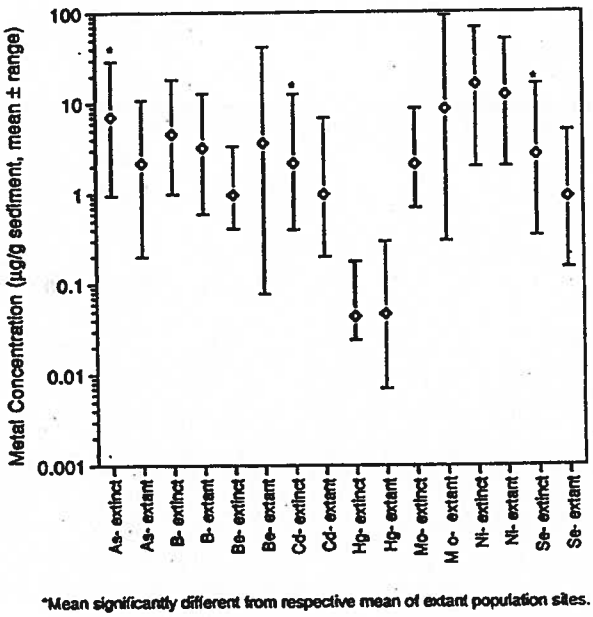
Chlorinated hydrocarbon analyte concentrations were below detection limits for sediment samples collected during 1994 from boreal toad sites (Table 6). Thus,

Table 5. Trace element concentrations in sediment collected at current and former Boreal toad sites in Colorado during 1994-1996. Concentrations are expressed in µg/g dry weight.

	Extant sites		Extinct sites	
	Mean ± std. dev.	Min. to Max	Mean ± std. dev.	Min. to Max.
Al	16423 ± 11803	1370 to 53000	17588 ± 6600	8900 to 27400
As	2.18 ± 1.94	0.2 to 6.6	7.14[a] ± 6.12	0.96 to 15
B	3.25 ± 1.88	0.6 to 6.5	4.63 ± 2.92	1 to 0.93
Ba	162.8 ± 96.8	15.9 to 354	152.6 ± 72.4	60.5 to 297
Be	3.64 ± 8.73	0.078 to 34.7	0.98 ± 0.39	0.41 to 1.4
Cd	0.985 ± 1.22	0.2 to 4.9	2.17[a] ± 2.56	0.4 to 8.4
Cr	19.2 ± 22.2	2.2 to 103	15.1 ± 6.59	3.4 to 24
Cu	33.6 ± 33	0.6 to 146	23.8 ± 15.6	3.8 to 59
Fe	19207 ± 10832	1980 to 37400	22878 ± 11828	4900 to 42900
Hg	0.047 ± 0.048	0.007 to 0.2	0.044 ± 0.023	0.024 to 0.089
Mg	5124 ± 3259	506 to 15450	4709 ± 1923	2440 to 8180
Mn	8880 ± 26723	38.1 to 10100	211[a] ± 115	58.7 to 392
Mo	8.57 ± 19.2	0.3 to 75	2.1 ± 1.53	0.7 to 4.6
Ni	12.4 ± 7.83	2 to 26	16.3 ± 9.92	2 to 35
Pb	45.4 ± 53.1	3 to 250	37.4 ± 20.5	9 to 86
Se	0.913 ± 0.88	0.15 to 3.1	2.66[a] ± 3.25	0.34 to 11
Sr	174.5 ± 397	1.9 to 1530	54.8 ± 39.5	18.9 to 143
V	36.3 ± 25.5	3.5 to 108	42.6 ± 17.8	16 to 72
Zn	396 ± 834	10 to 3420	190.1 ± 250	47.5 to 834

[a] Significantly different from the respective mean of extant site samples, $p \leq 0.05$, Student's t, calculated using log-transformed data.





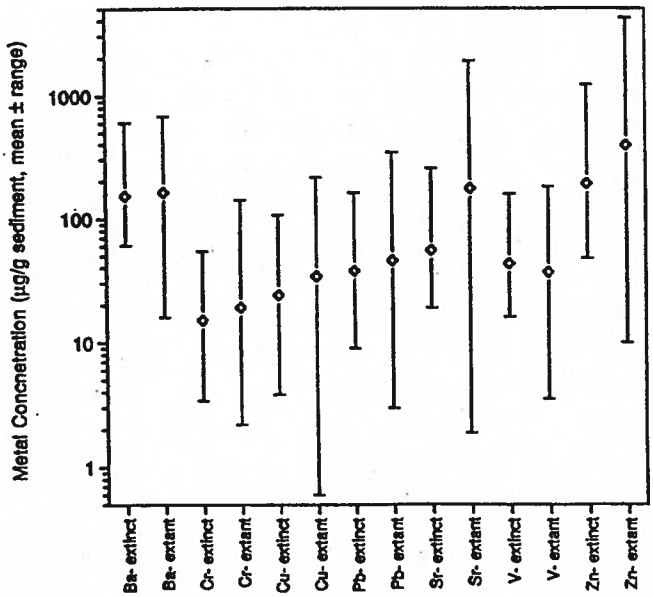


Figure 2. Comparison of metal concentrations in sediment between sites where boreal toad populations have gone extinct, and where they still occur.

Table 6. Chlorinated hydrocarbon analytes and detection limits for sediment and tissue samples from boreal toad breeding ponds.

Collection Sites	County	Status of boreal toads at site	Matrix[a]	Collection Date	Analyte	Detection Limit (µg/g wet weight)
Cunn. Res.[b]	Gunnison	Absent	Sediment	10/01/94	alpha BHC	<0.01
RdRkLk	Boulder	Absent	Sediment	10/15/94	alpha chlordane	<0.01
Tri.Pass	Gunnison	Present	Sediment	10/01/94	beta BHC	<0.01
Grgtown	Clear Creek	Present	Sediment	08/01/94	cis-nonachlor	<0.01
Herman Gulch	Clear Creek	Present	Sediment	08/01/94	delta BHC	<0.01
Caribou	Boulder	Extirpated	Sediment	08/15/94	dieldrin	<0.01
LostLk	Boulder	Present	Sediment	10/15/94	endrin	<0.01
MillCrk	Gunnison	Extirpated	Sediment	10/01/94	gamma BHC	<0.01
MudLk	Boulder	Absent	Sediment	10/15/94	gamma chlordane	<0.01
Cunn. Res.	Gunnison	Absent	Sal.lar.	10/01/94	heptachlor epoxide	<0.01
Denny Crk	Chaffee	Present	BT tad. w/guts	07/31/94	mirex	<0.01
Herman Gulch	Clear Creek	Present	BT tad. w/guts	08/01/94	o,p'-DDD	<0.01
					o,p'-DDE	<0.01
					o,p'-DDT	<0.01
					oxychlordane	<0.01
					p,p'-DDD	<0.01
					p,p'-DDE	<0.01
					p,p'-DDT	<0.01
					toxaphene	<0.05
					trans-nonachlor	<0.01

[a] Matrix-BT=Boreal toad, Sal. lar.=Tiger salamander larvae
[b] Site is salamander die-off location

sediment samples collected after 1994 were not analyzed for chlorinated hydrocarbons.

Tissue and Sediment Samples

Metal concentrations in amphibian tissues collected from boreal toad breeding ponds are presented in Table 7. Large variation in whole body metal concentrations exist between whole boreal toad tadpole carcasses among sites (Table 8). Highest metal concentrations were found in tadpoles from the Georgetown population, which bred in ponds directly below mine tailings. Breeding has not occurred at this site since the tadpoles were collected in 1994. The lead concentration in the Georgetown sediment sample was 300 ug/g and in the boreal toad tadpole sample was 245 ug/g. The background concentration for lead in sediments is about 39 ug/g (Church *et al.* 1993). Both sediment and boreal toad tadpoles taken from the 2 Pond site in Clear Creek county during 1995 contained high concentrations of manganese, molybdenum, strontium, cadmium, and zinc. The sediment sample contained 101,000 ug/g manganese, and the tadpole sample contained >150,000 ug/g manganese.

A significant relationship exists between whole body concentrations of boreal toad tadpoles and the sediment concentrations in the ponds from which the tadpoles were collected (Fig. 3). These values are heavily biased by

Table 7. Trace element concentrations (µg/g dry weight) in amphibian tissues from boreal toad breeding ponds.

Site	Species[a]	Collection Date	% Moisture	Al	As	B	Ba	Be	Cd	Cr	Cu	Fe	Hg	Mg	Mn	Mo	Ni	Pb	Se	Sr	V	Zn
Grgtown	BT tad. w/ guts	8\1\94	93.9	10200	---	3.9	168.0	0.33	2.06	45.90	39.0	14400	0.370	4010	1110.0	<1	12.3	245.0	2.60	29.6	23.0	316.0
Tri. Pass	BT tad. w/ guts	7\10\94	89.7	3400	---	<4	60.0	0.08	0.19	8.40	10.0	2500	0.200	2360	47.0	<5	3.0	9.2	1.70	17.0	10.0	120.0
Herman Gich	BT tad. w/ guts	8\1\94	94.1	7300	0.82	1.0	135.0	0.23	0.09	14.70	18.0	16400	0.190	2370	115.0	3.0	4.7	7.8	1.10	26.1	13.0	64.9
Denny Crk	BT tad. w/ guts	7\31\94	98.8	6470	0.53	<.9	125.0	0.18	0.22	22.40	9.8	10700	0.130	2540	148.0	<1	4.6	6.1	2.00	29.8	15.0	61.6
Kronke	BT tad. guts	8\24\95	84.3	8460	2.60	6.7	95.2	0.32	2.10	10.00	36.8	15000	0.130	2320	248.0	2.0	8.2	23.7	1.60	61.3	15.0	118.0
Kronke	BT tad. guts	8\24\95	88.2	8250	2.60	27.0	92.9	0.28	1.80	7.40	36.9	14500	0.130	2300	225.0	1.0	7.3	22.5	1.60	60.2	15.0	120.0
Kronke	BT tad. w/o guts	8\24\95	93.5	620	<.4	17.0	27.2	0.06	0.24	2.50	12.1	1180	0.068	2260	21.5	0.7	2.7	2.2	1.10	23.8	0.8	106.0
Kronke	BT tad.w/o guts	8\24\95	93.2	370	0.40	0.7	24.1	<.05	0.23	1.80	10.0	724	0.065	2060	72.2	<.3	3.9	1.3	1.30	21.0	0.7	88.9
Kronke	BT tad. w/ guts	8\24\95	93.3	2580	1.00	2.3	41.7	0.10	0.76	4.90	21.0	4590	0.120	2120	76.1	0.8	2.7	9.3	1.50	29.8	4.8	96.7
Pwr Alley	BT (M)	7\24\95	75.2	630	0.50	<.7	22.5	0.12	1.10	1.90	18.0	832	0.110	1420	278.0	1.0	0.5	7.4	0.88	73.9	<.5	189.0
2 Pond	B T (M)	7\24\95	79.2	48	<.3	<.7	18.3	0.29	0.63	0.60	8.8	207	0.079	1420	1580.0	1.0	1.2	7.7	0.60	136.0	<.5	164.0
2 Pond	BT tad. w/o guts	7\24\95	78.6	1800	<.4	<8	79.0	11.60	5.72	2.70	7.9	870	<.03	2710	157000.0	25.0	12.3	13.6	<.2	632.0	<20	2630.0
2 Pond	BT tad. guts	7\24\95	81.5	1900	<2	<5	73.0	13.00	5.70	2.50	8.2	910	<.2	2200	176000.0	26.0	13.0	12.2	<1	646.0	<10	2540.0
Chatfield	WH	6\1\95	77.0	675	0.40	2.8	33.9	0.06	0.72	2.20	13.4	544	0.100	1330	72.1	<.6	1.0	1.0	2.10	109.0	<1	110.0
Mag. Pond	Sal (f)	6\4\95	82.1	399	0.40	<.9	47.3	<.03	1.10	2.40	11.0	586	0.130	1160	26.2	<.6	0.9	3.1	2.00	58.2	<.7	145.0
Mag. Pond	CF	6\4\95	74.4	20	0.80	2.0	51.5	0.06	0.32	0.83	20.9	187	0.220	1630	92.5	<.6	0.4	1.2	1.20	79.1	<1	130.0
Mag. Pond	S. lar.	8\23\95	72.9	40	0.40	1.0	7.1	<.02	0.09	1.20	6.4	150	0.490	1170	4.1	<.6	1.1	0.4	1.30	30.0	<1	107.0
Henderson	BT toadlets	2\14\95	75.5	42	0.70	1.0	12.0	0.03	0.11	1.20	14.7	260	0.100	1370	133.0	<.6	0.9	0.5	1.50	37.3	<1	107.0
Mt. Bethel	BT tad. w/ guts	7\31\95	92.0	4360	0.90	2.6	88.2	0.22	0.36	15.70	17.8	9770	0.015	2100	203.0	2.2	5.9	8.6	0.47	12.1	7.4	73.3
Mt. Bethel	BT tad. guts	7\24\95	82.5	8030	2.00	6.0	168.0	0.36	1.10	25.50	25.9	21400	0.100	2820	375.0	4.7	11.0	16.7	0.90	21.1	16.0	89.0
Mt. Bethel	BT tad.w/o guts	7\31\95	89.4	1880	1.00	2.0	61.2	0.07	3.14	5.20	12.5	4670	0.150	1960	142.0	2.0	3.7	3.9	0.97	10.6	3.0	78.5
Pueblo	WH	8\7\95	66.7	47	<.3	1.0	11.0	<.03	0.51	1.10	4.4	234	0.021	1050	6.9	0.5	0.4	0.4	5.20	55.8	<.5	196.0
Craig's Cab	S. lar.	8\7\95	89.1	140	4.00	9.0	50.0	<.1	0.53	4.40	9.7	290	0.300	1990	78.0	<3	4.7	4.7	1.60	55.6	<9	124.0
Kronke	BT tad. w/ guts	8\24\96	93.3	2580	1.00	2.3	41.7	0.10	0.76	4.90	21.0	4590	0.120	2120	76.1	0.8	2.7	9.3	1.50	29.8	4.8	96.7
Kronke	BT tad. guts	8\24\96	84.3	8460	2.60	6.7	95.2	0.32	2.10	10.00	36.8	15000	0.130	2320	248.0	2.0	8.2	23.7	1.60	61.3	15.0	118.0
Kronke	BT tad. w/o guts	8\24\96	93.2	370	0.40	0.7	24.1	<0.05	0.23	1.80	10.0	724	0.065	2060	72.2	<.3	3.9	1.3	1.30	21.0	0.7	88.9
Kronke	BT tad. guts	8\24\96	88.2	8250	2.60	27.0	92.9	0.28	1.80	7.40	36.9	14500	0.130	2300	225.0	1.0	7.3	22.5	1.60	60.2	15.0	120.0
Kronke	BT tad. w/o guts	8\24\96	93.5	620	<.4	17.0	27.2	0.06	0.24	2.50	12.1	1180	0.068	2260	21.5	0.7	2.7	2.2	1.10	23.8	0.8	106.0
Limon	S. lar.	8\3\96	88.5	95	<.3	<.7	19.3	<.03	<.05	2.20	64.7	171	0.300	1230	6.2	<.4	0.8	0.4	1.90	18.2	<.5	73.1

[a] Species-BT=Boreal toad, WH=Woodhouse toad, CF=Chorus frog, S=Salamander

Table 8. Concentrations of trace elements (µg/g dry weight) in boreal toad tadpoles (whole body) collected from Grgtown, Tri Pass, Herman Gulch, Denny Crk, Kronke, and Mt. Bethel during 1994.

	mean ± std. dev.[a]	Min. to Max.
Al	5296 ± 3506	48 to 10200
As	0.64 ± 0.28	0.30 to 0.90
B	2.18 ± 1.53	0.70 to 4.0
Ba	99.1 ± 54.6	18.3 to 168
Be	0.35 ± 0.16	0.2 to 0.6
Cd	0.59 ± 0.74	0.09 to 2.06
Cr	18.0 ± 15.5	0.6 to 45.9
Cu	17.2 ± 11.4	8.8 to 39.0
Fe	8996 ± 6434	207 to 16400
Hg	0.047 [b]	0.015 to 0.079
Mg	2467 ± 853	1420 to 4010
Mn	534 ± 648	47 to 1580
Mo	2.2 ± 1.6	1.0 to 5.0
Ni	4.78 ± 3.9	1.2 to 12.3
Pb	10.6 ± 6.9	6.0 to 24.5
Se	1.41 ± 0.83	0.47 to 2.6
Sr	42 ± 47	12 to 136
V	11.5 ± 7.6	0.5 to 23.0
Zn	133 ± 97.9	61.6 to 316

[a] n=6
[b] Detected in only 2 of 6 samples.

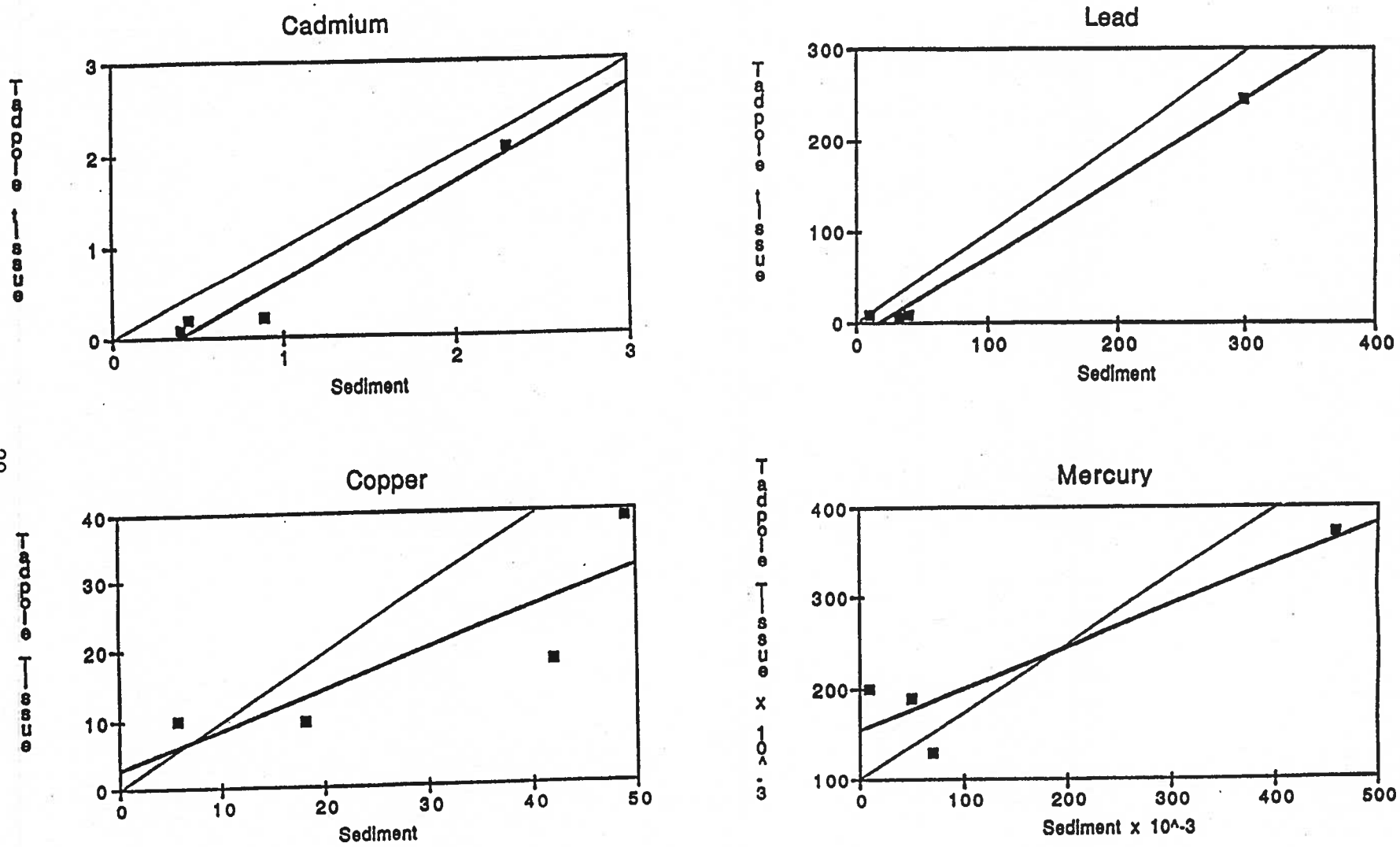


Figure 3. Correlation between metal concentrations in boreal toad tadpole tissue and sediment. The thin line is the equality line and the thick line is the regression line.

the gut contents, but these data show higher sediment metal concentrations of cadmium, lead, mercury, and copper result in higher exposure to metals through the gut. Because tadpoles feed on plant materials on the substrate, they are highly susceptible to metal contamination through feeding. Correlations for whole-body tissue levels and sediments are given for all metals in Table 9. In five out of six samples, the tadpoles and sediment were collected on the same day; in the sixth case, collections were made 5 weeks apart. Strong positive correlations existed between Al, Ba, Cd, Cr, Cu, Fe, Mg, Mn, Se, Sr, and Va. Too few analyses of water samples were collected concurrently with tadpole to test whether a correlation existed between tadpole whole body metal concentrations and water metal concentrations.

The sediment-carcass bioconcentration factors (mean ratio of carcass to sediment) for trace elements are also shown in Table 9. A value greater than one indicates a propensity of the organism to concentrate the metal in its tissues relative to the sediment. Values greater than one were observed only for selenium and mercury. In some samples of whole-bodies, high BCF values for beryllium and molybdenum also occurred, but the mean for all samples did not rise above one. If these values are omitted from the calculation, mean BCF's of 0.52 are obtained for these metals. Although calculation of BCF

Table 9. Correlation between sediment and tissue trace element concentrations for boreal toad sites.

Trace element	r[a]	Bioconcentration factor[b]
Al	0.73	0.32
As	-0.84[c]	0.82
B	-0.14	0.96
Ba	0.74	0.56
Be	-0.19	1.43
Cd	0.72	0.64
Cr	0.88	0.92
Cu	0.78	0.88
Fe	0.8	0.44
Hg	[d]	3.05
Mg	0.88	0.62
Mn	0.79	0.72
Mo	-0.39	1.47
Ni	0.41	0.39
Pb	0.08	0.49
Se	0.84[e]	2.13
Sr	0.99[e]	0.59
V	0.98[e]	0.34
Zn	0.24[e]	0.73

[a] Correlation coefficient calculated from least squares regression analysis. n=6 unless indicated otherwise
[b] Mean ratio of carcass to sediment concentration. (n was same as for correlation)
[c] n=4
[d] n=2
[e] n=5

relative to water samples would be more meaningful, the lack of sufficient water samples collected concurrently with tadpoles from each location rule out this analysis.

Only two collections of tadpoles were made in which guts were analyzed separately from the rest of the tissue at sites at which sediment samples were also taken. That small sample size precludes a meaningful correlation analysis between sediment and gut content, or sediment and the remaining carcass. For the four samples in which both guts and remaining carcass tissue were analyzed, significant correlations existed between the gut and remaining tissue concentration (Table 10). A perfect positive correlation between gut and remaining carcass tissue concentrations existed for Be, Mn, Mo, Sr and Zn. This correlation means that these metals are deposited in tissue at the same rate as they are taken into the gut.

Similar to sediment samples, all amphibian tissue samples collected during 1994 from boreal toad sites contained chlorinated hydrocarbon analyte concentrations below detection limits.

Table 10. Correlation between tadpole GI tract and remaining carcass trace element concentrations.

Trace element	r[a]
Al	-0.58
As	-0.58
B	0.87
Ba	0.1
Be	1
Cd	0.74
Cr	0.85
Cu	0.66
Fe	0.66
Hg	-0.85
Mg	-0.72
Mn	1
Mo	1
Ni	0.83
Pb	-0.91
Se	0.66
Sr	1
V	-0.96
Zn	1

[a] Correlation coefficient calculated from least squares regression. n=4

Metal Concentrations in Water Collected from Breeding Areas of Boreal Toads in Colorado

Of the metals that were screened for in water samples (Cu, Cd, Fe, Mn, Pb, and Zn), only iron and manganese were typically detected in most of the samples from a given site (Table 11). Copper was detected in water samples from most sites. Copper concentrations were at or below 2.7 ug/L at all sites except HESBO, where the mean copper concentration was 16 ug/L. The EPA chronic criterion for protection of aquatic life is 9 ug/L at 100 mg/L hardness (USEPA 1998). Cadmium and lead were infrequently detected and all concentrations were below the lethal concentrations (LC_{50}'s) given in Table 11. The EPA chronic criterion for protection of aquatic life for lead is 2.5 ug/L at 100 mg/L hardness (USEPA 1998). Water samples collected at Cucumber Gul. and Denny Cr.exceeded this lead criterion. Zinc concentrations in water samples ranged from 40.4 to 237 ug/L. The highest mean zinc concentration was found at Montezuma, but even this concentrations was below those that would be expected to cause acute toxicity to amphibian larvae (see references in Table 12). However, the EPA acute and

Table 11. Concentrations of trace elements in water samples (µg/L) collected at boreal toad sites during 1995.

Site	n[a]	Cu	Cd	Fe	Mn	Pb	Zn
Browns Cr	6	2.7 ± 1.6[b] 3[c]	nd[d]	80.3 ± 24.5 6	nd	nd	10 1
Cucumber Gul.	6	2.4 1	0.2 1	322 ± 269 6	47.0 ± 17.0 2	3.7 1	34 1
Montezuma	5	2.5 ± 0.9 3	0.40 ± 0.04 3	BTpub6 5	272 ± 113 5	1.8 ± 0.7 2	237 ± 78.5 5
N. Ten Mile	8	1.8 ± 0.4 2	nd	617 ± 190 8	31.4 ± 8.0 5	nd	nd
Denny Cr.	7	1.5 ± 0.4 2	nd	222 ± 212 7	37 1	3.9 1	nd
Hartenstein L.	6	2.5 ± 0.6 3	0.15 ± 0 2	112 ± 31 6	nd	nd	12 1
Kroenke L.	5	2.2 ± 1.3 2	nd	1314 ± 468 5	35.0 ± 19.8 5	nd	10 1
2 Pond	15	1.1 ± 0.2 3	nd	74.0 ± 31.1 2	3926 ± 4331 15	nd	34.7 ± 21.9 12
Kettle	1	nd	nd	nd	20	1	nd
HESBO	17	16 ± 29 4	0.1 1	252 ± 146 3	3087 ± 3837 17	nd	40.4 ± 40.8 14
Herman	1	nd	nd	1521	241	nd	15

[a] Total number of water samples analyzed, usually from different collection dates.
[b] Mean ± standard deviation of values exceeding the detection limit. When only a single value is presented, only one sample had a concentration above the detection limit.
[c] Number of samples with values exceeding the detection limit.
[d] Not detected in any of the samples from this site.

chronic criterion for the protection of aquatic life is 120 ug/L at 100 mg/L hardness (USEPA 1998), of which the Montezuma site exceeded.

Table 12. List of references which evaluate toxicity of metals to amphibians.

Metal	LC_{50} (mg/L)	References
copper	0.32 - 5	Rao and Madhyastha, Toxicol. Lett. 36: 205, 1987; Khangarot and Ray, Bull. Environ. Contam. Toxicol. 38: 523, (1987)
cadmium	2 - 12	Canton and Sloff, Ecotoxicol. Environ. Safety 6: 113, 1982
iron	>20 mg/l	Porter and Hakanson, Copeia 2: 327 1976
lead	~1	Dilling and Healey, Ann. Appl. Biol. 13: 177, 1926
zinc	20-26	Rao and Madhyastha, Toxicol. Lett. 36: 205, 1987; Khangarot and Ray, Bull. Environ. Contam. Toxicol. 38: 523, (1987)

Concentrations of manganese were below 500 ug/L at most sites. Much higher concentrations of manganese were observed at 2 Pond and HESBO. Mean manganese concentrations at 2 Pond and HESBO were 3926 and 3087 ug/L, respectively. It should be noted that samples collected at these two sites prior to July 18, 1995 had low (2 Pond) or non-detectable (HESBO) concentrations whereas the samples obtained after this time had very high concentrations of manganese. Manganese concentrations at other sites were more consistent during the summer. These observations suggest that a release of manganese-bearing water into these ponds occurred after July 18, 1995. It should be noted that these two sites, which are

within the Henderson Mine complex, had manganese concentrations that were one to two orders of magnitude higher than other sites.

Manganese was the only metal for which water concentration data were available that might generate concern regarding boreal toad embryo/larvae toxicity. The mean concentrations observed at HESBO and 2 Pond exceeded amphibian embryo lethal levels (1.4 mg/l; Birge 1979). The highest levels occurred in late summer when tadpole exposure might be of great concern, right before metamorphosis. A 96-h LC_{50} value for the Ornate rice frog (*Microhyla ornata)* of 15 mg/l has been reported (Rao and Madhyastha 1987). The 7-day LC_{50} for the Eastern narrow-mouth frog (*Gastrophryne carolinensis)* embryos exposed for 96 hr post-hatch was 1.4 mg/l (Birge et al. 1979). Since manganese was significantly lower in historical boreal toad habitat than in extant (due to the very high concentrations at HESBO and 2 Pond), manganese not thought to have contributed substantively to boreal toad die-offs.

Water concentrations for other metals in water do not approach LC_{50}'s for amphibian larvae (Table 11). The acute toxicity of cadmium to amphibian larvae ranges from 2-12 mg/l

(Canton and Sloff, 1982). For two-day old embryos of the African clawed frog (*Xenopus laevis)*, the LC50 was 1.5 mg/l, whereas the EC50 for inhibition of embryonic development was 0.65 mg/l (Canton and Sloff, 1982). In contrast, the LC_{50} for Eastern narrow-mouth frog embryos (exposed for 96 hr post-hatch) was 0.04 mg/l (Birge et al. 1979). The LC_{50} of selenium (as selenite) to *Xenopus* larvae was 8 mg/l. Concentrations of selenium in excess of 2 mg/l caused a high incidence of malformations in *Xenopus* larvae (Browne and Dumont, 1979). Selenium was much more toxic to *Gastrophryne carolinesis* embryos, with an LC_{50} of 0.09 mg/l. Arsenic also appears to have relatively low acute toxicity. An LC_{50} (96 hr) of 123 mg/l has been reported for sodium arsenate in cane toad (*Bufo marinus) tadpoles* (Johnson 1976). This concentration corresponds to an arsenic concentration of 71 mg/l. Therefore, one can conclude that, whatever the historical levels of metals in water were during the mass die-offs of boreal toads, current metal concentrations in extant boreal toad breeding ponds pose no obvious risk, unless they are exerting synergistic effects or sublethal effects that are currently undescribed. Note, however, that these values are for embryos, while the cause of boreal toad extinctions was mortality of metamorphosed individuals.

LC50's for metal exposures of adult amphibians are unavailable. Furthermore, note that these values are for single metal tests, whereas amphibians in the field are most likely exposed to a combination of metals (Fig. 2). The effects on adult amphibians of exposure to mixtures of metals have not been evaluated.

Correlations between Water and Sediment Metal Concentrations at Non-die-off sites.

Correlations analyses of water and sediment metal concentrations were performed at 10 sites with both types of data (Table 13). Concentrations were poorly correlated for copper, lead and zinc. Strong correlations were observed for manganese, cadmium and iron. However, it should be noted that sediment and water samples were not collected at the same time.

Conclusions Regarding Metal Data for Boreal Toads.

Small sample sizes and the lack of coordination between sediment and tissue preclude certain analyses. However, these data seem to suggest that acutely toxic concentrations

Table 13. Correlation between sediment and water metal concentrations for boreal toad sites.

Metal	r[a]
Cadmium	0.82
Copper	-0.54
Iron	0.8
Manganese	0.99
Lead	0.12
Zinc	0.06

[a] Correlation coefficient calculated from least squares regression analysis

of heavy metals did not exist during the years that boreal toad populations were experiencing mass-die offs in the Colorado Rockies. However, synergistic effects of combinations of metals and/or interaction between metals, low pH, cold temperatures, etc. may have caused immunosuppression or changes in the pathogen mix of soil/water fungi, viruses, or bacteria in a manner that could have induced disease.

The data also suggest that HESBO and 2 Pond may have concentrations of Mn at certain times during larval development that could be lethal or have lasting sublethal effects. If populations of toads have not been removed from these areas, this step could be considered as a possible

management strategy. Furthermore, if breeding ever occurs at the Georgetown site again, eggs/tadpoles should be removed to another location because of the extremely high levels of metals at that site.

Concentrations of Metals in Sediment and Salamander Tissues in Breeding Areas of Tiger Salamanders in Colorado

Sediment

With one exception, trace element concentrations were similar between salamander die-off and non-die-off sites (Table 14). Only mean strontium concentrations in sediment were significantly higher in die-off versus non-die-off sites. Strontium concentrations in the four die-off ponds ranged from 36.4-73.6 ug/g, while concentrations from the non-die-off sites were all lower, ranging from 12.5 to 22.8 ug/g. The toxicological significance of this finding is unclear, because so few data exist on effects of strontium exposure on amphibians. The only existing report is an LC_{50} value of 160 ug/l for 96 hr exposure levels of Eastern narrow-mouthed frog (Gastrophryne carolinensis) embryos (Birge et al. 1979). Effects on metamorphosing amphibians (the forms that experienced mortality) are unknown.

Table 14. Trace element concentrations (µg/g dry weight) in sediment samples collected at salamander sites in Colorado during 1994-1996.

	Non-die-off sites (n=3)		Die-off sites (n=4)	
	Mean ± std. dev.	Min. to Max.	Mean ± std. dev.	Min. to Max.
Al	8117 ± 6616	1140 to 14300	14138 ± 8803	2650 to 24100
As	1.14 ± 0.27	0.83 to 1.3	1.8 ± 0.9	0.6 to 2.7
B	3.4 ± 2.34	2.0 to 6.1	1.88 ± 1.36	0.7 to 3.5
Ba	89.3 ± 14.3	72.8 to 99	216 ± 123	46.3 to 329
Be	0.89 ± 0.57	0.27 to 1.4	0.86 ± 0.56	0.10 to 1.4
Cd	0.29 ± 0.25	0.10 to 0.58	0.45 ± 0.21	0.21 to 0.71
Cr	8.9 ± 4.5	5.5 to 14	12.2 ± 5.78	7.7 to 20
Cu	13.1 ± 10.3	7.2 to 25	12 ± 8.5	4 to 23
Fe	12436 ± 10116	6050 to 24100	16015 ± 8127	5460 to 25100
Hg	0.032 ± 0.016	0.018 to 0.049	0.029 ± 0.013	0.02 to 0.038
Mg	3223 ± 739	2560 to 4020	4410 ± 3035	710 to 7290
Mn	282 ± 259	72.1 to 571	203 ± 151	28.8 to 380
Mo	0.73 ± 0.49	0.40 to 1.3	0.63 ± 0.24	0.3 to 0.8
Ni	7.77 ± 3.67	5.5 to 12.0	8.9 ± 5.16	4.4 to 16
Pb	24.0 ± 13.7	12.0 to 39.0	21.5 ± 14.2	6.0 to 40
Se	0.79 ± 0.46	0.4 to 1.3	0.79 ± 0.50	0.20 to 1.3
Sr	18.6 ± 5.4[a]	12.5 to 22.8	51.5 ± 16.1[a]	36.4 to 73.6
V	23.0 ± 5.6	17.0 to 28.0	23.4 ± 16.3	0.7 to 37.0
Zn	44.0 ± 8.0	36.8 to 52.6	98.3 ± 55.9	44.5 to 172

[a] Significantly different, $p<0.01$, t-test of log-transformed

Tissues

In most cases, average metal concentrations were higher in carcasses from non-die-off sites than from carcasses from die-off sites, with the notable exception of strontium and vanadium (Table 15). Small sample sizes preclude statistical comparisons. The toxocological significance of these findings *is* unknown.

Correlations of Tissue and Sediment of Metal Concentrations for Salamander Samples

Correlations were performed using metal concentrations from sites with data for both sediment and tissue (Table 16). Because only two sets of values were available from sites where salamander die-offs have occurred, these could not be examined separately. When all sites are included in the analysis, the strongest positive correlations were observed for Mn, Se, and Sr. Stronger correlations were generally observed when only non-die-off sites were analyzed, but the value of an analysis containing only 3 samples is dubious anyway. In fact, several metals exhibited negative correlations when only non-die-off sites were analyzed, whereas positive correlations existed when all five data points were included. The only metals for which strong

Table 15. Trace element concentrations (µg/g dry weight) in tiger salamander tissues collected in Colorado during 1994 to 1996.

	All sites[a] (n = 5)	Non-die-off sites [a] (n = 3)	Die-off sites [b] (n = 2)
Al	255 ± 143	336 ± 68	25-240
As	0.33 ± 0.07	0.33 ± 0.06	<0.4-0.24
B	1.02 ± 0.56	1.13 ± 0.75	0.7-1.0
Ba	29.1 ± 14.6	35.0 ± 17.2	18.3-22.1
Be	0.054 ± 0.027	0.40 ± 0.010	<0.01-<0.05
Cd	0.248 ± 0.289	0.38 ± 0.32	0.048-0.06
Cr	2.14 ± 0.75	2.07 ± 0.12	1.2-3.3
Cu	7.47 ± 3.13	9.73 ± 0.25	3.5-4.7
Fe	531 ± 392	749 ± 347	108-301
Hg	0.112 ± 0.063	0.13 ± 0.06	0.047-0.06
Mg	1178 ± 116	1140 ± 140	1190-1280
Mn	46.6 ± 52.3	62.3 ± 65.6	7.3-38.8
Mo	0.50 ± 0.28	0.60 ± 0.35	<0.3-0.4
Ni	0.51 ± 0.18	0.59 ± 0.04	0.2-0.6
Pb	1.03 ± 1.08	1.57 ± 1.10	0.13-0.31
Se	1.25 ± 0.30	1.30 ± 0.26	0.55-1.5
Sr	43.6 ± 28.4	24.4 ± 5.5	73.6-86.5
V	6.54 ± 13.1	0.67 ± 0.29	0.7, 0.7
Zn	125 ± 92.4	167 ± 101	79.9-172

[a] Mean ± standard deviation
[b] Range

positive correlations existed were observed in both cases were Mn and Se.

Table 16. Correlation between trace element concentrations in sediment and Tiger salamander tissues collected in Colorado during 1994-1996.

Trace	All sites		Non-die-off sites
	(n = 5)		(n = 3)
Al	-0.63[a]		0.8
As	-0.21		0.5
B	-0.48		-0.52
Ba	-0.59		-0.99
Be	0.30		0.64
Cd	-0.58		-0.32
Cr	-0.36		-0.66
Cu	-0.30		-0.12
Fe	0.07		0.999
Hg	0.32		0.69
Mg	0.44		0.23
Mn	0.87		0.98
Mo	-0.50		-0.59
Ni	-0.76		0.24
Pb	0.21		0.81
Se	0.84		0.88
Sr	0.75		-0.998
V	-0.85		0.16
Zn	-0.69		-0.87

[a] *Values represent correlation coefficients (r)*

Conclusions about Metal Data for Salamander Die-offs

The use of sediment data to predict salamander tissue concentrations is probably not warranted in future studies. Since salamander larvae feed largely on aquatic insects rather than on plant material on the substrate as boreal tadpoles do, it is unlikely that they have much opportunity to absorb metals from the substrate.

The effect of exposure to Sr and Va on immune function in amphibians and on water-borne pathogens needs further evaluation to determine if these correlations might represent a potentially causal agent in salamander die-offs.

Metal Exposure Affects on Amphibian Immune Function

Unfortunately, exposure to 5C for 6 weeks during the summer was completely immunosuppressive to the frogs with the result that no lymphocytes were present in the blood nor was complement above detectable levels. Because time and financial support was limited for this study, it was impossible to rerun this test at a warmer temperature.

Conclusions about Immunosuppression in Frogs

Exposure to 5C for 6 weeks was completely immunosuppressive to frogs during this study. Thus, we were unable to determine immunosuppressive effects from low pH or metals. Future studies that investigate immunosuppression in amphibians should be run at warmer temperatures. Hopefully, the results from this study provide some foundation for more feasible, and hopefully successful studies.

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