FINAL REPORT

The Effects of Ammonium Perchlorate on Reproduction and Development of Amphibians

SERDP Project ER-1236

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Table of Contents

Page

List of Tables

Page

List of Figures

Acronym List

AP – Ammonium Perchlorate

EDC – endocrine disrupting chemical

FETAX – Frog Embryo Teratogenesis Assay-Xenopus

IPR – In Progress Review

K – Kalium

KI – Potassium Iodide

Mg – Magnesium

Na – Natrium (Sodium)

NIEHS – National Institute of Environmental Heath Sciences

SAB – Scientific Advisory Board

SERDP – Strategic Environmental Research and Development Program

UV – ultraviolet

Acknowledgements

On October 2, 2001, Dr. James Dumont, of the Department of Zoology at the Oklahoma State University, initiated a Strategic Environmental Research and Development Program (SERDP) project entitled *The Effects of Ammonium Perchlorate on Reproduction and Development of Amphibians* (ER-1236). Unfortunately, due to the sad and untimely passing of Dr. Dumont, this project was not completed as envisioned. The following document is a summary of the research that has been conducted to date on this project.

Executive Summary

The objectives of this SERDP project were to examine the long-term effects of perchlorate on developing amphibians (e.g., growth, metamorphosis) and on the general health and reproductive capacity of adult females. The studies examined the effects of perchlorate present in the water as well as perchlorate available through the food chain. Because perchlorate competes for iodine binding sites in the thyroid, the addition of iodine to culture water was examined to determine if perchlorate effects can be mitigated. Finally, perchlorate is known to affect normal pigmentation of amphibian embryos. The effects of ultraviolet (UV) irradiation on pigment-altered embryos were examined.

In range-finding tests, it was observed that concentrations of 1 mg/L were sufficient to inhibit or at least delay metamorphosis.

It appears that iodine is capable of blocking or overcoming the effects of ammonium perchlorate within certain concentration ranges but there appear to be species differences to this response. Further studies should be conducted to determine threshold concentrations of iodine. Long-term exposure of Xenopus embryos to AP concentrations above 100 mg/L is toxic and embryos at these concentrations both with and without iodine died after 80 days exposure. There was no significant difference in the snout-to-vent lengths of any of the newly metamorphosed froglets. *Hyla* sp. general response to treatment with potassium iodide and perchlorate appears to be similar to that of *Rana*, however, the *Hyla* appear to be much more sensitive and fragile as they reach metamorphic climax. As a result, mortality rates become high.

For the reproductive capacity of females, fecundity was reduced by 50% in test groups exposed to 2000 mg/L but was not significantly affected in other groups (10 to 1000 mg/L). Fertilization rates, however, were reduced by 3 to 18% in the exposed groups compared to the control animals while the percent of the fertilized eggs that reached hatching was reduced by 14 to 19% in the groups receiving perchlorate.

In addition to reduction in reproductive capacity, there may be trans-generational effects that influence normal development of the early embryos. High concentrations of perchlorate observed in the ovary suggest that some perchlorate may be incorporated directly into developing oocytes and hence eventually transferred to the embryos. Alternatively, low thyroxin levels in the females may be reflected in levels potentially present in the oocytes. In either case this raises a concern that the impact of perchlorate exposure may extend to the F_1 generations.

1.0 Objective

The objectives of this SERDP project were to examine the long-term effects of perchlorate on developing amphibians (e.g., growth, metamorphosis) and on the general health and reproductive capacity of adult females. The studies examined the effects of perchlorate present in the water as well as perchlorate available through the food chain. Because perchlorate competes for iodine binding sites in the thyroid, the addition of iodine to culture water was examined to determine if perchlorate effects can be mitigated. Finally, perchlorate is known to affect normal pigmentation of amphibian embryos. The effects of UV irradiation on pigment-altered embryos were examined.

This project provides basic information on the effects of perchlorate on developing amphibians and on its effects on the reproductive capacity of adult females. The data is useful for evaluating environmental risks from perchlorate-contaminated surface and ground water and, in addition, provides guidance to organizations that must use or dispose of ammonium perchlorate stockpiles. The use of a variety of native amphibian species allows inter-species comparisons of perchlorate sensitivity.

The effects of ammonium perchlorate on developing *Xenopus* embryos that were exposed for four days in a FETAX tests. The embryo at the top of the photo is a control. Those below it were exposed to 500, 300, 200, and 100 mg/L of ammonium perchlorate. Severe malformations are noted at 500 mg/L. The severity decreases in lower concentration. At 300 mg/L there is notable malformation of the head and brain and an abnormal curvature of the tail. All exposed embryos do not achieve normal growth.

2.0 Background

Ammonium perchlorate (AP) has applications in munitions, primarily as an oxidizer for solid rocket, missile propellants and pyrotechnic devices. It is also used as an air-bag inflator in the automotive industry, in fireworks, and is a component of agricultural fertilizers, feed additives, herbicides and a variety of manufacturing processes. Because of these uses and its high solubility, chemical stability, and persistence, it has become widely distributed in surface and ground water systems particularly in the southwestern United States, and, it is difficult to remediate.

There is little information about the effects of perchlorate in these systems on the aquatic life that inhabits them. It is a prevalent component in some human water sources and is thought to be the cause of some developmental abnormalities. Perchlorates are known to inhibit thyroxin production by competitively blocking iodine uptake by the thyroid; for example, perchlorate is an endocrine disrupting chemical (EDC) that interferes with normal thyroid function and that, in vertebrates, thyroid dysfunction impacts both growth and development. Because of its prevalence and persistence in the environment and its known effects on a wide range of biological processes (e.g., hormones, growth, development, neuro/muscular system, reproduction) perchlorate is potentially a threat not only to human health but also to the health of ecosystems and the organisms that inhabit them.

3.0 Materials and Methods

This project examined the effects of perchlorate on adult female reproduction using the common laboratory amphibian, *Xenopus laevis,* as a surrogate for native species. Adult females were induced to breed and the quality of egg clutches, i.e., number oviposited, percent fertilized, and survivability of resulting embryos, were determined. These adults were then exposed to concentrations of perchlorate for four months−the time required to complete another cycle of vitellogenesis (oocyte development). At the end of the treatment, they were bred again to the same males and again the quality of egg clutches examined. During exposure general health, food consumption, locomotor activity and body weights were recorded. Following exposure the females were necropsied to determine organ health/weights, thyroid histology, and tissues analyzed for perchlorate burden. The effects of long-term exposure of developing embryos (tadpoles) were studied by rearing young embryos of *Xenopus* and local Rana and Bufo species, in concentrations of perchlorate to examine embryo-lethal and abnormal development endpoints. These studies included the ability of such exposed embryos/tadpoles to grow normally and metamorphose−a thyroxin-dependent process. Since perchlorate has been shown to affect pigmentation, treated tadpoles were exposed to UV irradiation to determine the effects on altered pigmentation on UV sensitivity. This parameter has potentially important consequences for survival of young amphibians in contaminated environments and has been related to the worldwide decline of amphibian species. Since some plants, which are important food sources for some amphibian tadpoles, bioaccumulate perchlorate. To address this issue, native amphibian species were reared on perchlorate-laden food (e.g., hydroponically grown lettuce) and their growth and development monitored. Thyroid histology and thyroxin levels and perchlorate body burdens were monitored in treated embryos and adults. Finally, since iodine uptake by the thyroid is reduced in perchlorate-exposed animals, laboratory tests were conducted with culture medium and native contaminated water with Xenopus and native species to determine if increased iodine levels mitigate the effects of perchlorate on this endocrine system.

3.1 Task Structure.

The original task structure of the project is presented in Table 3-1.

Table 3-1. Task Structure.

Task	Description
1	Effects of Continuous Long-term Perchlorate Exposure on Developing Amphibians
1.1	Tests with Xenopus and Native Species
1.2	Tests with Xenopus
1.3	Tests with Native Species
2	Mitigation of Perchlorate Effects by the Addition of Potassium Iodide (KI)
2.1	Range Finding Tests for KI
2.2	Tests with Xenopus
2.3	Tests with Native Species
3	Effects of Perchlorate Derived from Food Sources on Amphibian Development

Table 3.1 (Continued)

3.1	Initiate Lettuce Growth
3.2	Tests with Native Species
4	Pigmentation and Sensitivity to Ultraviolet Radiation
4.1	Tests with Xenopus and Native Species
5	Effects of Naturally Occurring Perchlorate-contaminated Water on Amphibian Development
5.1	Water Procurement and Begin Tests with Native Species
6	Effects of Perchlorate on Reproductive Capacity of Female Xenopus.
6.1	Range Finding for Adult Toxicity
6.2	Initiate Four Month Exposure
6.3	Remate and Assess Reproductive Capacity
7	Effects of Perchlorate on Developing Embryos Following Exposure and Reproduction of Adult Females
7.1	Milestones to be Determined
8	Histology and Thyroxin Analysis
8.1	On-going During Course of Study
9	Final Report to SERDP
9.1	Final Report
Additional Task 1	The Effects of Perchlorate on Male Fertility
Additional Task 2	Examination of Sex Ratios in Metamorphosed Treated and Control Froglets

3.2 Experimental Design Protocol

Task 1: Effects of Continuous Long-term Perchlorate Exposure on Developing Amphibians.

Studies in our laboratory on the effects of perchlorates (ammonium, magnesium [Mg], potassium, and sodium) on early amphibian development have shown that they, and AP in particular, are embryotoxic and significantly inhibit growth in early 96-hr embryos of *Xenopus laevis* and in native amphibian species, e.g. *Rana utricularia* and *Hyla sp.* (Harvey and Dumont, 2000). The sensitivity to ammonium perchlorate displayed by all of these amphibian species is virtually the same. Although overt terata are usually not seen following perchlorate exposure, there is a dramatic abnormal reduction in growth. In all species tested, all of the surviving 96-hr embryos exposed to higher concentration of the perchlorates are smaller that the mean length of control embryos. Failure to thrive is likely to severely reduce survival in native habitats. Since the objective of these early studies was to determine the effects on early development. The effects of long-term continuous exposure to ammonium perchlorate at various concentrations, beginning with early stages (Stage 8-10) and continuing through metamorphosis of *Xenopus* and other available native species were studied.

Task 2: Mitigation of Perchlorate Effects by the Addition of KI.

Perchlorate inhibits the production of thyroxin and thus growth and metamorphosis. The presence of iodine would be expected to mitigate the effects of perchlorate since an abundance of iodide would compete with perchlorate for binding sites in the thyroid. It is important to know what levels of iodide might be required and if these levels are near those that might be found under environmental conditions. Early stage *Xenopus* embryos (and available native species) were exposed to a concentration series of perchlorate and KI by standard Frog Embryo Teratogenesis Assay-xenopus (FETAX) methodology. Prior to testing, a range-finding test for KI was conducted to determine its embryotoxicity.

Task 3: Effects of Perchlorate Derived from Food Sources on Amphibian Development.

Very preliminary tests indicate that *Rana* tadpoles fed perchlorate-laden lettuce grow more slowly that controls. However, the tadpoles were not reared to metamorphosis, and therefore the extent to which perchlorate derived from food effects development, growth, and metamorphosis is unclear. Leaf lettuce was grown in sand under green house conditions and nourished with a hydroponic nutrient medium (30% Hoagland's solution). Near maturity, the plants were watered for two weeks with 150 mg/L ammonium perchlorate added to the Hoagland's Solution to allow bioaccumulation of perchlorate. The plants were harvested and frozen until needed. Local amphibian tadpoles were captured, brought in to the laboratory and reared on a diet of perchlorate-laden lettuce. The tadpoles were photographed and length measurements taken with computer software (SigmaScan®). Control groups consisted of tadpoles collected from the same areas and reared on lettuce that has not been treated with perchlorate. Test animals were anesthetized with MS-222, fixed in Bouin's solution and prepared for histological sectioning to examine the condition of the thyroid gland. Tissue was collected from others and used to conduct T_3 and T_4 analysis (see Task 7).

Task 4: Pigmentation and Sensitivity to UV Radiation.

Alterations in pigmentation have important consequences for the developing embryo, i.e., protective coloration and protection from detrimental effects of UV radiation. Exposure to UV can be managed in the laboratory with an array of calibrated UV lamps and daily doses can be adjusted to mimic those typically found seasonally or geographically (Bruggeman, et al., 1998). Initially, normal *Xenopus* embryos were used to establish a UV dose-response curve and normal pigmentation. Once established, perchlorate-treated embryos were exposed and followed to determine UV sensitivity, i.e., mortality, abnormality and growth with standard FETAX methods. As local native species became available, similar tests were done to determine possible changes in UV sensitivity following perchlorate exposures.

Task 5: Effects of Naturally Occurring Perchlorate-contaminated Water on Amphibian Development.

The effects of perchlorate-contaminated water on amphibian development was observed, to determine biological effects of naturally occurring contamination. Water from a naturally contaminated site was collected in collaboration with Dr. Greg Harvey, and used in standard FETAX tests with *Xenopus* and/or native amphibians. The native species selected depended upon the time during which water samples are available. The water samples were analyzed for perchlorate content. Control groups were reared in FETAX solution. Developmental progress were followed and mortality, growth, and other abnormalities were recorded. The Tail Resorption Assay was used to expose later stage embryos and determine effects on metamorphosis.

Task 6: Effects of Perchlorate on Reproductive Capacity of Female *Xenopus.*

Adult female *Xenopus* were exposed to three concentrations of perchlorate that were determined by a range-finding test to evaluate adult toxicity levels. Each group consisted of 15 females and a control group maintained in the absence of perchlorate. All were fed salmon starter (Rangen Corporation). Prior to exposure, each adult was tattooed (Bantle, et al., 1998) for identification purposes and the females were mated to untreated tattooed males. This mating introduced a new cycle of oogenesis and vitellogenin synthesis and uptake. The success of the mating was measured by recording the number of eggs laid, the number fertilized, and the general health of the embryos. Females that failed to successfully mate or produce eggs were removed from the study and substituted with those that were successful. The females were then exposed to perchlorate for four months. Observations on the status of each were made daily and each animal was weighed weekly during the course of exposure. Animals that became moribund or otherwise exhibited life-threatening pathology were anesthetized in MS-222 and necropsied. In these cases organ weights (liver, gonads, spleen, fat body, kidney) were recorded and blood and thyroid samples taken for hormone and histological analysis. Following the four-month exposure the females were repaired to the same untreated males and induced to breed. Again, the success of the mating was measured by recording whether or not amplexus was successful, the number of eggs laid, the number fertilized, and the general health of the embryos. Some embryos from each pair were reared in FETAX solution to determine abnormalities, viability, and survivor success. The females were anesthetized in MS-222, weighed, necropsied, and organ weights recorded. Blood samples were collected for thyroxin analysis and thyroid tissue samples for histological examination.

Task 7: Histology and Thyroxin Analysis.

In order to examine the functionality of the thyroid, thyroxin analyses were conducted on embryos, tadpoles, and adults. These studies were concurrent with all objectives and the organisms selected for thyroxin analysis were determined based on data obtained. Thyroxin (T_3 and T_4) analyses were performed by the Clinical Laboratory, college of Veterinary Medicine, Oklahoma State University, using a chemiluminescent immunoassay procedure. The assay required only 130 µL of blood that was easily obtained from adults. Young embryos were homogenized in 0.8% saline and 6-N-propylthiouracil to block endogenous deiodinase activity. The homogenate was centrifuged and the supernatant was analyzed directly for thyroxin levels. Larger tadpole

or froglets were anesthetized with MS-222, dissected to remove the gastrointestinal tract and the tissue prepared as described above.

Additional Task 1: Examination of Sex Ratios in Metamorphosed Treated and Control Froglets.

It has been reported that exposure to perchlorate alters the sex ration in favor of males (Goldman, W. L., et al., Env. Tox. Chem., 21:590, 2002. We have an archived collection of metamorphosed froglets, e.g., Hyla, Xenopus, Rana and Bufo, from a variety of perchlorate exposures from which the sex can be determined. The specimens were preserved in either formalin or Bouin's fixative. These specimens were examined to determine possible relationships between exposures and species sensitivities.

Additional Task 2: The Effects of Perchlorate on Male Fertility.

Studies were conducted on the fecundity of females exposed to perchlorate. After exposure, these females were mated to untreated males in FETAX solution without perchlorate. These studies were extended to include a short study on the possible effects of perchlorate on the ability of sperm to fertilize eggs. Ovulation was induced in untreated females, their ovulated eggs collected, and *in vitro* fertilization conducted. Healthy ovulated eggs were selected and placed in Petri dishes containing FETAX solution and environmental relevant levels of perchlorate, e.g., in the ppb range. The testes from untreated males were excised and macerated in FETAX to create a suspension of sperm that will be added to the unfertilized eggs. Jelly was not removed from the eggs, as it is required to activate the sperm and prepare it for fertilization. At least 50 eggs were used for each control and each concentration of perchlorate from at least five females. The percent of eggs fertilized, the size and survivability of the resulting 96 hr old embryos as well as the number of abnormal embryos were recorded.

4.0 Results and Accomplishments

Results and accomplishments of the project are discussed in terms of the task structure presented in Section 3.0.

Task 1. Effects of Continuous Long-term Perchlorate Exposure on Developing Amphibians.

1.1 Tests with Xenopus and Native Species.

Tests with native species, Rana utricularia, Rana catesbeiana, and Hyla versicolor took longer to complete because of an underestimation of the time required for these species to metamorphose. Tests continued until metamorphosis was complete. Note: Bullfrogs require a long metamorphic period.

1.2 Tests with Xenopus.

Embryos exposed at the neural stage (15-18-hrs old) of development to 1 mg/L perchlorate displayed delayed growth. Embryos exposed at Stage 46 (96-hrs old) to 1 mg/L did not show differences in growth compared to controls. However, those exposed to 100 mg/L display delayed development. Exposure of pre-metamorphic embryos (Stage 55/56, ~ 32 days old) all showed a delay in metamorphosis relative to controls. These data suggest that sensitivity to perchlorate varies with their stage of development. Interestingly, the neurula were sensitive to 1 mg/L, long before the thyroid glands had differentiated. Similarly, embryos exposed to FETAX protocol (age ~14 to 96-hrs old) were also sensitive to perchlorate (see Objective 2).

These tests were still in progress and the data sets are large and final analysis was not completed. However, concentrations of 1 mg/L clearly were sufficient to inhibit or at least delay metamorphosis.

1.3 Tests with Native Species.

Two native species, *Rana utricularia* and *Hyla* sp. were collected and tests to measure growth during perchlorate exposure (control, 1, 10, 100, 200, and 400 mg/L ammonium perchlorate) were conducted. One set of measurements has been made that indicates a dose-dependent retardation of growth. These tests were continued until metamorphosis when snout-vent length measurements of the metamorphosed froglets was made.

Hyla versicolor: The time to metamorphosis was longer than anticipated. Most have metamorphosed but some concentrations of perchlorate and iodide were slower. Only 1 to 5 tadpoles remained in these groups, but the experiment was not completed (except for control groups).

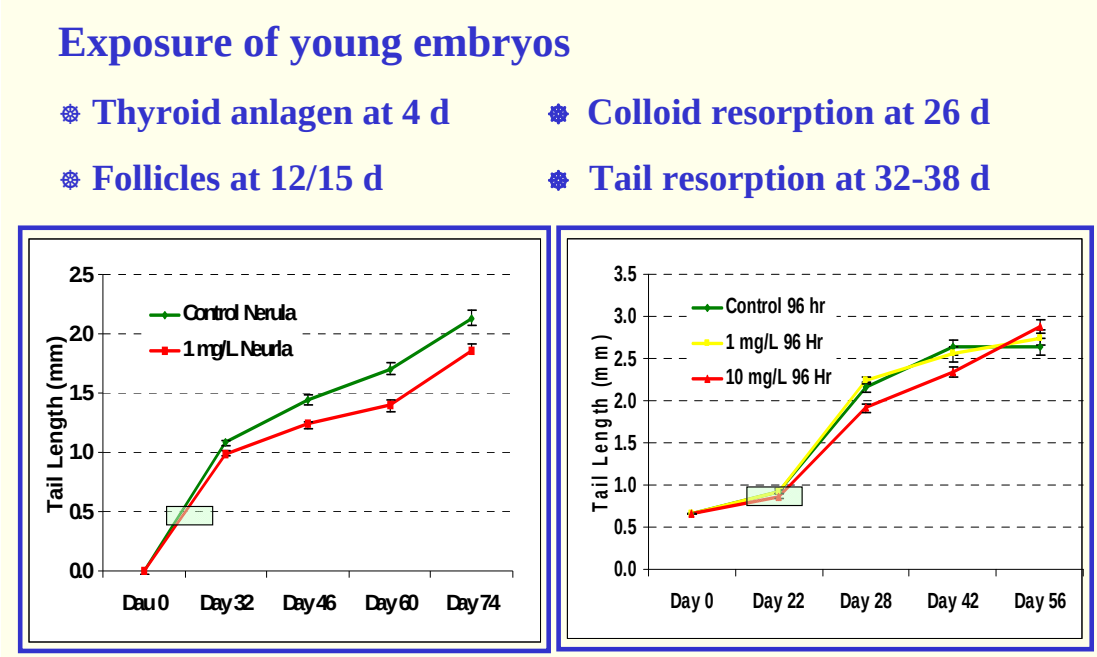


Figure 4-1. Long-term Effects on Development of *Xenopus*. Exposure of Young Embryos. Thyroid Anlagan at 4 days; Follicles at 12 to 15 days; Colloid Resorption at 26 days; Tail Resorption at 32 to 28 Days.

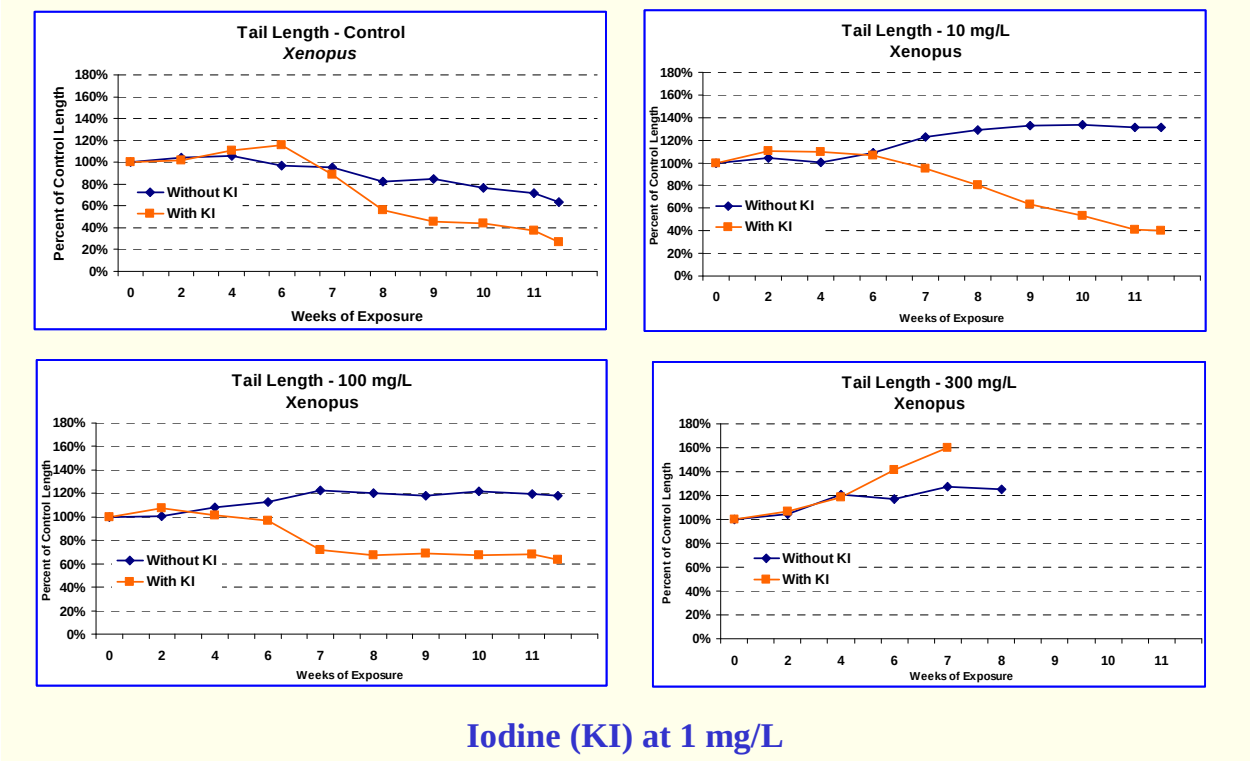


Figure 4-2. Long-term effects on development – *Xenopus*.

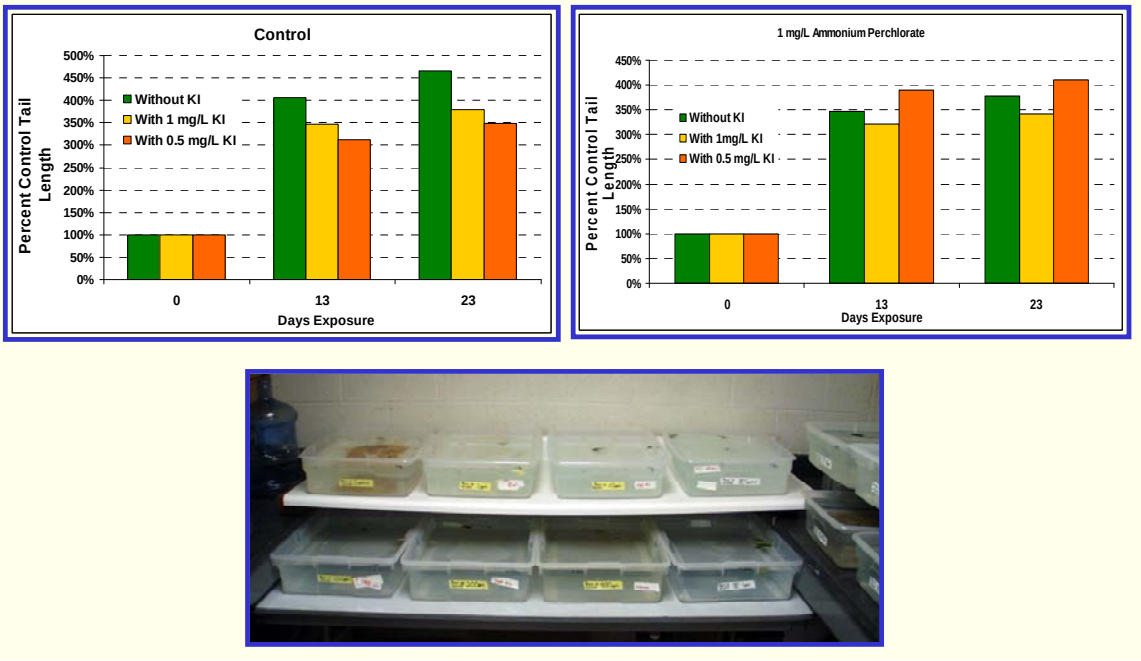


Figure 4-3. Long-term Effects on Development – *Rana utricularia* and *Hyla sp.*

Rana catesbienna: Bullfrog tadpoles that were collected last fall have now begun to metamorphose from the control and 25 mg/L groups. Exposure groups in addition to controls are 25, 50, 100, and 300 mg/L of AP. After 280 days of exposure (bullfrogs require two seasons to metamorphose) there was no significant difference in the growth (tail length) of controls and those exposed to 25, 50, and 100 mg/L of AP. Five hundred mg/L was lethal. However the tail length of those exposed to 300 mg/L was significantly shorter. The average weight of the tadpoles decreased in a dose-dependent way.

On day 280 ten tadpoles from each group were removed and placed into the same concentrations of AP with the addition of 1 mg/L of KI to determine if the addition of iodine would mitigate the effects of AP and if recovery is possible. It was too early to determine if the embryos are responding to the addition of iodine. Previous studies have shown that recovery is possible in some native species simply if removed from AP.

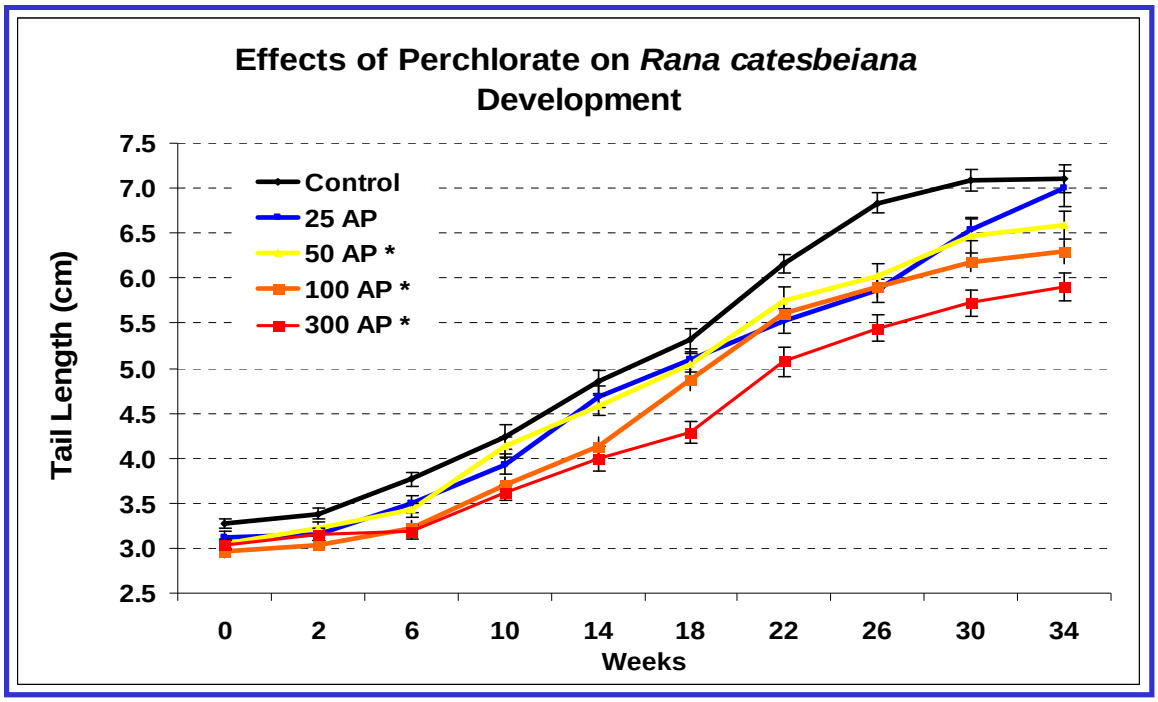


Figure 4-4. Long-term Effects on Development – *Rana catesbienna*.

Task 2. Mitigation of Perchlorate Effects by the Addition of KI.

2.1 Range Finding Tests for KI.

Range finding tests to determine toxicity to Xenopus embryos were completed. Iodine clearly has a "protective" effect even in these very young embryos that were exposed prior to the time when their thyroids are active.

2.2 Tests with Xenopus.

Tests with Xenopus were begun using KI in addition to AP Completed FETAX tests suggest that even in the young embryos (blastula/gastrula to 96hrs) KI has a mitigating effect by increasing the LC50 concentration of perchlorate.

Xenopus embryos were exposed to ammonium perchlorate concentrations of 10, 100, and 300 mg/L of AP with and without 1 mg/L of KI (~0.75 mg/L iodine). In the case of controls (no AP) those reared in the presence of iodine metamorphosed more quickly that controls without iodine suggesting that the addition of iodine to the FETAX saline culture medium my stimulate thyroid function. The addition of 1 mg/L of iodine to 10 mg/L and to 100 mg/L AP permitted metamorphosis comparable to the iodine controls. The LC50 for controls is ~ 650 mg/L, for 1 mg/L and for 5 mg/L KI it is ~ 900 mg/L. The EC50 (concentration at which 50% of the survivors are abnormal) for controls is ~500 mg/L and 600 mg/L for KI-exposed embryos. This demonstrates that the addition of iodine will overcome the effects of these concentrations of AP. However long term exposure of Xenopus embryos to AP concentrations above 100 mg/L is toxic and embryos at these concentrations both with and without iodine died after 80 days exposure. There was no

significant difference in the snout-to-vent lengths of any of the newly metamorphosed froglets.

2.3 Tests with Native Species.
Similar tests with locally collected *Rana utricularia* were initiated. These tadpoles were exposed to 1, 10, 100, 200, and 400 mg/L perchlorate. Two concentrations of KI were used: 1 and 0.5 mg/L. *Hyla* was collected and was treated with the same concentration of perchlorate and 1 mg/L KI. Because of the smaller number of embryos available, tests with 0.5 mg/L could not be initiated.

Rana utricularia: Native R. utricularia tadpoles were collected and exposed to a concentration series of AP of 1, 10, 100, 200, and 400 mg/L. One series contained 1.0 mg/L KI and the other 0.5 mg/L KI and the third no iodine. Those subjects in 1 mg/L AP and 1 mg/L KI were initially delayed but were not at the same stage of tail regression and others in this AP concentration, i.e., at 1 mg/L it appears that the presence of iodine will mitigate the effects of AP. Data showed little difference in growth rates (defined as reduction in tail length, i.e., resorption) between control and iodine treatment or between 1 and 10 mg/L perchlorate with or without iodine. Progress toward metamorphosis is slowed by100-mg/L perchlorate with 1.0 mg/L KI being more effective than 0.5 mg/L. Perchlorate concentrations of 200 and 300 mg/L essentially stop metamorphosis but the addition of 1.0 mg/L KI allows it to continue – 0.5 mg/L is ineffective. Interestingly, there was little difference in the size (snout-to-vent length) of froglets between treatments.

Bufo sp.: Note: the species were not identified because metamorphs had not matured enough to make positive identification. Toad embryos were exposed to 10 and 50 mg/L of AP with and without 1 mg/L of iodine. Control embryos had nearly completed metamorphosis. The 1 mg/L of iodine controls did not reach the size of controls and their metamorphosis seemed somewhat delayed. The addition of 1 mg/L of iodine has speeded metamorphosis in embryos exposed to 10 and 50 mg/L AP compared to those that were not exposed to iodine. This test was still in progress but it appears that 1 mg/L AP can mitigate the effects of AP.

Rana catesbeinana: Bullfrog tadpoles were collected locally and placed in pans containing 25, 50, 100, and 300 mg/L of AP. Tail length was measured weekly for nearly on year – a time when the onset of metamorphosis was imminent. While growth was significantly slowed in 300-mg/L tadpoles growth in lower concentrations was close to that of controls. After one year, each exposure group was divided into two. One group remained in its original perchlorate concentration while1.0 mg/L KI was added to the second group. During the ensuing four months control animals began to metamorphose and those in KI continued to grow slowly for 60 days and then began metamorphosis. The addition of iodine to those in 25 mg/L of perchlorate had little effect on growth relative to those without KI. In the remaining thee groups, KI sped the onset of metamorphosis, most significantly in the 50-mg/L group.

Hyla versicolor: Embryos were submitted to a test regime as described above for *Rana utricularia*. However, an additional concentration of iodine, i.e., 0.1 mg/L, was added in an effort to examine the effects of lower concentrations. Ammonium perchlorate was added in concentrations of 1, 10, and 100 mg/L. Concentrations of perchlorate at 0.5 and 1.0 mg/L had no effect on the progress of metamorphosis relative to controls but the presence of iodine speeds resorption. Perchlorate in concentrations of 10 and 100 mg/L caused mortality after 60 days of exposure. These data suggest that, at an appropriate ratio between iodine and perchlorate, progress through metamorphosis is possible. Higher iodine concentrations are required for higher perchlorate concentrations. The effects of iodine and response levels appeared to differ with the species.

To summarize, it appears that iodine is capable of blocking or overcoming the effects of AP within certain concentration ranges but there appears, at this juncture, to appear that there are species differences to this response. Further studies should be conducted to determine threshold concentrations of iodine. *Hyla* sp. general response to treatment with potassium iodide and perchlorate appears to be similar to that of *Rana*, however, the *Hyla* appear to be much more sensitive and fragile as they reach metamorphic climax. As a result, mortality rates become high.

A summary of this task is presented in Table 4-1.

Table 4-1. The Effects of Iodine in Mitigating AP Exposure.

Treatment	LC50	EC50	TI
AP Only	575	450	1.3
AP + 0.5 mg/L KI	800	525	1.5
AP + 1 mg/L KI	875	700	1.3
AP + 5 mg/L KI	950	750	1.3

Task 3. Effects of Perchlorate Derived from Food Sources on Amphibian Development.

Effects of perchlorate derived from food sources on amphibian development were studied. Tadpoles of two native species, *Rana utricularia*, and *Hyla* sp. were fed control and perchlorate-laden lettuce. The embryos were treated in 4 groups: (1) control being fed non-perchlorate contaminated lettuce, (2) iodide controls being fed non-perchlorate contaminated lettuce but with the addition or 0.5 mg/L iodide, (3) Perchlorate lettuce group being fed perchlorate-contaminated lettuce (80 mg/kg), and (4) a group being fed perchlorate-contaminated lettuce with the addition of 0.5 mg/L iodide.

3.1 Initiate Lettuce Growth.

Lettuce, grown in the presence of ammonium perchlorate, accumulated perchlorate to a concentration of 81 mg/kg. A supply of the perchlorate-laden lettuce was grown.

3.2 Tests with Native Species.
Perchlorate-laden lettuce was fed to tadpoles of toads (*Bufo americanus*) and frogs (*Pseudacris streckerii* and *Rana utricularia*). In the case of *Bufo* and *Pseudacris* there did not appear to be any difference in the response. However, in the case of *Rana*, it appeared that tail resorption was accelerated in tadpoles that ingested perchlorate-laden lettuce. The snout-vent length of newly metamorphosed froglets of *Pseudacri*s and *Rana* were larger than controls while the toadlets of *Bufo* were smaller. Based on the present status of these tests the data would suggest species differences in the responses to ingested perchlorate.

In the case of *R. utricularia* fed perchlorate-laden lettuce (80 mg/kg) there was no significant difference between controls (non-perchlorate-laden lettuce) and the test group in terms of time to metamorphosis. However the weight of the froglets from the test group was slightly less than controls (controls = 0.9g = +/- 0.1025g; test groups = 0.8g +/- o.0435g). Similarly the snout/vent length of controls is larger that test group (control = 2.16 cm +/- 0.1051 cm; test = 2.00 +/- 0.0506 cm).

Early embryos of the frog *Hyla veriscolor* were acquired and feeding tests were begun. Two additional regimes were added to the test – one with 0.1 mg/L of iodine and another with 0.5 mg/L KI addition to the control lettuce group was toxic and caused 100% mortality. The control group completed metamorphosis in 31 weeks while those fed perchlorate-laden lettuce with the addition of KI completed metamorphosis in 21 weeks. Clearly, in this case the addition of KI mitigated the effects of perchlorate. Those fed perchlorate-laden lettuce without the addition of KI completed metamorphosis in 35 weeks.

Task 4. Pigmentation and Sensitivity to UV Radiation.

Tests using UV radiation (with and without Mylar and acetate filters to screen wavelengths) was planned using *Xenopus* embryos in various stages of development. However, a request was submitted and approved to delete this objective and substitute an objective that examined progeny of AP treated adult females (see Objective 6). This request was submitted because of differences noted in size and a growth rate of embryos derived from such exposed females and because it was felt that a duplication of effort was being expended by other investigators. Therefore, this task was deleted and substituted with Task 7.

Task 5. Effects of Naturally Occurring Perchlorate-contaminated Water on Amphibian Development.

Effects of naturally occurring perchlorate-contaminated water on amphibian development were studied. Arrangements were made for the collection of water from Lake Meade Nevada. An aliquot was submitted for perchlorate analysis. Standard FETAX tests using *Xenopus* were conducted. Local species were tested as they became available.

This task was not completed.

Task 6. Effects of Perchlorate on Reproductive Capacity of Female Xenopus.

Ammonium perchlorate is a pervasive pollutant primarily from rocket fuel and fertilizers. Although much is known about the effects of perchlorate on amphibian larval development and metamorphosis, little is known about its affects on the reproductive capacity of adults.

6.1 Range Finding for Adult Toxicity.
A control group and four experimental groups of ten adult female *Xenopus* were exposed to four different concentrations of perchlorate – 10, 100, 1000, and 2000 mg/L for a period of four months. Prior to exposure the females were induced to ovulate and mated to marked males. Body weight, fecundity (number of eggs oviposited), fertilization rates, hatching success, and 96-hr growth achieved by the embryos were measured. After the four-month exposure the females were again induced to ovulate, mated to the same males, and body weight, fecundity, fertilization rates, hatching success, and embryo growth re-measured. Organ weights were also measured following the final ovulation.

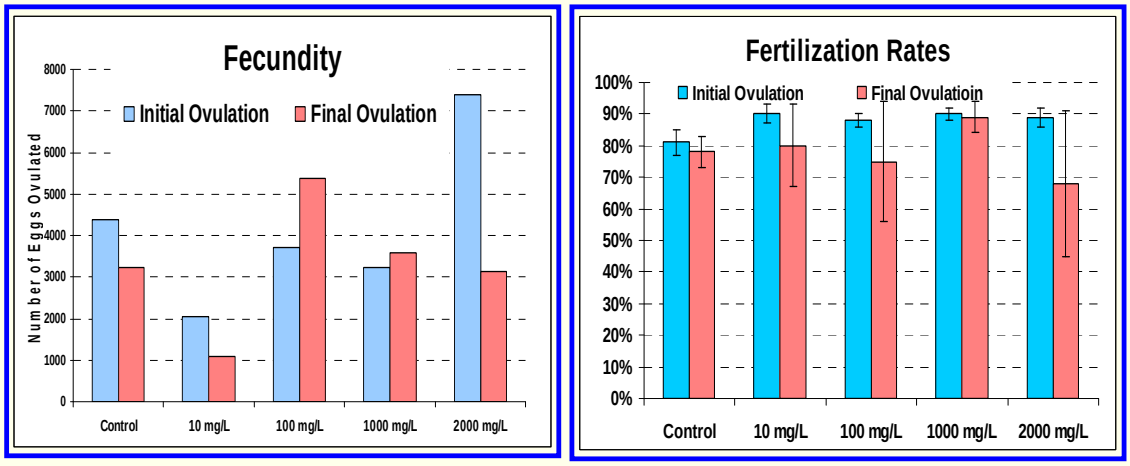
6.2 Initiate Four Month Exposure.
Body weights were not significantly affected by the treatments nor were significant differences in organ weights noted. Perchlorate analysis of organs (carcass, liver, and ovary) indicated the highest accumulations of perchlorate in the ovary, e.g., from the 2000 mg/L exposure group: carcass, 15 mg/kg; liver, 155 mg/kg; ovary, 311 mg/kg (analyses of tissues from other concentrations are in progress). Fecundity was reduced by 50% in the groups exposed to 2000 mg/L but was not significantly affected in other groups. Fertilization rates, however, were reduced by 3%, 9%, 11%, and 18% in the exposed groups compared to the control animals while the percent of the fertilized eggs that reached hatching was reduced by 14%, 10%, 13%, and 19% respectively in the experimental groups.

6.3 Remate and Assess Reproductive Capacity.
Experiments were not completed. However observations were made that lead the researchers to suspect that AP exposure of the adult female affected the progeny. The results of this experiment let to a proposal to study to examine the long term success of the progeny in Objective 8.

To date the data show:

- The number of eggs ovulated decreased 62%*, 33%*, 16%, and 42% in 10, 100, 1000 and 2000 mg/L AP respectively
- Fertilization rates decreased 15-20% following exposure
- The number of abnormal increased to 13% and 15% from females exposed to 1000 and 2000 mg/L AP
- All females showed about a 5% weight loss
- No significant difference in organ weights, e.g., liver, ovary, oviduct, spleen, and fat bodies in exposed over control female

- Initial perchlorate analysis results for females exposed to 100 mg/L-exposed females indicate a concentration of perchlorate of 32 mg/kg in the ovary, 6 mg/kg in the liver, and 0.11 mg/kg in the carcass. For females exposed to 2000 mg/L of perchlorate the concentrations are 311 mg/kg, 113 mg/kg, and 14 mg/kg respectively. We have submitted samples of ovulated eggs for analysis as well.



Final ovulation data sets are incomplete,

Control, 7; 10 mg/L, 3; 100 mg/L, 5; 1000 mg/L, 5; 2000 mg/L, 3

Figure 4-5. Effects on Female Reproduction – Fertilization Rate.

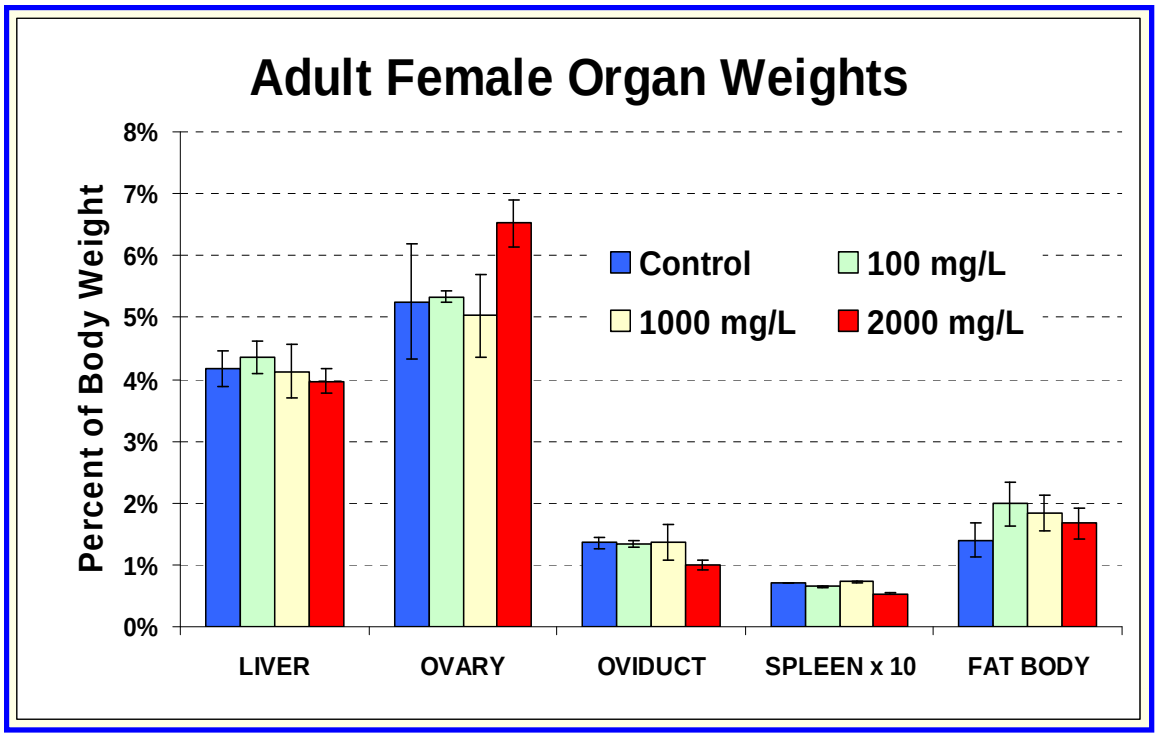


Figure 4-6. Effects on Female Reproduction.

Task 7. Effects of Perchlorate on Developing Embryos Following Exposure and Reproduction of Adult Females.

The 96-hr length achieved (growth) by the hatched embryos obtained at the end of Task 6 was significantly smaller than that of the controls. The abnormality rates of those surviving 96-hr embryos was decreased 4%, 3%, 13%, and 15% in the experimental groups compared to the control group. Finally, the surviving 96-hr embryos were reared for 14 weeks in water in the absence of perchlorate. The data show an increase in mortality directly related to the concentration of perchlorate to which the females were exposed. The presence of thyroxin in amphibian oocytes and early embryos has been reported. The data suggest that, in addition to reduction in reproductive capacity, there may be trans-generational effects that influence normal development of the early embryos. The high concentrations of perchlorate in the ovary suggest that some perchlorate may be incorporated directly into developing oocytes and hence eventually transferred to the embryos. Alternatively, low thyroxin levels in the females may be reflected in levels potentially present in the oocytes. In either case this raises a concern that the impact of perchlorate exposure may extend to the F_1 generations.

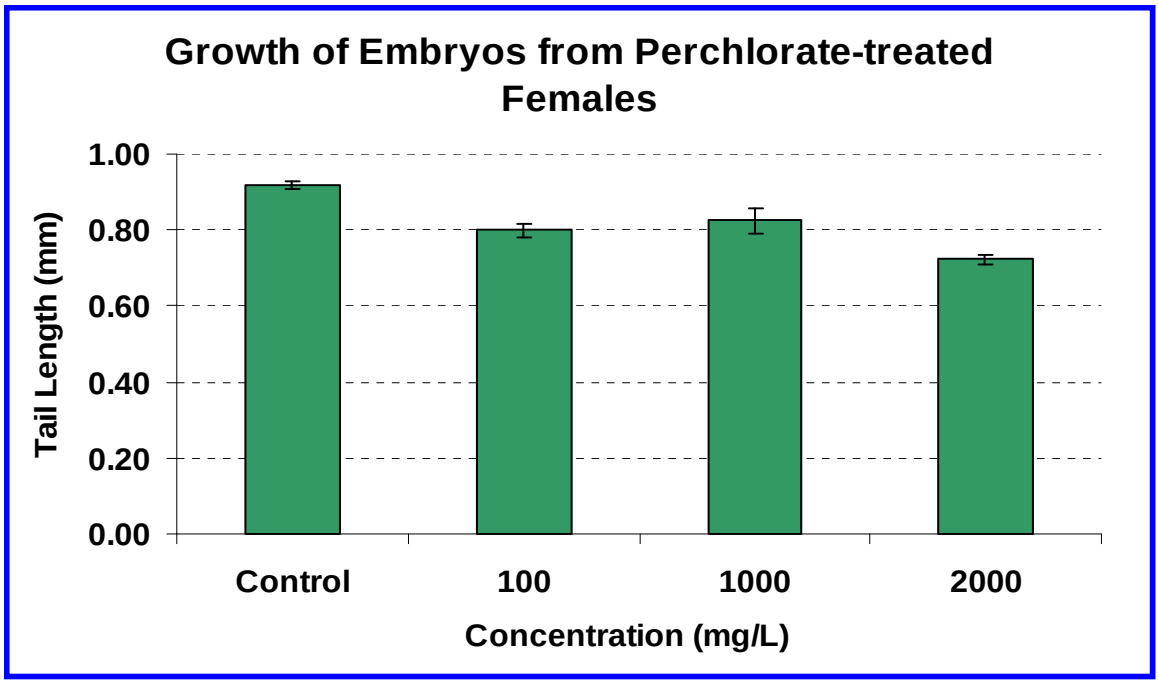


Figure 4-7. Effects on Female Reproduction – 96 hr Embryo Growth.

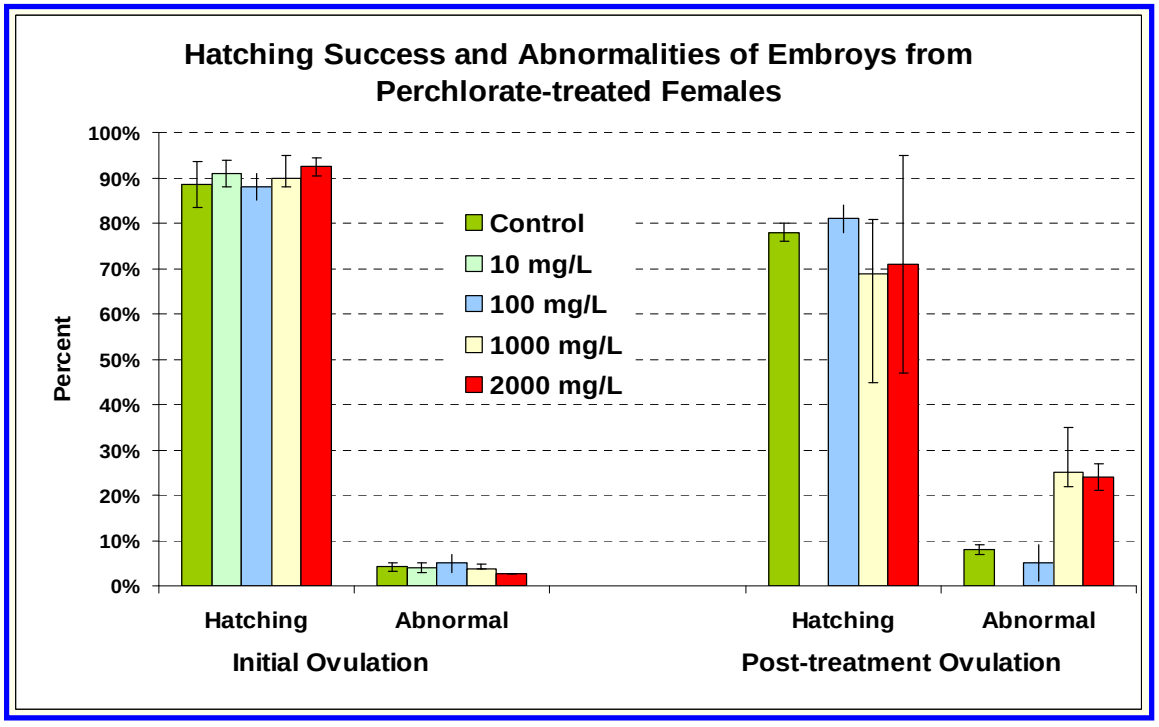


Figure 4-8. Hatching and Development.

Task 8. Histology and Thyroxin Analysis.

Effects of perchlorate on reproductive capacity of females was studied, whereby twenty-four females having undergone initial ovulations were exposed to perchlorate. Others were ovulated in a staggered fashion to complete the exposure regime.

8.1 On-going during Course of Study.
Embryos derived from AP treated females were reared and observed for long-term growth and survival. Although these data sets were incomplete, preliminary data indicated that that at four days of age, embryos derived from females in all exposure groups were significantly smaller (shorter) than those of control females. Interestingly, (though data are incomplete) it appeared that after about 28 days embryos derived from treated females appeared to be growing faster (larger) than controls. The reason for this is unclear at present but if there is perchlorate in the oocytes from which they are derived, it is possible that this represents a hormetic effect (response of an organism in an apparent attempt to overcome or compensate for low levels of toxicants). In any case, it is believed this is a demonstration of a trans-generational effect of perchlorate, i.e., an effect on progeny from a maternal source.

Additional Tasks
In addition to the new tasks to examine naturally occurring perchlorate contaminate dewater from Lake Mead (which was not completed), the revised proposal submitted in August requested a no-cost extension for the grant deadline (approved verbally, awaiting written confirmation). In this regard two additional tasks were proposed:

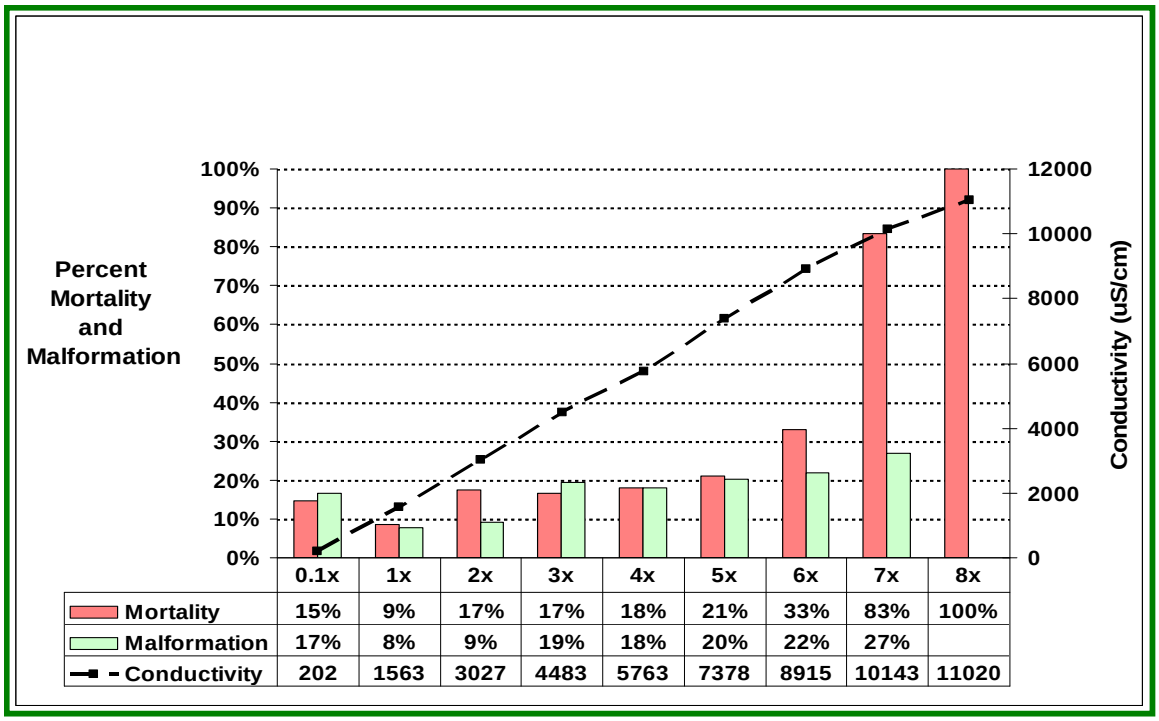
- Additional Task 1: examination of sex ratios of embryos derived from perchlorate-treated females, and
- Additional Task 2: a study on the effects of perchlorate on fertilization rates.

The latter is designed to examine the possible effects of perchlorate on sperm during the spawning process. Untreated males and females would be used and fertilization accomplished in vitro in the presence of perchlorate concentrations.

*These tasks were not completed.

Studies with Sodium Perchlorate
At the Fall 2002 In Progress Review (IPR), data was presented showing the effect of using sodium perchlorate in place of ammonium perchlorate. The following observations were made:

- Sodium perchlorate increase both Na and Cl concentrations
- Sodium perchlorate required the addition of NaCl controls
- Sodium perchlorate increases the concern for excessive ion concentrations – imbalances can be toxic
- Sodium perchlorate lessens the concern for the presence of ammonium\



	0.1x	1x	2x	3x	4x	5x	6x	7x	8x
Mortality	15%	9%	17%	17%	18%	21%	33%	83%	100%
Malformation	17%	8%	9%	19%	18%	20%	22%	27%	
Conductivity	202	1563	3027	4483	5763	7378	8915	10143	11020

Figure 4-9. The Relationship Between *Xenopus* Mortality, Malformations, and Conductivity in Increasing Concentrations of NaCl.

5.0 Cost and Performance

The cost outlay for this project is presented in Table 5-1.

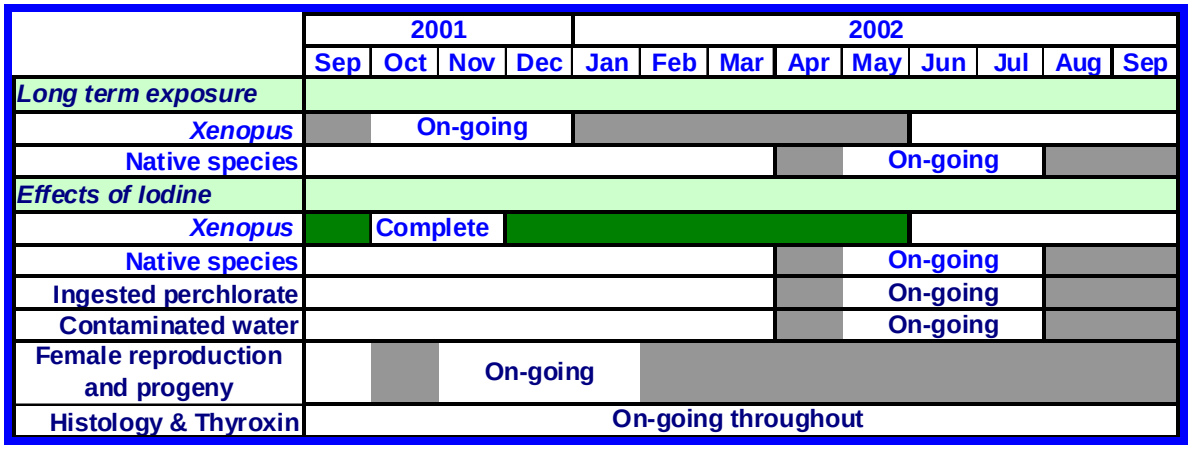
Table 5-1. Funding History and Outyear Estimates.

FY	Proposal Requested ($K)	Planned ($K)	Total Amount Distributed ($K)
2001	No Value Entered	$137.00	$137.00
TOTAL	$0.00	$137.00	$137.00

(Start date: October 2, 2001.)

Budget for proposed work: The budget includes all of the tasks described in the original proposal.

Schedule: The project schedule below reflects the major milestones for the proposed project as of the IPR meeting in fall 2002.



6.0 Conclusions

The objectives of this SERDP project were to examine the long-term effects of perchlorate on developing amphibians (e.g., growth, metamorphosis) and on the general health and reproductive capacity of adult females. The studies examined the effects of perchlorate present in the water as well as perchlorate available through the food chain. Because perchlorate competes for iodine binding sites in the thyroid, the addition of iodine to culture water was examined to determine if perchlorate effects can be mitigated. Finally, perchlorate is known to affect normal pigmentation of amphibian embryos. The effects of UV irradiation on pigment-altered embryos were examined.

In range-finding tests, it was observed that concentrations of 1 mg/L were sufficient to inhibit or at least delay metamorphosis.

It appears that iodine is capable of blocking or overcoming the effects of AP within certain concentration ranges but there appear to be species differences to this response. Further studies should be conducted to determine threshold concentrations of iodine. Long-term exposure of Xenopus embryos to AP concentrations above 100 mg/L is toxic and embryos at these concentrations both with and without iodine died after 80 days exposure. There was no significant difference in the snout-to-vent lengths of any of the newly metamorphosed froglets. *Hyla* sp. general response to treatment with KI and perchlorate appears to be similar to that of *Rana*, however, the *Hyla* appear to be much more sensitive and fragile as they reach metamorphic climax. As a result, mortality rates become high.

For the reproductive capacity of females, fecundity was reduced by 50% in test groups exposed to 2000 mg/L but was not significantly affected in other groups (10 to 1000 mg/L). Fertilization rates, however, were reduced by 3 to 18% in the exposed groups compared to the control animals while the percent of the fertilized eggs that reached hatching was reduced by 14 to 19% in the groups receiving perchlorate.

In addition to reduction in reproductive capacity, there may be trans-generational effects that influence normal development of the early embryos. High concentrations of perchlorate observed in the ovary suggest that some perchlorate may be incorporated directly into developing oocytes and hence eventually transferred to the embryos. Alternatively, low thyroxin levels in the females may be reflected in levels potentially present in the oocytes. In either case this raises a concern that the impact of perchlorate exposure may extend to the F_1 generations.

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Appendix A: Technology Transfer

The following paper was accepted for presentation at the SETAC meetings in Salt Lake City, Utah in November, 2002:

The Effects of Ammonium Perchlorate on Reproduction of Xenopus Females. Dumont, JN, J. Burkhart, V.A. Nzengung, and J.D. Collier.

Abstract: Ammonium perchlorate is a pervasive pollutant primarily from rocket fuel and fertilizers. It is know, among other things, to affect thyroid function by competing with iodine binding sites. Although much is known about the effects of perchlorate on amphibian larval development and metamorphosis, little is know about its affects on the reproductive capacity of adults. We exposed four experimental groups of adult female Xenopus to four different concentrations of perchlorate: 10, 100, 1000, and 2000 mg/L. Prior to treatment the females were induced to ovulate and mated to marked males. Fecundity, fertilization rates, and hatching success of the embryos were measured. After exposure the females were again induced to ovulate, mated to the same males, and fecundity, fertilization rates, and hatching success re-measured. Following treatment there is a reduction in all three parameters. Embryos derived from these post-treatment breedings show a significantly reduced growth (length) after 96-hr. Further they also show an increased rate of developmental abnormalities. The presence of thyroxin in amphibian oocytes and early embryos has previously been reported. The data suggest that in addition to reduction in reproductive capacity, there may be trans-generational effects that influence normal development of the early embryos. None of the adults show significant changes in body weight over the exposure period. Analysis of organ weights indicates a slight increase in liver weight in those treated with 2000 mg/L perchlorate. Analyses of blood thyroxin levels and body/organ burdens of perchlorate are in progress and will be reported. This research was supported by SERDP.

Appendix B: Research Accomplishments (Chronological)

The Effects of Ammonium Perchlorate on Reproduction and Development of Amphibians (SERDP ER-1236)

2001–OCT

This project has a start date of October 2, 2001. The initial matings for the long term female reproduction task have begun. Because of the large number of animals needed, the breedings are being staggered to spread the workload now and at the termination of the task. Preliminary work on establishing parameters for the UV exposure task have also begun.

2001–OCT

Matings for the control female reproduction task group have been completed. The mating success has been measured, i.e., number of eggs laid, percent fertilized, and hatching success. Matings for experimental groups to determine control (untreated) reproductive success for test groups in progress.

A group of bullfrog (Rana catesbiena) tadpoles has been collected and established in groups and exposures to perchlorate have begun. Preliminary tests to determine exposure doses of UV continue using Xenopus embryos.

2001–OCT

Task 1. Observations continue on Bullfrogs exposed to perchlorate. Differences in growth are noted but since metamorphosis is a long (D.I.Y.) process the test continues. Effects of long-term perchlorate exposure on Xenopus have been established and are on-going. Two exposure sets have been established, one with iodine and one without. Though early in the test, it appears that iodine mitigates some of the effects of perchlorate.

Task 2. Two FETAX analysis of perchlorate with and without iodine have been completed. Iodine clearly has a "protective" effect even in these very young embryos that are exposed prior to the time when their thyroids are active.

Task 4. Effects of UV. Preliminary tests are in progress to determine effective doses of UV.

Task 7. Histological preparations have been completed on thyroids from some exposed animals. Distinct histological differences related to exposures are seen.

Task 8. Effects of perchlorate on reproductive capacity of females. Twenty-four females have undergone initial ovulations and are currently being exposed to perchlorate. Others will be ovulated in a staggered fashion to complete the exposure regime. This is a long and labor-intensive test, hence ovulations are being staggered to spread the work load.

2002–JAN

Quarterly Report January 15, 2002

Task 1. Perchlorate (50, 100, and 300 mg/L) exposures of bullfrog tadpoles continue, as do tests with Xenopus. We are preparing now to collect native species and begin long-term growth and metamorphosis tests and FETAX analysis with the eggs and embryos collected.

Task 2. Range finding tests to determine the toxicity of potassium iodide have been completed. Tests with Xenopus have been begun using KI in addition to ammonium perchlorate. Completed FETAX tests suggest that even in the young embryos (blastula/gastrula to 96-hrs) KI has a mitigating effect by increasing the LC50 concentration of perchlorate. Similar tests will be established when native species become available in the spring.

Task 4. Tests using UV radiation (with and without Mylar and acetate filters to screen wavelengths are in progress using Xenopus embryos in various stages of development.

Task 6. Tests are well underway on the four-month study of the reproductive effects of perchlorate on adult females. Four concentrations of ammonium perchlorate have been chosen: 10, 100, 1000, and 2000 mg/L. Initial ovulations have been induced in the control group and in the 1000 mg/L group (each group contains ten adult females). Induced ovulations have been spread over time because of the number of test animals involved. The number of eggs ovulated and the percent fertilized are being recorded as well as the hatching rate. Pre- and post-ovulation and then weekly weights of the females are recorded.

In general, all tasks are on schedule and on budget. Many tests of local species will be initiated as soon as they become available. Discussions with Jody Wireman have successful resolved the issue of the number of adults in the reproductive test and the use of ammonium perchlorate instead of perchlorate salts of Natrium (Na), Magnesium (Mg), or Kalium (K).

2002–APR

NOTE: Because of the dry and cold spring only two native species of amphibians, Rana utricularia and Hyla sp. have been available to date. We expect, however to collect other local frog species as well as toad species, i.e., Bufo sp.

Objective 1. Effects of continuous long-term perchlorate exposure on developing amphibians. Studies with Xenopus long-term exposures have all been established and some are nearly complete. Embryos exposed at the neurla stage (15 – 18-hrs old) of development to 1 mg/L perchlorate display delayed growth. Embryos exposed at Stage 46 (96-hrs old) to 1 mg/L do not yet show differences in growth compared to controls. However, those exposed to 100 mg/L display delayed development. Exposure of pre-

metamorphic embryos (Stage 55/56, ~ 32 days old) all show a delay in metamorphosis relative to controls. These data suggest that sensitivity to perchlorate varies with their stage of development. Interestingly, the neurla are sensitive to 1 mg/L, long before the thyroid glands have differentiated. Similarly, embryos exposed to FETAX protocol (age ~14 to 96 hr old) are also sensitive to perchlorate (see Objective 2).

Two native species, Rana utricularia and Hyla sp. have been collected and tests to measure growth during perchlorate exposure are in progress (exposures: control, 1, 10 100, 200 and 400 mg/L AP. One set of measurements has been made that indicates a dose-dependent retardation of growth. These tests will be continued until metamorphosis when snout-vent length measurements of the metamorphosed froglets will be made.

Bullfrog (Rana catesbienna) tadpoles collected last fall are still under test. This species requires two years to metamorphose so this test is of longer duration. Exposure groups are: control, 25, 50, 100, and 300 mg/L perchlorate. Five hundred mg/L was lethal. Data to date indicate no significant difference in growth rates between controls, 10, 25, 50, and 100 mg/L. However, those exposed to 300 mg/L show a significant retardation in growth rates.

Objective 2. Mitigation of perchlorate effects by the addition of potassium iodide. Tests with Xenopus embryos (mid-blastula to mid-gastrula stages) using the 96-hr FETAX protocol have been completed using 1 mg/L and 5 mg/L potassium iodide and controls have been completed. The data indicate a "protective" effect of iodine even in these young "pre-thyroid" embryos. The LC50 for controls is ~ 650 mg/L, for 1 mg/L and 5 mg/L KI it is ~ 900 mg/L. The EC50 (concentration at which 50% of the survivors are abnormal) for controls is ~500 mg/L and 600 mg/L for KI-exposed embryos.

Tests with pre-metamorphic stages of Xenopus using 10, 100 and 300 mg/L perchlorate with and without 1 mg/L KI are almost complete. Data to date shows that in all cases in which 1 mg/L KI is present the tadpoles metamorphose faster than controls. This is true even in control groups to which iodine has been added. In those cases where KI treated tadpoles have metamorphosed, the snout-vent length of the froglets is smaller than controls.

Similar tests with locally collected Rana utricularia have been initiated but it is too soon to determine effects. These tadpoles are being exposed to 1, 10, 100, 200, and 400 mg/L perchlorate. Two concentrations of KI are being used: 1 and 0.5 mg/L. Hyla has also been collected and is being treated with the same concentration of perchlorate and 1 mg/L KI. Because of the smaller number of embryos available, tests with 0.5 mg/L could not be initiated.

Objective 3. Effects of perchlorate derived from food sources on amphibian development. A supply of lettuce has been grown in the presence and absence of perchlorate. Samples of this lettuce have been sent for perchlorate analysis but the analyses have not been completed. Tadpoles of two native species, Rana utricularia, and Hyla sp. are being fed control and perchlorate-laden lettuce. When other species become

available we expect to begin similar tests with those.

Objective 4. Pigmentation and sensitivity to ultraviolet radiation. We have conducted some preliminary tests with Xenopus using UV radiation but the results are equivocal. Because of the large number of other tests that are ongoing and currently being initiated with local species, the start of additional UV studies has been delayed so that local species collection and completion of current tests can be achieved. However, we do not anticipate any problem with completing this objective and the milestones have been rescheduled.

Objective 5. Effects of naturally occurring perchlorate-contaminated water on amphibian development. Arrangements have been made for the collection of water from Lake Meade wash. The samples are expected later this month. An aliquot will be submitted for perchlorate analysis. Standard FETAX tests using Xenopus will be conducted. Local species will be tested as they become available.

Objective 6. Effects of perchlorate on reproductive capacity of female Xenopus. These tests are progressing well. Initial ovulations and exposures of all groups of ten adult females each (control, 10, 100, 1000, and 2000 mg/L perchlorate) have been completed. Re-evaluation after four months of the control group in terms of fecundity, fertilization and hatching rates have been completed. None of these parameters show a significant difference from the data from the initial ovulation. After four months, the females were euthanized and blood samples taken and submitted for thyroxin analyses. Body and organ weights (liver, spleen, ovary, oviduct, and fat body) have been recorded. Organs and carcass are quickly frozen in liquid nitrogen and are sent for perchlorate analysis.

Six adults from the 1000 mg/L exposure group have completed their four-month exposure. They were re-mated and the data indicate that, on average, the fecundity (number of eggs ovulated) had the fertilization rate has remained about the same, ~ 90%. The hatching success after 96-hr however decreased from 90% pre-treatment to 69% post-treatment. Further, the number of abnormal embryos increased from 4% pre-treatment to 25% post-treatment. Although it is too soon to draw finite conclusions, it appears, based on this very preliminary data, that exposure may not greatly affect fecundity and fertilization but that hatching success and normal development of resulting embryos may be affected.

In terms of organ weight differences, it appears that liver, spleen, ovary, and oviduct weights are not affected. However, there seems to be a trend toward increasing weight of the fat body.

This experiment is on schedule and progressing according to plan.

Objective 7. Histology and thyroxin analysis. Regularly tissue samples from tadpoles and adults are taken for histological preparation for thyroid examination. These are being prepared by the Oklahoma Animal Disease Diagnostic, Oklahoma State University. Blood serum samples are being analyzed for thyroxin levels by a colleague at the

National Institute of Environmental Health Science (NIEHS). Although control and 1000 mg/L exposure samples have been submitted, at this writing there has been insufficient time for analysis.

2002–JUL

The following has been accepted for presentation at the SEATC meetings in Salt Lake City in November:
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Burkhart, J
Nzengung, VA
Collier, JD
Key Words: perchlorate, Xenopus, reproduction, fecundity
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The Effects of Ammonium Perchlorate on Reproduction of Xenopus Females. Dumont, JN1*, Burkhart2, J, Nzengung, VA3, Collier, JD1. , Oklahoma State University, Stillwater, Oklahoma; 2 NIEHS, Research Triangle Park, Raleigh, North Carolina; 3University of Georgia, Athens, Georgia. Ammonium perchlorate is a pervasive pollutant primarily from rocket fuel and fertilizers. It is know, among other things, to affect thyroid function by competing with iodine binding sites. Although much is known about the effects of perchlorate on amphibian larval development and metamorphosis, little is know about its affects on the reproductive capacity of adults. We exposed four experimental groups of adult female Xenopus to four different concentrations of perchlorate.10, 100, 1000, and 2000 mg/L. Prior to treatment the females were induced to ovulate and mated to marked males. Fecundity, fertilization rates, and hatching success of the embryos were measured. After exposure the females were again induced to ovulate, mated to the same males, and fecundity, fertilization rates, and hatching success re-measured. Following treatment there is a reduction in all three parameters. Embryos derived from these post-treatment breedings show a significantly reduced growth (length) after 96-hr. Further they also show an increased rate of developmental abnormalities. The presence of thyroxin in amphibian oocytes and early embryos has previously been reported. The data suggest that in addition to reduction in reproductive capacity, there may be trans-generational effects that influence normal development of the early embryos. None of the adults show significant changes in body weight over the exposure period. Analysis of organ weights indicates a slight increase in liver weight in those treated with 2000 mg/L perchlorate. Analyses of blood thyroxin levels and body/organ burdens of perchlorate are in progress and will be reported. (Research supported by SERDP)

2002–JUL

Objective 1. EFFECTS OF CONTINUOUS LONG-TERM PERCHLORATE EXPOSURE ON DEVELOPING AMPHIBIANS. Xenopus laevis: These studies are nearing completion. Xenopus embryos at four different ages, neurula, 2 days, 4 days and per-metamorphic tadpoles (~ 36 days old), were exposed to perchlorate to examine sensitivity at different stages of development. Neurulae exposed to 1 mg/L perchlorate show no differences in growth from the controls after 80 days of exposure. Embryos exposed to 100 mg/L of AP at two days of age (Stage 35/36) have not begun to metamorphose while metamorphosis is complete in the control group. The other groups show similar responses. These tests are still in progress and the data sets are large and final analysis is as yet incomplete. However, concentrations of 1 mg/L clearly are sufficient to inhibit or at least delay metamorphosis. Snout-to-vent length and weight of newly metamorphosed froglets are being recorded to determine these aspects of growth.

Rana catesbienna: Bullfrog tadpoles that were collected last fall have now begun to metamorphose from the control and 25 mg/L groups. Exposure groups in addition to controls are 25, 50, 100, and 300 mg/L of AP. After 280 days of exposure (bullfrogs require two seasons to metamorphose) there is no significant difference in the growth (tail length) of controls and those exposed to 25, 50 and 100 mg/L of AP. However the tail length of those exposed to 300 mg/L is significantly shorter. The average weight of the tadpoles decreases in a dose-dependent way. On day 280 ten tadpoles from each group were removed and placed into the same concentrations of AP with the addition of 1 mg/L of KI to determine if the addition of iodine will mitigate the effects of AP and if recovery is possible. It is too early to determine if the embryos are responding to the addition of iodine. Previous studies have shown that recovery is possible in some native species simply if removed from AP.

Objective 2. MITIGATION OF PERCHLORATE EFFECTS BY THE ADDITION OF POTASSIUM IODINE. Xenopus laevis: Xenopus embryos were exposed to ammonium perchlorate concentrations of 10, 100, and 300 mg/L of AP with and without 1 mg/L of KI (~0.75 mg/L iodine). In the case of controls (no AP) those reared in the presence of iodine metamorphosed more quickly that controls without iodine suggesting that the addition of iodine to the FETAX saline culture medium my stimulate thyroid function. The addition of 1 mg/L of iodine to 10 mg/L and to 100 mg/L AP permitted metamorphosis comparable to the iodine controls. This demonstrates that the addition of iodine will overcome the effects of these concentrations of AP. However long term exposure of Xenopus embryos to AP concentrations above 100 mg/L is toxic and embryos at these concentrations both with and without iodine died after 80 days exposure. There was no significant difference in the snout-to-vent lengths of any of the newly metamorphosed froglets.

Rana utricularia: Native R. utricularia tadpoles were collected and exposed to a concentration series of AP – 1, 10 100, 200, and 400 mg/L. One series contained 1.0 mg/L KI and the other 0.5 mg/L KI and the third no iodine. The controls with no iodine and both of the iodine controls (no AP) were not significantly different from each other in terms of progress toward metamorphosis.

At 1 mg/L AP those without iodine and those in 0.5 mg/L of iodine are progressing together. Those in 1 mg/L AP and 1 mg/L were initially delayed but are not at the same stage of tail regression and others in this AP concentration, i.e., 1 mg/L it appears that the presence of iodine will mitigate the effects of AP. In 10 mg/L of AP those without iodine and those with 1 mg/L of iodine are progressing together while those in 0.5 mg/L of iodine show a reduced tail resorption rate. In other words, 0.5 mg/L iodine appears to overcome the effects of 1 mg/L AP while at 10 mg/L of AP, 1mg/L of iodine is required. All embryos in 100, 200, and 400 mg/L AP with and without iodine are progressing together but at a much slower rate than those noted above.

Bufo sp.: (Species has not yet been identified because metamorphs have not matured enough to make positive identification). Toad embryos were exposed to 10 and 50 mg/L of AP with and without 1 mg/L of iodine. Control embryos have nearly completed metamorphosis. The 1 mg/L of iodine controls did not reach the size of controls and their metamorphosis seems somewhat delayed. The addition of 1 mg/L of iodine has speeded metamorphosis in embryos exposed to 10 and 50 mg/L AP compared to those that are not exposed to iodine. This test is still in progress but it appears that 1 mg/L AP can mitigate the effects of AP. To the extent to which this is successful must await completion of the test.

Hyla versicolor: We have recently received some Hyla versicolor embryos and these will be submitted to a test regime as described above for Rana utricularia above. However we plan to add an additional concentration of iodine, i.e., 0.1 mg/L, in an effort to examine the effects of lower concentrations. Ammonium perchlorate will be added in concentrations of 1, 10, and 100 mg/L.

To summarize, it appears that iodine is capable of blocking or overcoming the effects of AP within certain concentration ranges but there appears, at this juncture, to appear that there are species differences to this response. Further studies should be conducted to determine threshold concentrations of iodine.

Objective 3. EFFECTS OF AMMONIUM PERCHLORATE DERIVED FROM FOOD SOURCES ON AMPHIBAIN DEVELOPMENT. Lettuce, grown in the presence of ammonium perchlorate accumulated perchlorate to a concentration of 81 mg/kg. This was fed to tadpoles of toads (Bufo americanus) and frogs (Pseudacris streckerii and Rana utricularia). In the case of Bufo and Pseudacris there does not appear to be any difference in the response. However, in the case of Rana, whose test ongoing, it appears that tail resorption is accelerated in tadpoles that ingested perchlorate-laden lettuce. The snout-vent length of newly metamorphosed froglets of Pseudacris and Rana was larger than controls while the toadlets of Bufo were smaller. Based on the present status of these tests the data would suggest species differences in the responses to ingested perchlorate.

Early embryos of the frog Hyla veriscolor have recently been acquired and we have begun a feeding test with this species as well. We plan to add two additional regimes to the test – one with 0.1 mg/L of iodine and another with 0.5 mg/L

Objective 4. EFFECTS OF UV LIGHT ON PRECHLORATE TREATED TADPOLES. A request was submitted and approved to delete this objective and substitute an objective that examined progeny of AP treated adult females (see Objective 6). This request was submitted because of differences noted in size and a growth rate of embryos derived from such exposed females and because we felt it was a duplication of effort being expended by other investigators.

Objective 5. EFFECTS OF NATURALLY OCCURING AMMONIUM PERCHLORATE-CONTAMINATED WATER ON AMPHIBIAN DEVELOPMENT. We are in the process of preparing and expanding this objective in response to an action item from the Scientific Advisory Board (SAB). We have been in contact with Mr. Ken Covey, Las Vegas, Nevada, to develop a sampling plan for the collection of contaminated water from Lake Meade. We have tentatively identified three potential sites at Lake Meade that are known to be contaminated with perchlorate and where some endocrinological work has been done and where there is data on water chemistry. This revised objective will be submitted by July 31 in accordance with the action item.

Objective 6. THE EFFECTS OF AMMONIUM PERCHLORATE ON REPORDUCTIVE CAPACITY OF FEMALE XENOPUS. The portion of this study dealing with the reproductive capacity of females exposed to AP is nearly complete. Five adults remain to be studied in the 10 mg/L group and 2 in the 100 mg/L group (*). To date the data show:
(1) The number of eggs ovulated decreased 62%*, 33%*, 16%, and 42% in 10, 100, 1000, and 2000 mg/L AP respectively
(2) Fertilization rates decreased 15-20% following exposure
(3) The number of abnormal increased to 13% and 15% from females exposed to 1000 and 2000 mg/L AP
(4) All females showed about a 5% weight loss
(5) No significant difference in organ weights, e.g., liver, ovary, oviduct, spleen, and fat bodies in exposed over control female
(6) Initial perchlorate analysis results for females exposed to 100 mg/L-exposed females indicate a concentration of perchlorate of 32 mg/kg in the ovary, 6 mg/kg in the liver, and 0.11 mg/kg in the carcass. For females exposed to 2000 mg/L of perchlorate the concentrations are 311 mg/kg, 113 mg/kg, and 14 mg/kg respectively. We have submitted samples of ovulated eggs for analysis as well

These observations, especially those dealing with hatching success and the increase in abnormal embryos from females exposed AP, coupled with the fact that a very high concentration of perchlorate was detected in the ovary, lead us to suspect that AP exposure of the adult female affects the progeny. Thus we were led to propose the study to examine the long term success of the progeny in Objective 8.

Objective 7. HISTOLOGY, THYROXIN AND AMMONIUM PERCHLORATE ANALYSES. Blood samples have been sent to NIEHS (Dr. Burkhart) and we are awaiting analysis for thyroxin levels. Histological preparations are in process to examine

thyroid histological changes. Tissues (organs) of females, embryos, and water samples are routinely submitted to Dr. Valentine Ngunzung, University of Georgia, for perchlorate analysis. These analyses continue in progress.

Objective 8. POTENTIAL TRANS-GENERATIONAL EFFECTS ON GROWTH AND DEVELOPMENT OF EMBRYOS DERIVED FROM PRECHLORATE TREATED FEMALS. Embryos derived from AP treated females are being reared and observed for long-term growth and survival. Although these data sets are still incomplete, preliminary data indicates that that at 4 days of age, embryos derived from females in all exposure groups are significantly smaller (shorter) than those of control females. Interestingly, (though data are incomplete) it appears that after about 28 days embryos derived from treated females appear to be growing faster (larger) than controls. The reason for this is unclear at present but if there is perchlorate in the oocytes from which they are derived, it is possible that this represents a hormetic effect (response of an organism in an apparent attempt to overcome or compensate for low levels of toxicants). In any case we believe this is a demonstration of a trans-generational effect of perchlorate, i.e., an effect on progeny from a maternal source.

Comment: We agree with the comment that the use of a single laboratory species not exposed to potentially mitigating factors in the environment such as iodine is not optimal. However, to our knowledge such studies with amphibians have not been done and we believe our studies at least demonstrate the potential for trans-generational effects following exposure to AP. The use of other amphibian species, i.e., Rana or Bufo, for such studies under laboratory conditions is virtually impossible because of the breeding cycle of these amphibians. Unlike Xenopus, that can be easily reared and bred in the laboratory, other amphibian species cannot. Because of these characteristics it would be very difficult to conduct laboratory studies such as this with other species. We believe that to get data from other species would entail extensive field studies conducted over several seasons and in environments that had fairly stable perchlorate concentrations.

2002–OCT

Objective 1: Complete

Objective 2: This task remains ongoing with Hyla versicolor. It is progressing well but delayed somewhat because of an unexpectedly long time for the tadpoles to reach metamorphosis. We are of course continuing to collect data as they progress. Their general response to treatment with potassium iodide and perchlorate appears to be similar to that of Rana, however, the Hyla appear to be much more sensitive and fragile as they reach metamorphic climax. As a result, mortality rates become high. They are being treated with concentrations of perchlorate ranging from 0.25 to 10 mg/L and iodide concentrations ranging from 0.1 to 1 mg/L. These lower concentrations are being tested to achieve greater environmental relevance.

Objective 3: Tests with Rana utricularia are complete and tests with Hyla versicolor remain ongoing for the reasons stated above. The embryos are being treated in 4 groups:

(1) control being fed non-perchlorate contaminated lettuce, (2) iodide controls are being fed non-perchlorate contaminated lettuce but with the addition or 0.5 mg/L iodide, (3) Perchlorate lettuce group being fed perchlorate-contaminated lettuce (80 mg/kg), and (4) a group being fed perchlorate-contaminated lettuce with the addition of 0.5 mg/L iodide.

Objective 4. Task deleted and substituted with Task 7.

Objective 5. This task has been revised at the request of SERDP and the revision was submitted in August. We are awaiting review and approval before proceeding.

Objective 6. This task has been completed and will be reported at the upcoming SETAC meetings in Salt Lake City. The abstract was submitted as part of this report last quarter.

Objective 7. This task has been completed and will be and data reported at the Salt Lake City SETAC meetings. (see abstract submitted last quarter)

In addition to the new tasks to examine naturally occurring perchlorate contaminate dewater from Lake Mead, the revised proposal submitted in August also requested a no-cost extension or the grant deadline (approved verbally, awaiting written confirmation). In this regard two additional tasks were proposed: (1) examination of sex ratios of embryos derived from perchlorate-treated females and (2) a study on the effects of perchlorate on fertilization rates. The latter is designed to examine the possible effects of perchlorate on sperm during the spawning process. Untreated males and females would be used and fertilization accomplished in vitro in the presence of perchlorate concentrations. These tasks have not begun as the university is awaiting confirmation of the no-cost extension in order to release needed funds. Though this will impact the proposed starting and completion dates, the delay will not impact the eventual completion of the tasks except for determining the sex of embryos derived from perchlorate-treated females. Such embryos show reduced growth and increased abnormality rates after 96 hours of development. These parameters are directly related to the concentration of perchlorate to which the females were exposed. Further, as they develop over the next 14 week period, their growth is reduced and mortality increased compared to controls.

2003–JAN

Basically most tests are near completion, we are awaiting metamorphosis of the final few tadpoles in those tests with higher concentrations of perchlorate and/or iodine.

2003–APR

April 15, 2003. All tests have been completed except those described for the bullfrog tadpoles that have been removed from perchlorate. We would like to determine if they can recover from the long-term exposure to perchlorate and will continue to run this task until the project end date.