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FOREWORD

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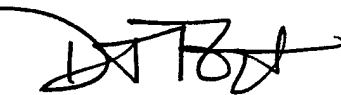
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INTRODUCTION

Objectives

The purpose of this project was to develop and validate a reproductive toxicity test using the gametes and embryos of the South African clawed frog, *Xenopus laevis*. The use of *X. laevis* provides an alternate species to standard mammalian tests and uses the same species as the Frog Embryo Teratogenesis Assay - *Xenopus* (FETAX). The specific objectives of this project were:

1. Develop and evaluate exposure methods that effectively deliver toxicants to adult frogs. Four experimental exposure methods were evaluated: direct via culture media; uptake of the toxicant by ingestion in food (oral); intraperitoneal (i.p.) injection (parenteral); and subcutaneous (s.c.) injection into the dorsal lymph sac.
2. Develop a reproductive toxicity assay battery using *X. laevis* focusing on gametogenesis (oogenesis/spermatogenesis), reproductive performance (mating success, fertilization), and embryonic development (embryonic viability and developmental toxicity).
3. Develop a preliminary protocol and validate the system boric acid (B) (Ku et al., 1993; Weir and Fisher, 1972; Lee et al., 1978; Fail et al., 1991; Price et al., 1996; Heindel et al., 1992; Price et al., 1991; Bussi, 1995), cadmium (Cd) (Vogiazis and Loumbourdis, 1997; Barr, 1973; Webster, 1990; IARC, 1993; Kostial, 1986; Watanabe et al., 1977; De et al., 1993), and ethylene glycol monomethyl ether (EGME) (Chapin and Sloane, 1997; Nagano et al., 1979; Nagano et al., 1981), three toxicants shown to possess varying degrees of reproductive toxicity in mammals.

Significance of the Current Research

Cellular and molecular processes differ among the various stages of an organism's life cycle. These differences can cause a varied sensitivity to xenobiotics, where some life stages may be more sensitive because of specific cell receptors or differences in metabolism. Therefore, it is important that toxicity screening assays account for the different stages of the life cycle, especially the most sensitive stages such as within reproduction and development. Reproduction and development are complex and specialized processes that begin with gametogenesis, continue

through fertilization and embryogenesis, and end in the sexual maturation of the organism. In designing a reproductive toxicity assay it is critical to assess toxicity at the stages in an animal's life cycle when sensitivity to toxicants occur (Mattison and Thomford, 1989). For example, gametes can be affected by toxicants at levels well below those of adults. Many current short term reproductive and developmental toxicity tests may be missing significant toxic events by excluding the effects on gametes, and full life cycle studies are not practical in most vertebrates due to their long life spans. It may be wiser to use a battery of short-term tests to assess toxicity to vertebrates. It is more economically feasible to use the same species for as many tests as possible. Therefore, the reproductive toxicity test we are developing complements the more extensively developed FETAX developmental toxicity test and a thyroid disruption assay currently being developed using *X. laevis*. In order to achieve a thorough assessment of hazards to an organism, toxicity testing must take into account the different stages in the organism's life cycle. Testing should, therefore, incorporate all aspects of toxicity, including immunotoxicity, neurotoxicity, developmental toxicity, and reproductive toxicity. Reproductive toxicity tests should examine the effects of xenobiotics on gametes, fertilization, and progeny.

Historically, much of reproductive toxicity testing had centered primarily on the male reproductive system. Concern with male reproduction has escalated following the discovery of declining sperm counts over the past 50+ years by some investigators, although this is controversial. Research has been conducted on the processes of spermatogenesis as well as changes in testes morphology (Mori et al., 1992; Majumder and Kumar, 1995; Llobet et al., 1995; Pant et al., 1995; Boockfor and Blake, 1997). Female reproductive toxicity was not vigorously studied until recently and as such there are very few validated assays that test toxicant effects on the female reproductive system and impact on developmental processes in their progeny (Kamrin et al., 1994). The preponderance of female reproductive toxicity tests are conducted with mammals, specifically on events occurring after fertilization (Levin and Miller, 1980; Rice, 1981; Wier et al., 1990; De et al., 1993). For example, researchers have examined the effects of toxicants on post-fertilization events such as the implantation of embryos (Rice, 1981; Eisenmann and Miller, 1994) and interaction between mother, placenta, and fetus (Wier et al., 1990). Additionally, the cellular processes such as gap junction communication during muscular contractions in the uterus have been reviewed by Kamrin et al. (1994). Paksy et al.

(1997) showed that Cd interfered with cell-cell junctions and cell adherence in rats. Studies have also been conducted with vertebrates other than mammals. Michibata et al. (1987) have examined the survivability of teleost fish eggs exposed to varying toxicant concentrations, the amount of toxicant accumulated by the egg, and the location of the toxicant within the egg and surrounding membranes.

The endpoints that can be evaluated in a reproductive toxicity test are numerous. Adult animals can be exposed and their gonads and/or gametes examined for morphological changes (Lohiya, 1976; Mattison et al., 1983; Sato and Koide, 1984; Jarrell et al., 1988; Toth et al., 1992; Domingo, 1995; Kramer et al., 1998). Cross-over breeding studies in which treated females are bred with untreated males, and the reciprocal cross, can assess impact on reproductive performance characteristics. Transgenerational effects on the progeny of exposed adults can be assayed using the endpoints from FETAX. FETAX endpoints include mortality, malformation, growth inhibition, behavior, pigmentation, locomotion, and feeding ability. Additionally, a 96-h LC50 (mortality) and 96-h EC50 (malformation) can be determined by probit analysis of the data collected from FETAX. A teratogenic index (TI) can then be calculated by dividing the LC50 by the EC50. The TI is a comparison of observed toxicities between an adult and embryo and is a measure of developmental hazard. A compound with a TI < 1.5 is interpreted to have a low teratogenic hazard whereas higher values signify an increase in the potential teratogenic hazard.

Although the use of *X. laevis* as a model system for evaluating reproductive and developmental toxicity is currently being assessed by the Endocrine Disruptor Screening and Testing Advisory Council (EDSTAC) as a Tier 2 test, standardized and validated methods of evaluating reproductive toxicity in amphibians have not yet been fully developed. Because standard mammalian reproductive toxicity models rely on either gonadal or post-fertilization effects, a toxicity model providing information on the effects of potential toxicants on reproductive performance and gametogenesis is warranted. Amphibians represent a suitable model for simultaneously monitoring reproductive performance and gametogenesis. In particular, *X. laevis* is well suited for female reproductive studies because of its fecundity and continuous asynchronous oogenesis. A new reproductive cycle can be initiated *in vitro* during any season by administration of hormones (Dumont, 1972). The study of *X. laevis* oogenesis has led to our understanding of the molecular mechanisms of human oogenesis (Muller, 1997).

Thus, *X. laevis* also serves as an excellent model to evaluate early embryonic development (Dumont et al., 1983; Fort et al., 1988; Dawson et al., 1988; Fort et al., 1989; Fort and Bantle, 1990). Collection of simultaneous information on the effects on gametogenesis and reproductive outcome not only provides hazard assessment information, but also provides mechanistic clues concerning the modes of action of reproductive toxicants. Additionally, an extensive knowledge base has been developed through its use for research in developmental and molecular biology.

Rationale for Gametogenesis-Based Endpoints in Assay

Design

Gametogenesis and fertilization are highly specialized processes. Meiosis is the reduction of chromosomes from a diploid to a haploid number and takes place only during gametogenesis. Interruption of meiosis can result in aneuploidy or polyploidy as well as other chromosomal defects of a hazardous nature.

At the onset of amphibian oogenesis, the oocytes begin meiosis but are arrested in the diplotene stage of prophase I. During the diplotene stage, the chromosomes become highly extended into a lampbrush configuration and are very active in RNA synthesis (Gilbert and Raunio, 1997). These specialized chromosomes direct the metabolic activities of the developing cell. Along with the lampbrush chromosomes, gene amplification also occurs during this stage (Gilbert and Raunio, 1997). During gene amplification ribosomal rna (rRNA) is replicated for the synthesis of ribosomes, which is necessary for synthesizing proteins. Proteins are needed to supply the growing oocyte as well as carry the embryo through the first stages of development.

Diplotene is also a period of oocyte growth and differentiation. The oocytes grow mainly by accumulating yolk through vitellogenesis. Vitellogenin may be an important route of exposure to toxicants, functioning as a carrier protein for inorganic phosphates, lipids, carbohydrates, and metals that ultimately get incorporated into the oocyte (Ghosh and Thomas, 1995). Once incorporated into the oocyte, vitellogenin is converted into yolk proteins (Wallace and Bergink, 1974). A majority of the yolk accumulates in the vegetal hemisphere of the growing oocyte. This hemisphere is yellowish in color and contains mRNAs essential for organism development. The other half of the oocyte, the animal hemisphere, is darker in

pigmentation and contains the nucleus, or germinal vesicle. During development, most of the organs come from the animal hemisphere (Gilbert and Raunio, 1997). The end product of oogenesis, an oocyte, is a highly polarized cell. This cell contains enough material to form a complete embryo in the absence of sperm (Gilbert and Raunio, 1997). Additionally, the oocyte contains numerous morphological and physiological adaptations that permit rapid and controlled embryonic development.

Oocyte maturation begins when luteinizing hormone (LH) stimulates the follicle cells around the oocyte to produce progesterone (Gilbert and Raunio, 1997). Upon oocyte maturation, meiosis resumes and the germinal vesicle breaks down (GVBD). During GVBD microvilli retract, nucleoli disintegrate and the lampbrush chromosomes contract. The oocyte divides and becomes arrested for a second time in metaphase II. The course of *X. laevis* oocyte maturation and the associated biochemical changes have been extensively researched (Sato and Koide, 1984; Cicirelli and Smith, 1985; Taylor and Smith, 1987). Cicirelli and Smith (1985) found that levels of cyclic adenosine monophosphate (CANT) declined upon oocyte maturation. Exposure to toxicants that interfere with the decline in CANT levels cause the oocyte to remain in meiotic arrest, thereby impeding maturation. Fertilization is the next signal that allows completion of meiosis and the subsequent fusion of pronuclei. The new embryo utilizes its store of histones, energy, mRNA, and proteins to proceed rapidly through development. Missing genetic information or the effect of the toxicant on early development will be quickly translated into malformed, stunted or dead embryos.

Preliminary or Related Studies

Reproductive Toxicity

Previous work was conducted in this laboratory in which the reproductive toxicity of JP-4 contaminated soil was examined using *X. laevis*. Tests were conducted with known reproductive toxicants to evaluate endpoint selection, and the reproductive toxicity of the contaminated soil was examined. Adults were exposed either orally or dermally for a period of 60 d. Although the direct exposure method was successful in this study, we concluded that direct i.p. or s.c. injection should be explored in the future. Additionally, attempts should be made to shorten the length of

exposure. More research with positive controls was recommended. Analysis of oocytes *in vitro* proved to be a valuable endpoint; however, we concluded that two groups of female frogs should be used, one for oocyte analysis and the other for breeding so that the impact of effects on oogenesis or oocyte maturation on reproductive performance could be determined.

BODY

Experimental Methods

Preliminary Studies

Route of Administration Evaluation

In an effort to determine the most efficacious route of test material administration, eight adult *X. laevis* (four male and four female) were administered either boric acid (B), CdCl₂ (Cd), or EGME via the culture water, food, or by i.p. or s.c. injections. Efficacy of the delivery method for the purposes of the present study was determined not only by which route delivered the most toxicant to the reproductive organs, but also by ease of administration and the environmental relevance of the route. For exposure via the culture water, 100 mg B/L, 2.5 mg Cd/L, and 1.0 mg EGME/L were provided via the culture water (FETAX Solution). Culture water was renewed every 48 h for a total exposure period of 30 d. Enteral toxicant administration was provided using B-, Cd- or EGME-enriched beef liver. The total B, Cd, or EGME administered was 10 mg/Kg/d, 2.5 mg/Kg/d, or 1.0 mg/Kg/d, respectively, for 30 d. I.p. or s.c. injections of 100 mg B/Kg/d, 2.5 mg Cd/Kg/d, or 1.0 mg EGME/Kg/d for five concurrent d were performed. For the s.c. route, the injection was made directly into the dorsal lymph sac. At the conclusion of the administration studies and immediately prior to euthanasia, the frogs were weighed and anesthetized with 1% w/v 3-aminobenzoic acid, ethyl ester (MS-222). A 10% solution (w/v) of MS-222 at a dose of 0.2 mL per 50 g of body weight was injected into the dorsal lymph sac of the animal to be euthanized (Bantle et al., 1998). Gonadal and liver tissue from each specimen was collected and analyzed for each respective test material.

Initial Determination of Test Endpoints Using Cadmium

Mature female and male *X. laevis* were obtained from *Xenopus* I (Dexter, MI) and allowed to acclimate for a period of 14 d before being number branded with liquid nitrogen for identification. Each experiment was composed of one control group and two treatment groups; each group contained the same number of frogs. Frogs were randomly divided into treatment groups and housed them in six identical heavy plastic laundry tubs containing 15 L of filtered,

non-chlorinated water. No more than 4 frogs were housed per tub. The water was changed three times weekly; temperature was kept between 20 and 22° C and monitored daily. Frogs were housed in a room with a photoperiod of 12 h light:12 h dark. They were fed a diet of ground beef liver and lung containing vitamins three times per week. Frogs were weighed weekly and behavior was monitored routinely.

Frogs were injected s.c. via the dorsal lymph sac every other d for 21 d with either 0.7% saline or Cd as cadmium chloride (CdCl_2) (Aldrich Chemical CAS #10108-64-2) in saline solution at the dose levels (mg Cd/Kg body weight). Frogs in experiments 1 (3.0 and 5.0 mg Cd/Kg) and 2 (0.5 and 5.0 mg Cd/Kg) were injected with Cd immediately following the acclimation period. Frogs in experiments 4 (0.5 and 3.0 mg Cd/Kg), 5, and 6 (0.75 and 1.0 mg Cd/Kg) were induced to ovulate 20 d prior to beginning Cd exposure by injection with 800 IU human chorionic gonadotropin (hCG) (Sigma #CG-5).

After the final injection, frogs were anesthetized with MS-222 until unresponsive and then killed by cervical dislocation. The ovaries, livers and spleens were immediately removed and weighed. Livers and spleens were stored in 3% formalin while ovaries were placed in Barth's medium (Smith et al., 1991). The organ weight to body weight ratio for ovaries, livers, and spleens was calculated and the data analyzed for non-stimulated and stimulated frogs. Necropsies were performed, noting any gross lesions or tumors. A lobe from the ovary was removed. Ova were isolated according to Smith et al. (1991), staged by diameter (using an ocular micrometer) and morphology as per Dumont (1972) and stored in 3% formalin. Ova at each stage of oogenesis were compared between control frogs and treated frogs.

Treated and control females were mated with untreated males. Animals were induced to breed by injection with hCG (800 μmL - females, 400 μmL - males). Mating behavior was observed and deviations from normal recorded. Following successful mating, 200 eggs from each pair were double sorted according to ASTM E1439-91 (ASTM, 1998) procedures and allowed to grow for 96 h in an incubator at 24°C. After 4 d (stage 46), mortality of the embryos was assessed, embryos were anesthetized in 4% MS-222 and fixed in 3% formalin. Malformation and length data were gathered as in standard FETAX protocol.

For analysis of Cd in ovaries, eggs and embryos, samples were homogenized and acidified with trace-metal grade nitric acid (HNO_3). Samples and blanks were sent to the U.S.

Army Center for Environmental Health Research (USACHER) for Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) analysis using a Hewlett Packard Model 4500 ICP-MS I. Techniques for sample processing and microwave digestion were conducted to maximize recovery and to minimize extraneous metal contamination using Environmental Protection Agency Method 6020. Once the samples arrived at Ft. Detrick they were transferred to acid-washed Teflon digestion vessels. Additional HNO_3 was added to make the final volume 10 ml. Samples were digested by microwave using the CEM Microwave Sample Preparation System (CEM Corp., Matthew, NC). Following digestion, the samples were diluted to 40 ml with distilled water and filtered through a 0.45 μM Teflon membrane. Cadmium standards were prepared at 0, 1, 10 and 100 $\mu\text{g/L}$. An internal standard of 89Y at 1 mg/L was added prior to nebulization. Following standardization the calibration curve was verified by analyzing a 100 $\mu\text{g/L}$ calibration standard and a blank. The check standard was repeated at a 10% frequency during the analysis and after the analysis was completed. The acceptable range for the check standard was $\pm 10\%$ of the actual value. Final Cd concentrations were corrected for internal standard recovery, analysis dilution, digestion volume and the original mass of the sample. The lower limit of sensitivity was 0.1 $\mu\text{g/g}$ wet weight of the samples.

Statistical analysis

All data were initially tested for normality and homogeneity of variance. To determine differences in oogenesis and organ-to-body weight ratios between control and treated frogs, either a one-way ANOVA or Kruskal-Wallis one-way ANOVA on ranks, depending on normality, was performed (Zar, 1984). Multiple comparison procedures were performed using either Dunnett's or Dunn's Test (Zar, 1984). The data were analyzed using a two-way ANOVA to evaluate the time of experiment and exposure effects (Zar, 1984). Analysis of embryo mortality and malformation rates was performed using the Chi-Square test of equal proportions and Bonferroni confidence interval procedure. The Student's t-test was used to examine the differences in embryo length between the exposure groups (Zar, 1984). The data from the 0.5 mg Cd/Kg exposure groups was deleted for mortality, malformation, and length analysis due to the small sample size ($n = 1$).

Concentrations below the detection limits of the ICP-MS were represented as zero for statistical analysis. All statistical analysis were performed at the $p < 0.05$ level of significance. ANOVAs and multiple comparison procedures were performed with the SigmaStat software from SPSS Science, Chicago, IL. The Student's t-test, Chi-square test, and Bonferroni procedure were performed using the Statistical Analysis Software from SAS Institute (Cary, NC).

Reproductive Toxicity Evaluation

Female Assessment

Exposure, Sample Collection and Breeding

Healthy female frogs were superovulated and bred by injecting 750 IU of human chorionic gonadotropin (hCG) into the dorsal lymph sac s.c. (ASTM, 1991; Bantle et al., 1998; Fort et al., 1999). Within 3-4 h post injection, ovulation commenced. For breeding, females were paired with males injected with ~500 IU hCG s.c. Each mating pair was placed in a polyethylene breeding chamber and allowed to breed overnight. In order to ensure that each female fully discharged the majority of mature oocytes, each female was squeezed gently along the flanks at the anterior portion of the ovary with posterior movement down the oviduct so as to strip the ovary of oocytes not yet released. Each female was then placed into her respective treatment for 30 d, at which time a thorough evaluation of reproductive status was performed. Four of the eight females were paired with unexposed males to evaluate breeding response as previously described. The females were weighed and anesthetized with 1% w/v MS-222. The ovaries, oviducts and livers were surgically removed prior to euthanasia. A 10% solution (w/v) of MS-222 at a dosage level of 0.2 mL per 50 g of body weight was injected into the dorsal lymph sac of the animal to be euthanized. The weight of each organ was determined, and the ovary and liver were further processed for necropsy.

Gametogenesis

Oocyte staging was performed in accordance with the method of Dumont (Dumont, 1972), in which stages are based on maturity and numbered from I to VI, with I representing the most immature oocytes and VI representing fully mature, banded oocytes. A thorough count of

all oocytes was then performed. Once this information was collected, this process was repeated twice to verify the data collected. The number of necrotic oocytes was also determined.

***In Vitro* Oocyte Maturation - GVBD Assay**

Ovaries were excised and placed in sterile, chilled Ca^{+2} /phenol red-free Hanks balanced salts solution, pH 7.6, and diluted to 230 milliosmoles (mOsm) with distilled water (Hanks O) in accordance with the methods of Pickford and Morris (1999) and Dumont (1972). Ovarian tissue was then cut into strips, rinsed, and incubated overnight at 4°C in Hanks O. Tissue was then digested for 75 min. in a 0.2% solution of collagenase D in Hanks O until all of the follicle cell layer was removed from the oocytes. After sufficient digestion, the oocytes were rinsed again in fresh Hanks O and transferred to a disposable 100-mm Petri dish containing modified defined nutrient oocyte medium (mDNOM, pH 7.6) (Dumont, 1972, and Pickford and Morris, 1999).

Large, banded, pre-ovulatory stage VI oocytes were selected by hand using a sterile Pasteur pipette under a dissecting microscope and plated 20/well in sterile 36-well Pyrex glass culture plates in 2 mL mDNOM. One μM progesterone was added and the plates were incubated in a shaker at room temperature (20-23°C) for 24 h. The medium was then aspirated and the oocytes were fixed in 5% (w/v) trichloroacetic acid. Maturation was visible externally as a white "Roux" spot that indicates where the spindle has anchored to the plasma membrane at the animal pole of the oocyte. GVBD was verified by cracking open representative samples from the fixed oocyte. The maturational response of 20 oocytes in each well was expressed as the percentage exhibiting GVBD, and the mean maturational response for each treatment combination represents a minimum of four replicate wells.

Male Assessment

Breeding and Sample Collection

Following 30 d of exposure, four male adults were collected and used to breed with respective females. The remaining four adult males were also anesthetized at 30 d of exposure. At this time the testes and liver were removed and the specimen euthanized. One testis was used exclusively for determining sperm counts, whereas the other, which was sectioned rather than

minced/homogenized, was used to determine rates of dysmorphology and for histopathological examination (Hausen and Riebesell, 1991). The frogs were anesthetized as described previously for the females. The testes were removed and any remaining fat was trimmed from the testis. Each testis was weighed and the information recorded.

Sperm Count

For the total sperm count, saline-merthiolate-Triton (SMT) (1 mL/10 mg tissue [0.9% (w/v) NaCl, 0.01% (w/v) merthiolate, 0.05% (v/v) Triton X-100]) was used to maintain the tissues during homogenization (Hausen and Riebesell, 1991). Testes were placed in a clean scintillation vial with SMT, minced with scissors, and homogenized (Powergen® 125, Fisher Scientific, Houston, TX) for two min. A sample was then placed into a hemacytometer and the spermatids counted. At least three chambers were counted for each sample. If the totals were not within 10%, the samples were recounted.

Dysmorphology Assessment

Dysmorphology was assessed by fixing the testis in 10% (w/v) formalin, pH = 8.0. The preserved sperm cells were then surveyed. The total number of abnormal sperm was counted and the types of abnormalities recorded. Several testes were surveyed per testis to ensure that the rates fell within 10% of one another.

Data Analysis

Reproductive status, including ovary and testis weights and pathology, total egg count, oocyte necrosis, oocyte stage distribution, maturation capacity, sperm counts, and sperm dysmorphology rates were determined for each adult. Breeding success, fertilization rates, and embryonic viability were also determined. Comparisons of reproductive fitness evaluations were performed using ANOVA (Dunnett's Test, $P < 0.05$).

Chemical Analysis

B concentrations were measured in the biological samples using inductively coupled plasma-mass spectrometry (ICP-MS) analysis. Cd and EGME were determined by graphite

furnace-atomic absorption spectroscopy (AAS-GF) and mass selective gas chromatography (GC-MS), respectively. Practical quantitation limits (method detection limit * dilution factor) for B, Cd, and EGME were 10.0 µg/Kg, 17.5 µg/Kg, and 50.0 µg/Kg, respectively, for the biological samples.

Results

Preliminary Studies

Route of Administration

Results from the route of administration studies are presented in **Table 1**. With each of the three test materials, the parenteral routes, specifically s.c. injection into the dorsal lymph sac, was the most effective in terms of the amount accumulated in the gonads. For each toxicant, however, similar trends of accumulation were observed in the gonads via the enteral and parenteral routes. Because each toxicant accumulated when administered via the culture water, exposure via the culture water was selected for the reproductive toxicity test protocol development and preliminary validation since it represented an environmentally realistic route of exposure.

Effect of Cadmium on Organ Weight

Analysis of the ratio of the organ weight to body weight of the liver, spleen and ovary indicated that in non-stimulated frogs there was a significant increase in the spleen-to-body weight ratio in the 0.5 and 3.0 mg Cd/Kg groups ($p < 0.0001$) (**Table 2**). Statistical analysis revealed no differences in the ratios for the liver, spleen, or ovary in stimulated frogs. Both non-stimulated and stimulated frogs exposed at 3.0 and 5.0 mg Cd/Kg exhibited a higher incidence of edema due to quantities of yellowish fluid within the body cavity.

Initial Determination of Effect of Cadmium on Oogenesis

Non-stimulated *X. laevis*

Cd had a significant effect on oogenesis in non-stimulated and stimulated *X. laevis* (Figures 1 - 4). In non-stimulated *X. laevis* there was a concentration-dependent decrease in the percentage of stage II oocytes (Figure 1). This decrease was significant at 3.0 and 5.0 mg Cd/Kg ($p < 0.0001$). There was a decrease in stage III *X. laevis* oocytes exposed at 5.0 mg Cd/Kg ($p < 0.0001$) while a slight increase in stage III oocytes at 0.5 mg/Kg frogs was exhibited ($p > 0.05$). All exposure groups exhibited a decrease in the percentage of stage IV oocytes, but only the 5.0 mg Cd/Kg treatment group was significantly different at $p < 0.0001$. Although the percentage of stage V oocytes increased at all exposures, only at 5.0 mg Cd/Kg was the increase significant ($p = 0.0013$). The percentage of stage VI oocytes in all exposure groups was different from controls ($p < 0.0001$). Furthermore, there was an increase in atretic eggs in all exposure groups; however, only at 3.0 and 5.0 mg Cd/Kg was the increase significant ($p < 0.0001$).

Estimation of the total number of oocytes/g ovary at each stage (Figure 2) revealed the same trends and statistical differences as in the percentage data (Figure 1) for stages II, III, IV and atretic oocytes. Statistical examination of the total number of oocytes revealed no significant difference in the number of stage V oocytes at all exposures, while the number of stage V oocytes at all exposures decreased in the 3.0 and 5.0 mg Cd/Kg exposure groups ($p < 0.0001$).

Gonadotropin Stimulated *X. laevis*

Cadmium also affected oogenesis in hCG-stimulated frogs (Figures 3 and 4). There was a decrease in the percentage of stage II oocytes in the 3.0 mg Cd/Kg exposure group ($p = 0.04$) (Figure 3). The percentage of stage III oocytes was also decreased. The decrease was significant at 0.75 mg and 3.0 mg Cd/Kg ($p = 0.007$). There was a significant difference in the percentage of stage IV oocytes between the exposure groups ($p < 0.001$) but not between control and exposed frogs. The percentage of oocytes in the 0.75 and 1.0 mg Cd/Kg groups increased over the control while there was a decrease in the 0.5 and 3.0 mg Cd/Kg groups. The percentage of stage V oocytes at 0.5 mg and 3.0 mg Cd/Kg were decreased ($p = 0.03$). Statistical analysis of the stage VI oocytes revealed a significant difference ($p = 0.006$); however, because very few stage VI oocytes were produced during the exposure period, it was difficult to make comparisons

between control and experimental groups. There was a significant increase in the percentage of atretic oocytes in all exposure groups ($p < 0.0001$). Atretic oocytes at the lowest concentration exhibited a mottled and swirled appearance, while at 3.0 mg Cd/Kg, the ovaries of most frogs contained mostly completely degenerated oocyte follicles (**Figure 5**).

Estimation of the total number of oocytes/g ovary revealed basically the same trends in all stages across all exposure groups (**Figure 4**). There was a significant decrease in stage II oocytes at 3.0 mg Cd/Kg ($p = 0.04$); however, the decrease was not significant in stage III oocytes ($p = 0.06$). In stage IV oocytes, there was a difference between the 1.0 mg Cd/Kg and 0.5 and 3.0 mg/Kg exposure groups only ($p = 0.0001$). There was no significant difference in the number of stage V oocytes although there is a distinct decrease in the population of oocytes. There was a decrease in stage VI oocytes ($p = 0.03$); however, due to the high incidence of zeros in the data set, a multiple comparison test was not valid. All exposure groups exhibited a significant increase in atretic oocytes ($p = 0.0004$).

Initial Evaluation of the Transgenerational Developmental Toxicity of Cadmium

The percentage of successful matings between Cd-exposed females and non-exposed males was significantly decreased from control frogs ($p = 0.02$). In four experiments, 12 out of 22 (54.5 %) control animals mated successfully, 1/9 (11 %) at 0.5 mg Cd/Kg, 4/14 (28.6%) at 0.75 mg Cd and 1.0 mg Cd/Kg, and 0/8 (0%) animals mated successfully at 3.0 mg Cd/Kg. The results of the modified FETAX test on the progeny from females that did mate successfully are shown in **Table 3**. Because the percentage of successful matings was extremely low at 0.5 mg Cd/Kg (1 out of 9) and zero at 3.0 mg Cd/Kg statistical analysis of the mortality, malformations, and growth was limited to the control, 0.75 mg Cd, and 1.0 mg Cd/Kg groups. Mortality and malformations appeared to decrease with increasing Cd concentration. The Chi-square test indicates that the probability of mortality is dependent on the level of exposure ($X^2 = 252.1289$, $p < 0.001$). The Bonferroni confidence interval procedure showed that the 0.75 mg Cd/Kg exposure group is statistically different than the control group. The probability of malformations also depended on the level of exposure ($X^2_2 = 44.4026$, $p < 0.001$), and once again the difference lies between the control and 0.75 mg Cd/Kg exposure group. Statistical analysis of the head-to-

tail length data revealed no significant difference between progeny from exposed females and controls ($p = 0.60$).

In malformed progeny from control females, edema was observed most frequently (66.7%), with gut malformation being the second most frequent malformation (40%). The most common malformations in embryos from Cd-exposed females, including the 0.5 mg Cd/Kg group, are shown in **Figure 6**. The highest incidence of malformations in the 0.75 and 1.0 mg Cd/Kg groups was in the notochord (61.3 % and 61.9%, respectively). Notochord malformations mainly occurred at the proximal end, causing the body axis of the embryo to curve either vertically or horizontally (**Figure 7**). Because the tails were malformed, embryos swam abnormally, i.e. in circles, after 72 h. Embryos with and without notochord deformities from females exposed at 0.75 mg Cd/Kg were allowed to grow past 92 h. All embryos with notochord deformities died within 116 h postfertilization while the non-malformed embryos from the same exposure group survived beyond 140 h. Cadmium also increased the incidence of gut malformations. In the 0.75 mg Cd /Kg group 38.7% of embryos and 57.1 % of embryos from the 1.0 mg Cd/Kg group exhibited loosely coiled guts (**Figure 8**). Eye deformities were also observed which included misshapen lenses and abnormal eye placement. Other malformations observed included misshapen and shortened heads and deformed mouths.

Ovarian and Embryo Cd Burdens

ICP-MS analysis was conducted to examine the Cd concentration in frog ovaries in both stimulated and non-stimulated frogs. **Table 4** shows the ovary Cd concentrations in non-stimulated and stimulated frogs and also the percentage of Cd injected that was accumulated by the ovary. The Cd concentration in the ovaries of non-stimulated frogs was significantly different from control frogs at the 5.0 mg Cd/Kg exposure level ($p = 0.004$). In stimulated frogs, there was a difference at the 0.5 mg and 3.0 mg Cd/Kg levels ($p < 0.0001$). Cadmium concentrations within the ovaries ranged from 2.29 to 15.08 $\mu\text{g/g}$ wet. The percentage of Cd injected that was accumulated by the ovary appeared to decrease with increasing Cd dosage. Cadmium was readily detected by ICP-MS analysis in progeny of Cd-exposed females (**Table 5**). Statistical analysis of these preliminary results indicated no significant difference in fertilized egg or embryo Cd concentrations between control and exposed frogs. The data indicated that

there was a greater concentration of Cd in embryos at 24 h than in fertilized eggs. The data suggested that the embryo Cd concentration decreased over the length of the FETAX assay, based on measurements at 24, 48, 72, and 96 h (i.e. stages, 26, 37, 42, and 46, respectively) (Nieuwkoop and Faber, 1967). Although the decrease was not statistically significant (Chi Square/Borferoni Test, $p < 0.05$) the small sample size caused the variances to be extremely high.

Reproductive Toxicity Evaluation

Boric Acid

Female Assessment

Although some B accumulation was found in the livers of females (control = 43.2 ± 17.2 $\mu\text{g B/Kg}$; 1,000 mg B/L treatment = 108.0 ± 23.2 $\mu\text{g B/Kg}$) exposed to 1,000.0 mg B/L, no overt pathology was noted. The effects of 30-d B exposure via culture water on female reproductive endpoints are provided in **Table 6**. Results indicated that a significant reduction (Dunnett's Test, $P < 0.05$) in ovary weight occurred in specimens exposed to 500.0 mg B/L. General necrosis and histopathology of the ovary was observed at 1,000.0 mg B/L. A significant reduction (Dunnett's Test, $P < 0.05$) in the total number of oocytes was found in females exposed to 500.0 mg B/L for 30 d via the culture water. Increased rates of oocyte necrosis were noted in the 50.0 mg B/L treatment, with increasing necrosis occurring with increasing B concentration. Oocytes collected from females exposed to 1,000.0 mg B/L were all necrotic. In addition, the proportion of oocytes (> stage III) decreased in a concentration-dependant manner, with a significant effect (Dunnett's Test, $P < 0.05$) noted at 50 mg B/L.

Successful breeding responses as determined by amplexus occurred with each of the females exposed to B concentrations ranging from 0.0 to 100.0 mg/L. Three of the four females (75%) exposed to 500.0 mg B/L for 30 d via the culture media successfully bred. None of the four females exposed to 1,000.0 mg B/L initiated breeding activity. Interestingly, no effect on fertilization rates was observed with females exposed to B concentrations ranging from 0.0 to 500.0 mg/L for 30 d in the culture media; however, a significant reduction (Dunnett's Test, $P < 0.05$) in 96-h embryo viability was detected at both the 100.0 and 500.0 mg B/L treatments.

The effect of maternal B exposure on early embryo-larval development is provided in **Table 7**. Although no statistically significant (Dunnett's Test, $P < 0.05$) increases in mortality were detected, a significant increase (Dunnett's Test, $P < 0.05$) in abnormal development was observed in fertilized embryos collected from females exposed to 100.0 and 500.0 mg B/L for 30 d and cultured in FETAX Solution for 4 d. Female exposure to 500.0 mg B/L for 30 d induced abnormal development of the craniofacial region, gut, kinking of the notochord, and microencephaly.

Male Assessment

The effect of boric acid on male reproductive endpoints and reproductive performance is presented in **Table 6**. As with the female *X. laevis* exposed to 1,000.0 mg B/L, some accumulation of B was noted (control = $31.5 \pm 15.2 \mu\text{g B/L}$; 1,000.0 mg B/L = $136.3 \pm 29.4 \mu\text{g B/L}$); however, no overt pathological effects were observed in the male specimens. Exposure to 50.0 mg B/L for 30 d significantly reduced (Dunnett's Test, $P < 0.05$) testis weight, although overt pathology was only noted in males exposed to 1,000.0 mg B/L. The sperm count was significantly reduced and the rate of sperm dysmorphology increased at a concentration of 500.0 mg B/L. The rate of sperm dysmorphology in males exposed to 1,000.0 mg B/L for 30 d was $18.3 \pm 1.2\%$. No effect on breeding response was noted except at 1,000.0 mg B/L, at which level amplexus was not induced in any of the four males bred. In addition, no significant reduction (Dunnett's Test, $P < 0.05$) in fertilization rates or embryonic viability at 96 h was observed.

Cadmium

Female Assessment

Cd accumulation in the liver of females exposed to 10.0 mg Cd/L was appreciable (control = $< 30.0 \mu\text{g Cd/Kg}$; 10 mg Cd/L treatment = $192.3 \pm 38.6 \mu\text{g Cd/L}$), and moderate pathological effects, including decreased weight and discoloration, were noted; however, no liver pathology was observed in animals exposed to concentrations $< 10.0 \text{ mg Cd/L}$. The effects associated with 30-d Cd exposure via the culture water on female reproductive endpoints are presented in **Table 8**. A significant (Dunnett's Test, $P < 0.05$) reduction in ovary weight was observed in females exposed to 5.0 mg Cd/L. Overt pathology was noted in ovaries examined

from females exposed to either 5.0 mg Cd/L or, to a greater extent, 10.0 mg Cd/L. A significant reduction (Dunnett's Test, $P < 0.05$) in total oocytes was found at 2.5 mg Cd/L. A significant increase (Dunnett's Test, $P < 0.05$) in the incidence of necrotic oocytes was observed in females exposed to 1.0 mg Cd/L in the culture water for 30 d. All oocytes collected from females exposed to ≥ 5.0 mg Cd/L in the culture water for 30 d were necrotic. A decrease in the proportion of $>$ stage III oocytes was observed in females exposed to 1.0 mg Cd/L, but more notably in females treated with 2.5 mg Cd/L.

Breeding response was not affected in females exposed to either 0.5 or 1.0 mg Cd/L for 30 d. Half of the females exposed to 2.5 mg Cd/L bred, while none of the females treated with either 5.0 or 10.0 mg Cd/L bred with healthy untreated males. A significant reduction (Dunnett's Test, $P < 0.05$) in the fertilization rate was noted in oocytes collected from females exposed to 2.5 mg Cd/L for 30 d. A reduction in 96-h embryo-larval viability was observed in embryos obtained from females exposed to 1.0 mg Cd/L for 30 d. A concentration-dependent decrease in embryo-larval viability was observed, although no embryos were available to evaluate the 5.0 and 10.0 mg Cd/L treatments.

The specific effects of Cd exposure to adult female *X. laevis* on embryo-larval development and teratogenesis are given in **Table 9**. The effects of maternal Cd exposure prior to breeding were predominantly embryo-lethal; however, malformations induced in 4-d larvae from females exposed to 2.5 mg Cd/L for 30 d prior to breeding included visceral edema, skeletal kinking of the notochord, craniofacial defects, ruptured pigmented retina, and microencephaly.

Male Assessment

As with the females, Cd accumulation in the liver of males exposed to 10.0 mg Cd/L was appreciable (control = <30.0 $\mu\text{g Cd/Kg}$; 10 mg Cd/L treatment = 139.5 ± 21.6 $\mu\text{g Cd/L}$), and moderate pathological effects, including decreased weight and discoloration, were noted; however, no liver pathology was observed in animals exposed to <10.0 mg Cd/L. The effects of 30-d Cd exposure via the culture water on selected male gametogenesis endpoints and reproductive performance are provided in **Table 8**. A significant reduction (Dunnett's Test, $P < 0.05$) in testis weight and sperm count was observed in males treated with 2.5 and 5.0 mg Cd/L,

respectively. An increase in the proportion of abnormal sperm was detected in the 1.0 mg Cd/L treatment.

A slight reduction in breeding response (three of four pairs successfully bred) was noted in males exposed to 2.5 mg Cd/L. No breeding response was observed in the 10.0 mg Cd/L treatment. A reduction in fertilization rates was seen only in the 10.0 mg Cd/L treatment (Dunnett's Test, $P < 0.05$). A slight reduction in embryo-larval viability was noted in oocytes fertilized by males treated with 1.0 – 5.0 mg Cd/L, whereas a marked decrease (Dunnett's Test, $P < 0.05$) in viability was noted in the 10.0 mg Cd/L treatment.

Ethylene Glycol Monomethyl Ether

Female Assessment

Although some accumulation of EGME (control = $< 50 \mu\text{g EGME/Kg}$; 5.0 $\mu\text{g EGME/L}$ treatment = $158.3 \pm 58.2 \mu\text{g EGME/Kg}$) was noted in liver tissue from females exposed to 5.0 mg EGME/L and a slight increase in liver weight was noted, no pathology was detected. The effects of 30-d EGME exposure via culture water on female reproductive endpoints are provided in **Table 10**. Results indicated that a significant reduction (Dunnett's Test, $P < 0.05$) in ovary weight occurred in specimens exposed to 0.75 mg EGME/L. General necrosis and histopathology of the ovary was observed at 1.0 mg EGME/L. A significant reduction (Dunnett's Test, $P < 0.05$) in the number of oocytes was found in females exposed to 0.5 mg EGME/L for 30 d via the culture water. An increased rate of oocyte necrosis was noted in the 0.5 mg EGME/L treatment, with increasing necrosis occurring with increasing EGME concentration. Oocytes collected from females exposed to 5.0 mg EGME/L were all necrotic. In addition, the proportion of oocytes ($>$ stage III) decreased in a concentration-dependant manner, with a significant effect (Dunnett's Test, $P < 0.05$) noted at 0.5 mg EGME/L.

Successful breeding responses as determined by amplexus occurred with each of the females exposed to EGME concentrations ranging from 0.0 to 0.5 mg/L. Two of the four females (50%) exposed to either 0.75 or 1.0 mg EGME/L for 30 d via the culture media successfully amplexed. None of the four females exposed to 5.0 mg EGME/L initiated breeding activity. No effect on fertilization rates was observed with females exposed to EGME concentrations ranging from 0.0 to 0.5 mg/L for 30 d in the culture media; however, a significant

reduction (Dunnett's Test, $P < 0.05$) in fertilization rates was found in the 0.75 mg EGME/L treatment. A significant reduction (Dunnett's Test, $P < 0.05$) in 96-h embryo viability was detected at 0.5 mg EGME/L. Embryos from females treated with ≥ 7.5 mg EGME/L were completely inviable.

The effect of maternal EGME exposure on early embryo-larval development is provided in **Table 11**. A statistically significant (Dunnett's Test, $P < 0.05$) increase in embryo lethality was detected in 0.75 mg EGME treatment. In addition, a significant increase (Dunnett's Test, $P < 0.05$) in abnormal development was observed in fertilized embryos collected from females exposed to 0.5 mg EGME/L for 30 d and cultured in FETAX Solution for 4 d. Maternal exposure to >1.0 mg EGME/L for 30 d induced mal-development of the gut, pericardial edema, mal-development of the aorta, notochord kinking, hydroencephaly, and hemorrhage.

Male Assessment

Unlike females exposed to 5.0 mg EGME/L, males exposed to this concentration for 30 d more significantly accumulated EGME (control = <50 μg EGME/Kg; 5.0 mg EGME/L treatment = 304.9 ± 72.3 μg EGME/Kg) and exhibited significant liver necrosis, characterized by zonal necrosis. No adverse effects on the liver were noted in concentrations < 2.5 mg EGME/L. The effect of EGME on male reproductive endpoints and reproductive performance is presented in **Table 10**. Exposure to 0.5 mg EGME/L for 30 d significantly reduced (Dunnett's Test, $P < 0.05$) testis weight, although overt pathology was only noted in males exposed to 1.0 mg EGME/L. The sperm count was significantly reduced and the rate of sperm dysmorphology increased (Dunnett's Test, $P < 0.05$ for both) at concentrations of 0.5 and 0.75 mg EGME/L, respectively. The rate of sperm dysmorphology in males exposed to 5.0 mg EGME/L for 30 d was $100.0 \pm 0.0\%$. No effect on breeding response was noted in treatments ranging from 0.0 to 0.5 mg EGME/L. At 5.0 mg EGME/L amplexus was not induced in any of the four males bred. In addition, a significant reduction (Dunnett's Test, $P < 0.05$) in fertilization rates and embryonic viability at 96 h were observed in the 1.0 mg EGME/L treatment.

***In Vitro* Oocyte Maturation (GVBD)**

In an effort to determine if the increased proportion of immature oocytes found in toxicant-exposed females was the result of the lack of hormonal stimulation or the inability to be stimulated to mature, normal-appearing stage VI oocytes were removed from four females within the 100.0 mg B/L, 1.0 mg Cd/L, and 0.5 mg EGME/L treatments and co-cultured with 1 μ M progesterone. Results from these studies are given in **Table 12**. The majority of stage VI oocytes from the control females successfully completed GVBD when cultured with 1 μ M progesterone; however, a significant proportion (Dunnett's Test, $P < 0.05$) of the stage VI oocytes from the EGME-treated group did not undergo GVBD, indicating that they were not progesterone responsive. Cd or B-treated females were similarly responsive to progesterone as to control oocytes in terms of successfully completing GVBD.

RESEARCH ACCOMPLISHMENTS

Schedule	Accomplishments
1Q99	Completed study of i.p., s.c., oral (feed), and dermal routes with Cd and boric acid
1Q99	Selected endpoints for female and male
2Q99	Preliminary SOP developed for males and females
2Q99	Preparing for validation efforts; compounds selected (Cd) (performed in J. Bantle's laboratory)
2Q99	Completed following validation tests using cadmium, boric acid and EGME
1Q00	Prepared manuscript reviewed by USACHER and submitted to J. Appl. Toxicol. (currently in press)
2Q00	Final report submitted to USACHER

REPORTABLE OUTCOMES

Schedule	Non-publication Work Products
2Q99	Development of SOPs for use in finalizing standard protocols
2Q99	Completed and included in SOPs for reproductive studies in <i>X. laevis</i>
Manuscripts, Abstracts, Presentations	
Manuscripts	
1Q99	The Effect of Subcutaneously Injected Cadmium on Oogenesis and Progeny in <i>Xenopus laevis</i> : Applications for the Development of a Reproductive Toxicity Assay. Laila A. Lienesch, John A. Bantle and James N. Dumont. Chemosphere, in press.
1Q00	Douglas J. Fort, Enos L. Stover, John A. Bantle, James N. Dumont and Robert A. Finch. J. Appl. Toxicol. Evaluation of a Reproductive Toxicity Assay Using <i>Xenopus laevis</i> : Boric Acid, Cadmium, and Ethylene Glycol Monomethyl Ether. In press.
Abstracts and Presentations	
4Q99	Environmental Significance of Thyroid Disruption in Amphibians: Applications of the <i>Xenopus</i> Tail Resorption Assay. Douglas J. Fort, Enos L. Stover and James G. Burkhardt. Soc. Environ. Toxicol. and Chem., 1999.
4Q00	Effect of Citral and Phytolic Acid on Amphibian Development. D.J. Fort, R.L. Rogers, E.L. Stover, M.F. Miller, R. Paul, L.M. Morgan, J.A. White, P. Clark and J.G. Burkhardt. Soc. Environ. Toxicol. and Chem., 2000.
Degrees Obtained	
2Q00	L. Lien, M.S. degree conferred
2Q00	M.F. Miller, M.S. candidate
2Q00	R.R. Paul, M.S. candidate
Funding	
3Q00	Oklahoma Center for the Advancement of Science and Technology proposal

CONCLUSIONS

Route of Administration and Establishment of Endpoints

Preliminary results suggest that the most effective routes of exposure are parenteral. Although toxicant accumulation occurred, exposure via the culture water and per os administration delivered markedly less test substance to the gonads. These results are not necessarily unexpected because the majority of cadmium consumed via enteral routes will be cleared from the body and, thus, not be absorbed into gonadal tissue. Dermal administration of test substances is simply less effective in delivery than the direct parenteral routes due to additional barriers restricting absorption. Results from these studies also indicated that we can expect differences in accumulation based on sex and the toxicant being studied.

An additional consideration is the relevance of each route of exposure. Per os administration or exposure via culture water represents a more reasonable exposure route than parenteral administration in the environment. Feeding studies, however, require longer periods of exposure time and are more difficult to control in terms of external variables. Injection into the lymph sac is technically more simplistic and can be accomplished over a shorter period of time. Although it is not necessarily relevant as an environmental exposure route, it is a commonly used route in toxicological studies. Therefore, though all studies are not yet complete, it would appear that if environmental realism is not a concern, parenteral administration should be considered the primary means of toxicant administration. In circumstances where environmental realism is an issue, oral or culture water administration should be used.

Overall, our study found that the stages of oogenesis are a sensitive and relative endpoint in a reproductive toxicity assay. Dumont (1972) correlated the stages of oocyte development in *X. laevis* with the related physiological and biochemical data. By examining thousands of oocytes at each developmental stage Dumont (1972) determined the percentage of oocytes at each stage of oogenesis in non-stimulated frogs. The oogenesis data with control non-stimulated frogs in the present study are consistent with these findings.

At the onset of this study, female *X. laevis* were exposed to Cd immediately following the acclimation period. These animals/experiments are referred to as non-stimulated. Dumont (1972) suggested that oocytes from non-stimulated frogs were not as metabolically active as those from females stimulated with hCG. Our results indicate that the effect of Cd upon oogenesis is more severe in hCG-stimulated frogs. When hCG is administered to female *X. laevis*, they ovulate most of their mature oocytes and begin a period of vitellogenesis (Dumont, 1972). Therefore, hCG promotes an increase in protein incorporation (Wallace et al., 1970). Keem et al. (1979) measured the growth rate of oocytes in laboratory-maintained *X. laevis*. They noted that even when all environmental conditions were controlled, there still existed a large variability in the patterns of oocyte growth between non-stimulated and hCG-stimulated frogs. It took 16-24 weeks for oocytes from non-stimulated frogs to progress from stage III to stage VI, while in hCG-stimulated frogs the time period for the same growth was 9-12 weeks. The greatest amount of oocyte growth occurred 20 to 42 d after hCG-stimulation (Keem et al., 1979). In order to standardize reproductive status, decrease variability, and to achieve the greatest uptake of Cd by the ovaries while keeping the length of the toxicity assay as short as possible, it was decided that hCG-stimulated frogs would be used in the rest of the present study. Furthermore, to target the critical growth phase, exposure to Cd was begun 20 d after hCG-stimulation and the frogs were exposed for 21 d.

Ovaries from control frogs in the present study contained oocytes at all stages and most oocytes appeared healthy. For example, stage IV - VI oocytes had distinct animal and vegetal hemispheres and relatively few atretic oocytes were present. There was, however, a significant increase in the population of atretic oocytes in Cd-exposed frogs. In both non-stimulated and hCG-stimulated frogs, and at all concentrations of Cd tested, the ovaries contained a large portion of atretic oocytes. The morphology of these oocytes was consistent with that described by Dumont (1972). A high incidence of small, very darkly colored, spheres present only in the theca were common in the present study. Dumont (1972) categorized these as completely degenerated oocyte follicles containing densely packed pigment. In starvation studies with *X. laevis*, Dumont (1972) noted that only oocytes containing yolk were susceptible to atresia. Although no visibly degenerated stage II oocytes were found in this study, the population of stage II oocytes was decreased at every concentration (**Figures 1-4**) and the population of atretic

oocytes was significantly increased. This suggests that stage II oocytes either underwent atresia or there was no recruitment from the population of stage I oocytes. It is probable that Cd selectively removed oocytes from each stage of oogenesis, including stage III, thereby increasing the population of atretic oocytes.

The effects of Cd on oogenesis are much more apparent in hCG-stimulated frogs than non-stimulated frogs. As discussed by Dumont (1972), it appears that the ovaries of hCG stimulated frogs in the present study have a higher metabolic activity and thereby are more actively involved in taking up Cd. Such a phenomenon also occurs when trypan blue is injected into hCG-stimulated and non-stimulated frogs (Dumont, 1972). Trypan blue is actively taken up by oocytes in hCG-stimulated frogs; however, the uptake is virtually nonexistent in non-stimulated frogs. The overall metabolic activity of oocytes from non-stimulated frogs is markedly diminished. Therefore, although the morphology of oocytes is the same at the identical stages in non-stimulated and hCG-stimulated frogs, there are submicroscopic cytological and physiological differences (Dumont, 1972).

The interaction of cadmium and vitellogenin synthesis has been the focus of many studies. Sunderman et al. (1995b) have shown that Cd binds to the vitellogenin yolk protein lipovitellin 1 in *X. laevis* and could be a mechanism for reproductive toxicity of Cd following environmental exposure. They hypothesized that Cd absorbed by the female could bind to plasma vitellogenin in place of zinc (Zn), enter oocytes by endocytosis and become deposited in the yolk platelets. Cd, when is bound to vitellogenin, also becomes incorporated in the oocytes of Atlantic croakers (*Micropogonia undulatus*) (Ghosh and Thomas, 1995). Therefore, the binding of Cd to vitellogenin could significantly increase and accumulation of the metal. Our data indicate that Cd is incorporated into stage IV and V *X. laevis* oocytes, and atretic oocytes of Cd-exposed females (results not shown). In further studies, we will compare the amount of Cd in oocytes at various stages.

Watanabe et al. (1977) performed chromosome analysis on dd/YF mice. The number of females with abnormal oocytes increased in the Cd-treated groups in conjunction with an increase in the number of oocytes exhibiting chromosome aberrations. They concluded that Cd has the potential to be a mutagen in mammalian meiotic chromosomes. Watanabe et al. (1977) also exposed mice to Cd via a single s.c. injection of CdCl₂ and then examined the oocytes and

ovaries. They found a significant decrease in the number of oocytes recovered with increasing Cd concentration. We have shown that Cd caused a decrease in the oocyte population at most stages of oogenesis and at most concentrations of Cd tested (**Figures 1-4**). Davidson (1976) suggests that the *X. laevis* ovary contains a reserve of oocytes in stage II from which groups are selected to undergo continued oogenesis. If this is true, then the decrease in the population of stage II oocytes from Cd-exposed frogs results in a decrease in oocytes in later stages and an overall decline in fecundity. This may permanently affect the ability of the female to reproduce.

Although Cd caused a decrease in the oocyte population in most stages of oogenesis and in most exposure groups, the population of stage V (non-stimulated frogs) and stage IV (stimulated frogs) oocytes at some exposures was greater than in the controls. These findings may be due to the variation in the number of oocytes present in any given stage prior to treatment in captive female *X. laevis* (Dumont, 1972). Physiological changes caused by Cd at stage IV could preclude further oocyte development to later stages. Interestingly, frogs exposed to Cd at a dose of 3.0 mg/Kg did not breed (did not go into amplexus) and the percentage of frogs that bred at lower exposure concentrations was very low. Examination of the ovaries of the Cd-exposed frogs revealed that mature stage VI oocytes were present and, presumably, should have been ovulated. Frogs whose ovaries contained few or no stage VI oocytes did have stage V oocytes. Animals whose ovaries do not contain stage VI oocytes can still be induced to ovulate the most mature (stage V) oocytes upon hCG stimulation (Dumont, 1972). Therefore, Cd exposure may affect physiological or behavioral aspects of ovulation and breeding.

In dd/YF mice, Watanabe et al. (1977) found that the ovary Cd concentration increased relative to the dose. Additionally, the incidence of degenerated oocytes was higher in the treated groups. In the present study the concentration of Cd in the ovaries of Cd-exposed frogs was not positively correlated with dose. The ovaries of the lowest exposure group (0.5 mg/Kg) exhibited a higher Cd concentration than the 0.75 and 1.0 mg/Kg exposure groups. Additionally, the percentage of Cd that was accumulated by the ovaries decreased with increasing exposure concentration. It is possible that at the higher concentrations the ovary became overloaded with Cd, causing necrosis of the ovary and the subsequent inability to accumulate any more toxicant. **Figure 6** shows the dramatic loss of cells at higher Cd concentrations and illustrates that cell loss may well indicate why less Cd is present at high exposure doses. Alternatively, if vitellogenesis

is the main mechanism by which the oocytes incorporate Cd and Cd inhibits vitellogenesis, then, at the higher exposure groups, vitellogenesis was inhibited, causing a marked decrease in the uptake of Cd. Maternal transfer of toxicants to offspring has numerous biological implications such as toxicity to progeny and transgenerational accumulation of contaminants (Standley et al., 1994). Possible mechanisms of maternal transfer in oviparous animals include incorporation into the lipids forming the egg mass, passive diffusion into mature eggs and active transport via vitellogenin. Some toxicants, such as organochlorines, are transferred from female to egg via lipids in mayflies (Standley et al., 1994) and in lake trout (Miller and Amrhein, 1995). Sato et al. (1996) found that transfer of Cd to eggs of leghorn chickens was restricted even when high amounts of Cd accumulated in the maternal liver. Cadmium accumulated in higher concentrations in the follicle walls rather than the follicle yolks.

Reproduction Assay Validation

Results from the present studies clearly demonstrate the reproductive and developmental toxicity of boric acid, Cd, and EGME in *X. laevis*. Based on these studies, it appears that both male and female gonads and gametogenesis were affected by the selected toxicants and that accumulation of each toxicant occurred in varying levels in gonadal tissue. Results from these studies indicate that boric acid is capable of affecting both male and female gametogenesis and reproduction, but only at high concentrations (100.0 mg B/L/d for 30 d). Although effects on gametogenesis and the gonads were observed in the 50.0-250.0 mg B/L/d for 30 d range, impact on reproductive performance was not noted until a concentration of 1,000.0 mg B/L/d was reached. In addition, a 20.8% reduction in sperm count was detected in males exposed to 50.0 mg B/L/d, although little effect on breeding response or success (fertilization and embryonic viability) was noted. The fertilization rate of oocytes from females exposed to 500.0 mg B/L/d was not reduced; however, the 96-h viability of the fertilized oocytes was significantly lowered. Malformations induced in progeny by maternal exposure to 500.0 mg B/L/d included gut maldevelopment, craniofacial defects, kinking of the notochord and microencephaly. Interestingly, similar effects have been induced in normal-appearing *X. laevis* embryos from unexposed mothers exposed to 500.0 mg B/L in the FETAX model (Fort et al., in press). These results suggest that similar modes of action on the developing embryo are occurring, regardless of

whether exposure is provided through the mother to the oocyte or directly to the developing embryo following fertilization. The teratogenic effects noted in studies applying post-fertilization exposure are significantly more dramatic in terms of the incidence of malformation and the severity of effects (Bantle and Fort, in press).

Effects on the testis have been observed in both subchronic and chronic studies in rats and mice exposed to B (Hubbard, 1998). After 14 d of treatment, doses of 93 mg B/Kg/d caused a reduction in spermiation (Ku et al., 1993). Testicular atrophy, degeneration of seminiferous tubules, reduced sperm count, and a reduction in fertility was observed in a three-generation study of boric acid toxicity in rats at 58.5 mg B/Kg (Weir and Fisher, 1972). In male rats fed B in the form of borax for either 30 or 60 d at 100.0 or 200.0 mg B/Kg/d (NOAEL, 50 mg B/Kg/d), testis weight was reduced, testicular germ cells were depleted, selected testicular enzymes were affected, and fertility was reduced (Lee et al., 1978).

In a continuous breeding study of boric acid in mice, a dose-related effect on the testis (testicular atrophy and effects on sperm, motility, morphology, and concentration) was noted; fertility was partially reduced at 111 mg B/Kg/d, and totally reduced at 221 mg B/Kg/d (Fail et al., 1991). In rats, at non-maternally toxic doses, there was a reduction in fetal body weight and some skeletal anomalies (Povlsen et al., 1990). With the exception of shortened 13th rib, these malformations had reversed by postnatal d 21 at 13.7 and 28.6 mg B/Kg/d in a study designed to look at postnatal recovery (Price et al., 1996). At higher, maternally toxic doses, other indications of developmental effects were observed, including resorptions and visceral malformations; however, these may have been secondary to the maternal toxicity (Heindel et al., 1992, and Price et al, 1991). In mice, the key developmental effects observed were similar to those seen in rats, including reduction in fetal body weight at the mid-dose and an increase in skeletal variations, malformations, and resorptions at the highest, more maternally toxic dose (Price et al., 1991).

In rabbits, developmental effects were seen only in the high dose, where the majority of the embryos were resorbed and malformations were primarily visceral (major heart and/or great vessel defects) (Price et al, 1991). Some skeletal variations and malformations were observed at high doses, similar to rats (Bussi, 1995); however, these effects may have been secondary to the maternal toxicity. A small, insignificant decrease in fetal body weight was also observed. The

NOAEL for both maternal and developmental toxicity in the rabbit was 21.8 mg B/Kg/d (Bussie, 1995).

Similar to boric acid, but at much lower concentrations (2.5 mg/L/d), Cd exposure to female *X. laevis* for 30 d via the culture water appreciably affected ovary weight, total oocyte counts, oocyte maturity and necrosis. Greater concentrations rendered all of the oocytes necrotic. Reproductive effects in males were also observed at ≥ 2.5 mg Cd/L/d, including reduced testis weight, decreased sperm count, and increased proportion of abnormal sperm cells. Effects on breeding success, rate of fertilization, and 96-h embryo viability were substantially more marked in treated females than in treated males. The effect of maternal Cd exposure on embryo-larval development was primarily embryolethal, although terata, gut mal-development, visceral edema, skeletal kinking of the notochord, craniofacial abnormalities, ruptured pigmented retina, and microencephaly were induced in the 2.5 mg/L/d treatment.

Cadmium has been shown to accumulate in vertebrates, with slow elimination by the kidneys (Vogiazis and Loumbourdis, 1997). A variety of mammalian models have examined the reproductive toxicity of Cd to adults and fetuses (IARC, 1993). Reproductive tissues such as the gonads and uterus readily accumulate Cd (Kostial, 1986). Acute effects of Cd in mammals include testicular necrosis, ovarian hemorrhaging, and delayed embryo implantation (Watanabe et al., 1977, and De et al., 1993). An examination of oogenesis in mice indicated an increase in chromosomal anomalies in the ovulated oocytes and fewer oocytes ovulated, and showed an increase in necrotic oocytes (De et al., 1993).

Reproductive endocrine function was disrupted in adult female Atlantic croaker (*Micropogonias undulatus*) after Cd exposure (Thomas, 1990) and Cd altered vitellogenin metabolism in flounder (*Platichthys flesus*) (Povlsen et al., 1990). In *Medaka*, early stages of development were the most sensitive and fertilized eggs accumulated most of the Cd in the chorion (Michibata et al., 1987). Continuous exposure of rainbow (*O. mykiss*) and brown (*S. trutta*) trout to Cd for 30-d did not affect the survival and growth of the adults, although eggs from *O. mykiss* failed to develop to the fry stage at ca. 2 $\mu\text{g/L}$, while oogenesis in *S. trutta* was delayed at ca. 9 $\mu\text{g/L}$. Pramoda and Saidapur (Pramoda and Saidapur, 1986) found that the weight of ovaries and oviducts of CdCl₂-exposed Indian bullfrogs (*Rana tigerina*) was decreased and that the vitellogenic growth of oocytes was impaired.

Teratogenic activity of Cd has also been demonstrated (Barr, 1973, and Webster, 1990). Malformations induced in mammalian models included craniofacial and palate defects, eye defects, hydroencephaly, and various limb malformations (Padmanabhan and Hameed, 1990). Interestingly, Weir et al. (1990) found that perfusing human placenta with low concentrations of Cd caused a change in oxygen tension and markedly reduced glucose and amino acid utilization, as well as reducing the synthesis and release of hCG. Because gonadotropin activity in the frog is responsible for ovulation and breeding, it is possible that these endocrine-disrupting effects are responsible for the reproductive toxicity associated with Cd exposure in *X. laevis*.

In female *X. laevis*, EGME significantly reduced ovary and oviduct weight and increased the rate of oocyte necrosis at a concentration of 0.5 mg/L. Decreased maturation of normal-appearing oocytes was noted at an EGME concentration of 0.75 mg/L. Normal-appearing oocytes collected from adults exposed to 1 or 5 mg/L EGME were either partially responsive or completely unresponsive to progesterone, respectively, when cultured *in vitro*. Reduced testis weight and atrophy were noted in males at 0.5 mg/L. Reduced sperm counts and increased dysmorphogenesis rates were detected at 1.0 mg/L. Females exposed to 1.0 mg EGME/L/d for 30 d in the culture water had a much greater proportion of immature oocytes in stages I and II than control females, which had a greater proportion of mature oocytes in stages V and VI (mature).

Although the reproductive toxicity of EGME is not as well understood as for boric acid and Cd, sufficient literature exists to classify EGME as a reproductive toxicant and suspect developmental toxicant, depending on the dose and route of administration. EGME has been shown to reduce fertility indices, reduce epididymis, prostate and seminal vesicle weights, and increase the length of the estrous cycle in Swiss CD-1 mice (Chapin and Sloane, 1997). Nagano et al. (1979) also reported that EGME induced testicular atrophy in mice. Characteristic malformations induced in mammals by exposure to EGME include skeletal defects, cardiovascular defects, vertebral anomalies, exencephaly, and limb (paw) defects (Nagano et al., 1981). These defects were similar in type to the abnormalities induced in *X. laevis* larvae from females exposed to EGME.

Co-culture of progesterone with stage VI oocytes *in vitro* induced successful GVBD from control females, but not in the EGME-treated females. A significant reduction in the number of

stage VI oocytes that successfully completed GVBD following progesterone stimulation *in vitro* from Cd-treated females was also observed when compared to the controls. Adult females exposed to reproductively toxic levels of B had no appreciable effect on progesterone-induced GVBD *in vitro*. Interestingly, however, chronic B deficiency substantially reduced oocyte progesterone responsiveness (Hausen and Riebesell, 1991).

In the present study, incomplete maturation of "normal appearing" EGME-treated oocytes was marked by size and incomplete GVBD. Normal GVBD was observed in oocytes from control females. Unlike the oocytes from control females, addition of progesterone *in vitro* did not significantly stimulate GVBD in oocytes from EGME-treated females. The inability of the oocytes which were "normal appearing" to mature in the presence of progesterone *in vitro* suggests that EGME or Cd may disrupt the maturation process at either the progesterone receptor level, or possibly in the cascade of secondary and tertiary messenger events responsible for inducing the maturation of the oocyte.

Under natural conditions, gonadotropins released from the pituitary gland of female *X. laevis* trigger ovulation and the egg laying response. Maturation of the oocyte is triggered by the binding of progesterone produced by the follicle in response to gonadotropin stimulation. The progesterone receptor in the *X. laevis* oocyte is associated with the plasma membrane, rather than existing as an intracellular receptor, which is common for steroid hormones (Eckberg, 1988; Kishimoto, 1988; Smith, 1989;). The morphological effects associated with progesterone binding include the breakdown of the germinal vesicle and formation of the maturation spindle. A cascade of intracellular events associated with second messenger activity occurs shortly after progesterone binding, marked by a transient increase in free Ca^{+2} , decreased levels of cAMP and adenylate cyclase activity, membrane depolarization, rise in intracellular pH, and increase in protein synthesis and non-ribosomal protein phosphorylation immediately prior to germinal vesicle breakdown.

A maturation-promoting factor (MPF) has been identified as the link between the aforementioned events and progesterone binding (Kishimoto, 1988). MPF is formed from a pool of pro-MPF immediately prior to germinal vesicle breakdown, but the protein responsible for this activation process has yet to be identified. Purified MPF, however, has been shown to induce normal maturation without signal transduction when injected into immature *X. laevis* oocytes

(Smith, 1989). Similar experiments to identify the responsiveness of the oocytes from the EGME-exposed females to purified MPF are currently being performed, along with an evaluation of expression and structural integrity of the oocyte progesterone receptor. Preliminary findings indicate that progesterone levels in the adult females exposed to either EGME (>1.0 mg/L/d) are similar to control animals, and that administration of human chorionic gonadotropin (hCG) stimulates the ovulatory response and the production of progesterone. Thus, it is unlikely that the effects induced on oocyte maturation by EGME are the result of decreased hormone synthesis or effects on regulatory feedback loops, which are also being studied.

A greater proportion of previtellogenic oocytes was noted with exposure to high concentrations of each toxicant, in which greater proportions of immature oocytes were found. Since the process of vitellogenesis does not begin until the oocyte is ca. 400 μm (stage III), the greater proportion of previtellogenic oocytes in the toxicant-treated females is most likely a secondary effect of the inability of the oocytes to mature; however, the effect of these test materials, particularly at lower concentrations, on the fecundity of the oocyte membrane vitellogenin receptor responsible for binding vitellogenin and yolk platelet incorporation in the oocyte will require further evaluation.

Data regarding the potential effects of endocrine-disrupting contaminants (EDCs) on reproductive function in amphibians are particularly lacking. On a larger scale, female reproductive function in amphibians could be affected by EDCs at a number of target sites, including the brain, pituitary, gonad, liver, and oviduct. Gonadal effects of EDCs have considerable potential to impair the reproduction of female amphibians and have been reported in other lower vertebrate wildlife groups. Female juvenile alligators from pesticide-contaminated Lake Apopka, Florida, exhibit a number of ovarian abnormalities, including polynuclear oocytes and polyovular follicles (Guillette et al., 1994), suppressed synthesis of 17β -estradiol (E_2), and reduced aromatase activity *in vitro* (Crain et al., 1997; Guillette et al., 1995). Although these organizational alterations in the structure and/or function of reproductive tissues may have the greatest potential impact on the reproductive fitness of a population (Guillette et al., 1995), activational effects of EDCs such as the modulation of endocrine signaling in the adult gonad may also significantly impair reproduction. Polyaromatic hydrocarbons (PAHs) have been shown to impair various aspects of ovarian function in adult fish. PAHs inhibited oocyte growth,

caused increased follicular atresia, and prevented final oocyte maturation in the Atlantic croaker (Thomas and Budiantara, 1995). Furthermore, the organochlorine compounds kepone and o,p'-DDD inhibited *in vitro* final maturation of Atlantic croaker oocytes, which is induced by the steroid $17\alpha,20\beta,21$ -trihydroxy-4-pregnen-3-one (20β -S) (Ghosh and Thomas, 1995). In light of the current study, Pickford and Morris (1999) found that although a series of natural and synthetic estrogens did not alter progesterone-induced oocyte maturation, the proestrogenic pesticide methoxychlor was a surprisingly potent inhibitor of progesterone-induced oocyte maturation. This inhibitory activity was specific to methoxychlor, rather than to estrogenic contaminants or its metabolites, and was not antagonized by the estrogen receptor antagonist ICI 182,780, suggesting that this activity is not estrogenic. Further, the inhibitory activity of methoxychlor was found to be dose dependent, reversible, and early acting.

Results from these studies demonstrate the importance of concurrently monitoring effects on the reproductive organs, gametogenesis, reproductive performance, and developmental fitness. Although potentially toxic effects on the gonad or gametogenesis in males did not necessarily translate to effects on either reproductive performance or developmental fitness, adverse transgenerational effects of B and Cu deficiency in adult female *X. laevis* have also been detected in developing offspring (Fort et al., 1999). The present work demonstrated that transgenerational effects of adult toxicant exposure can be monitored with the *X. laevis* reproductive toxicity battery. These studies clearly demonstrate transgenerational effects associated with adult *X. laevis* female exposure to Cd and EGME. These transgenerational effects were also observed with female exposure to B, but not to the same extent as noted with EGME and Cd. The *X. laevis* reproductive toxicity assay battery appears to be capable of monitoring reproductive toxicity, providing potential connective and mechanistic information concerning effects at the gonad or gamete level through embryo-larval development. The use of the progesterone-responsiveness assay with immature oocytes may also provide links between gamete production and endocrine disruption.

Studies examining maternal transfer of toxicants in amphibians have been limited. Grillitsch and Chovanev (1995) performed a field study in which they measured the concentrations of heavy metals and pesticides in anuran spawn, tadpoles, water, and sediment. Spawn, as defined by the authors, are embryos at Gosner (1960) stage 15 ± 2 , (neurula). They

found Cd, copper (Cu), lead (Pb) and zinc (Zn) residues in spawn and tadpole samples of *Bufo bufo*, *Rana dalmatina*, and *Rana ridibunda* at higher levels than sediment concentrations.

Although maternal transfer could be a source of metal contamination in spawn, the possibility of water related metal contamination should not be discounted.

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APPENDIX I:
TABLES

Table 1. Effect of Route of Exposure/Administration on Boric Acid (B), Cadmium (Cd), or Ethylene Glycol Monomethyl Ether (EGME) Accumulation in Xenopus Gonadal Tissue¹

Route	B ²		Cd ²		EGME ²	
	Testes	Ovaries	Testes	Ovaries	Testes	Ovaries
Control	320.0 (52.3)	94.6 (19.6)	<30.0 ³ (0.0)	<30.0 ³ (0.0)	<50.0 ³ (0.0)	<50.0 ³ (0.0)
Culture Water ⁴	405.0 (44.0)	178.0 (76.9)	60.0 (8.0)	256.8 (3.0)	326.5 (73.2)	549.3 (31.5)
Food ⁵	633.2 (52.2)	191.3 (31.3)	118.5 (9.1)	496.2 (230.0)	273.6 (19.5)	466.7 (43.2)
i.p. ⁶	580.6 (60.2)	309.2 (117.0)	3823.0 (306.0)	5851.7 (468.0)	1374.2 (99.8)	1217.6 (100.2)
s.c. ⁶	792.0 (79.4)	451.2 (90.2)	4530.0 (270.0)	6461.7 (517.0)	2463.5 (117.3)	2991.4 (129.5)

¹ Total B (ICP-MS), Cd (AAS-GF), and EGME (GC-MS). N = 4 frogs per dose regimen/toxicant.

² Expressed as µg/Kg with SE in parenthesis.

³ Practical Quantitation Limit (detection limit * dilution factor).

⁴ 100 mg B/L, 2.5 mg Cd/L, or 1.0 mg EGME/L exposure provided via culture water (FETAX Solution). 48-h renewal for 30 days.

⁵ Administered via B-, Cd-, or EGME-enriched liver. Total B administered = 10 mg/Kg/day for 30 days. Total Cd administered = 2.5 mg/Kg/day. Total EGME administered = 1.0 mg/Kg/day.

⁶ 100 mg B/Kg/day, 2.5 mg Cd/Kg/day, or 1.0 mg EGME/Kg/day administered daily for 5 days.

Table 2. Effect of Maternal Cadmium Exposure on Organ:Body Weight Ratios In Non-Stimulated and Stimulated Frogs¹

	Exposure Group mg/Kg	Liver (g)	Spleen (g)	Ovary (g)
Non-stimulated frogs	Control	0.042 (0.150)	0.001 (0.001)	0.134 (0.033)
	0.5	0.044 (0.010)	0.003 ² (0.001)	0.152 (0.025)
	3.0	0.045 (0.005)	0.007 ² (0.015)	0.131 (0.027)
	5.0	0.041 (0.007)	0.001 (0.000)	0.139 (0.030)
Stimulated Frogs	Control	0.034 (0.005)	0.001 (0.001)	0.015 (0.028)
	.05	0.109 (0.151)	0.003 (0.002)	0.077 (0.032)
	0.75	0.042 (0.005)	0.001 (0.001)	0.109 (0.055)
	1.0	0.040 (0.008)	0.003 (0.004)	0.143 (0.061)
	3.0	0.0449 (0.013)	0.003 (0.002)	0.086 (0.040)

¹ Represents mean value with standard error in parenthesis.

² Indicated significant difference from the control (Dunnett's Test, $p < 0.0001$).

Table 3. Results of Modified Toxicity Testing in Progeny of Cadmium-Exposed Maternal *Xenopus laevis*

Exposure Group (mg/Kg)	Mortality ¹	Malformation ¹	Embryo Length ²
Control	8.8 (11)	1.63 (11)	9.18 (0.04) (2044)
0.50	50.0 (1)	59.3 (1)	8.01 (0.04) (15)
0.75	40.6 ³ (4)	8.91 ³ (4)	8.50 (0.07) (404)
1.00	13.5 (4)	3.52 (4)	8.96 (0.08) (597)

¹ Expressed as % with n value in parenthesis. Frogs exposed at 3.0 mg/Kg did not breed.

² Expressed as mean \pm standard error with n value in parenthesis.

³ Significantly different from control group (Chi Square/Bonferonni Test, $p < 0.05$).

Table 4. Total Cadmium Concentration in the Ovary Tissue of Non-Stimulated and Stimulated Frogs and the Percent Accumulated by the Ovary, Relative to the Total Quantity Injected

	Exposure Group (mg/Kg)	N	Cd Concentration ¹	% Accumulation ²
Non-stimulated Frogs	Control	7	0.06 (0.11)	--- (---)
	0.5	6	0.65 (0.29)	3.11 (1.65)
	5	6	1.53 ³ (0.29)	0.74 (0.21)
Stimulated Frogs	Control	3	0.07 (0.06)	--- (---)
	0.5	8	9.18 ³ (2.93)	22.21 (7.21)
	0.75	6	5.02 (2.66)	10.10 (5.14)
	1.00	8	6.40 (3.09)	9.13 (3.13)
	3.00	14	9.23 ³ (3.14)	5.79 (2.17)

¹ Expressed as mean µg/g weight with standard error in parenthesis.

² Calculation of % Cd injected that was accumulated by ovary: ovary wt. (g) * ovary Cd concentration (µg/g ovary) = total Cd content in ovary (µg) / total Cd in ovary/amt Cd injected (µg) * 100 = % Cd injected that ovary accumulated. Standard error in parenthesis.

³ Significantly different from control group (Chi Square/Bonferonni Test, p < 0/05).

Table 5. Cadmium Concentration in Fertilized Eggs and Embryos of Stimulated Frogs Collected From Maternal Adults Treated With Cadmium¹

Exposure Group (mg/Kg)	Fertilized Eggs	24 h	48 h	72 h	96 h
Control	0.057 (0.035)	0.467 (0.035)	0.365 (0.127)	0.126 (0.0350)	0.058 (0.060)
0.75	0.543 (0.670)	0.929 (0)	0.879 (0.103)	1.866 (2.411)	0.089 (0)
1.00	0.505 (0.203)	0.722 (0.417)	1.135 (0.658)	0.551 (0.523)	0.224 (0.243)

¹ Expressed as $\mu\text{g/g}$ wet weight with standard error in parenthesis.

Table 6. Effect of Boric Acid on Reproductive Endpoints in *X. laevis*

Concentration ¹ (mg/L)	Female Parameters			Male Parameters				Breeding			
	Ovary Weight ² (%)	Egg Mass		Testes Weight ² (%)	Sperm		Amplexus ⁴ (%) ⁵	Fertilization (%) ⁵	Embryo Viability at 96 h ⁶ (%) ⁵		
		< Stage ³ (%)	≥ Stage ³ (%)		Count (x10 ⁶)	Abnormal (%)					
0.0	0.11 (0.03)	3173.0 (463.0)	12.5 (4.6)	82.1 (5.3)	7.2 (1.9)	0.05 (0.01)	2.4 (0.2)	0.2 (0.1)	100.0/100.0	86.3/87.4 (7.2)/(6.3)	97.1/95.2 (4.2)/(5.6)
1.0	0.09 (0.02)	3238.0 (296.0)	8.2 (2.3)	78.6 (7.3)	13.2 (5.1)	0.04 (0.01)	2.2 (0.4)	0.5 (0.3)	100.0/100.0	84.7/89.2 (8.3)/(5.9)	91.9/90.3 (3.8)/(4.2)
10.0	0.08 (0.01)	3116.0 (401.0)	21.1 (5.6)	69.3 (7.8)	9.6 (3.9)	0.06 (0.02)	2.5 (0.3)	0.8 (0.4)	100.0/100.0	86.3/86.2 (5.7)/(9.2)	94.2/92.3 (5.6)/(4.6)
50.0	0.09 (0.02)	2912.0 (431.0)	25.2* (4.7)	58.3* (9.9)	16.5* (5.6)	0.03* (0.01)	1.9 (0.1)	1.2 (0.3)	100.0/100.0	82.2/87.1 (6.4)/(7.6)	89.3/90.1 (8.3)/(5.6)
100.0	0.10 (0.03)	2648.0 (412.0)	41.2* (6.9)	30.6* (5.8)	28.2* (9.1)	0.02* (0.01)	2.0 (0.3)	3.2 (0.9)	100.0/100.0	81.8/83.4 (9.2)/(8.2)	79.2*/86.1 (4.9)/(9.3)
500.0	0.05* (0.02)	1256.0* (396.0)	31.3* (7.8)	16.0* (4.9)	53.7* (8.9)	0.02* (0.02)	1.5* (0.2)	12.6* (0.5)	75.0/100.0	83.2/88.1 (7.3)/(5.3)	58.7*/92.3 (8.7)/(6.1)
1000.0	0.03* (0.01)	748.0* (427.0)	0.0* (---)	0.0* (---)	100.0* (0.0)	0.01* (0.01)	0.9* (0.1)	18.3* (1.2)	0.0/0.0	---/--- (---)/(---)	---/--- (---)/(---)

¹ Concentration (expressed as B) in FETAX Solution. Adults were exposed to B for 30 d prior to evaluation. N = 4 for reproductive endpoint evaluation and 4 for breeding response.

² Expressed as % of body weight.

³ Based on Dumont stages.

⁴ Breeding response.

⁵ Presented as treated females bred with untreated males/treated males bred with untreated females.

⁶ FETAX assessment culturing embryos in FETAX Solution alone.

* Statistically different than the control (Dunnett's Test, P > 0.05)

Table 7. Effect of Boric Acid on Embryo-Larval Development in *X. laevis*

Concentration ¹ (mg/L)	Mortality ² (%)	Malformation ² (%)	Type of Terata
0	1.2 (0.4)	2.5 (0.3)	Gut mal-development
1.0	1.6 (0.7)	6.5 (0.7)	Gut mal-development
10.0	5.4 (0.9)	2.4 (1.1)	Gut mal-development
50.0	3.7 (0.8)	7.0 (2.4)	Gut mal-development
100.0	5.2 (1.1)	16.6* (3.4)	Gut mal-development, craniofacial defects
500.0	7.5 (1.3)	33.8* (4.6)	Gut mal-development, craniofacial defects, kinking of the notochord, microencephaly
1000.0	--- ³	--- ³	---

¹ Concentration of boric acid (expressed as B) exposed to adult females via culture water for 30 d.

² Based on 96-h developmental studies in FETAX Solution. Embryos from successfully bred treated females and untreated males were collected; N = 4 except for 500 mg/L treatments, in which N = 3 adult females. 80 embryos/frog/concentration were evaluated.

³ Not performed due to lack of normal appearing embryos.

* Statistically different than the control (Dunnett's Test, P > 0.05).

Table 8. Effect of Cadmium on Reproductive Endpoints in *X. laevis*

Concentration ¹ (mg/L)	Female Parameters			Male Parameters			Breeding	Embryo Viability at 96 h ⁶ (%) ⁵		
	Ovary Weight ² (%)	Egg Mass		Testes Weight ² (%)	Sperm				Amplexus ⁴ (%) ⁵	Fertilization (%) ⁵
		Total	< Stage ³ 3 (%)		≥ Stage ³ 3 (%)	Count (x10 ⁶)				
0.0	0.13 (0.04)	2874.0 (452.0)	19.4 (3.2)	77.0 (5.9)	13.6 (5.2)	0.07 (0.02)	2.3 (0.3)	0.4 (0.1)	100.0/100.0	89.4/89.8 (6.2)/(2.1)
0.5	0.11 (0.02)	2654.0 (336.0)	23.5 (4.2)	61.3 (7.1)	15.2 (3.8)	0.08 (0.01)	2.5 (0.4)	6.1 (4.1)	100.0/100.0	91.2/95.2 (9.5)/(3.8)
1.0	0.11 (0.03)	1923.0 (492.0)	25.9* (6.1)	32.6* (8.3)	41.5* (4.1)	0.06 (0.03)	2.1 (0.2)	15.4 (6.9)	100.0/100.0	86.3/90.3 (10.2)/(3.1)
2.5	0.08 (0.02)	1231.0* (562.0)	21.8* (5.9)	5.0* (3.2)	73.2* (8.6)	0.02* (0.02)	1.9 (0.4)	39.6 (3.9)	50.0/75.0	51.6*/85.2 (19.3)/(8.3)
5.0	0.06* (0.02)	394.0* (196.0)	0.0* (---)	0.0* (---)	100.0* (0.0)	0.03* (0.01)	1.5* (0.3)	52.8 (5.6)	0.0/50.0	---/83.2 (---)/(7.2)
10.0	0.02* (0.01)	152.0* (36.0)	0.0* (---)	0.0* (---)	100.0* (0.0)	0.01* (0.01)	0.3* (0.1)	98.4 (1.6)	0.0/0.0	---/9.5* (---)/(10.7)

¹ Concentration (expressed as B) in FETAX Solution. Adults were exposed to B for 30 d prior to evaluation. N = 4 for reproductive endpoint evaluation and 4 for breeding response.

² Expressed as % of body weight.

³ Based on Dumont stages.²⁸

⁴ Breeding response.

⁵ Presented as treated females bred with untreated males/treated males bred with untreated females.

⁶ FETAX assessment culturing embryos in FETAX Solution alone.

*Statistically different than the control (Dunnnett's Test, P > 0.05).

Table 9. Effect of Maternal Cadmium Exposure Prior to Breeding on Early Embryo-Larval Development in *X. laevis*

Concentration ¹ (mg/L)	Mortality ² (%)	Malformation ² (%)	Type of Terata
0	3.8	5.2	Gut mal-development
0.5	5.2 (1.4)	6.2 (1.9)	Gut mal-development, visceral edema
1.0	22.5* (3.7)	11.7* (2.6)	Gut mal-development, visceral edema, skeletal kinking
2.5	62.5* (4.7)	25.8* (3.5)	Gut mal-development, visceral edema, skeletal kinking, craniofacial defects, ruptured pigmented retina, microencephaly
5.0	---3	---3	---
10.0	---3	---3	---

¹ Concentration of Cadmium (expressed as Cd) exposed to adult females via culture water for 30 d.

² Based on 96-h developmental studies in FETAX Solution. Embryos from successfully bred treated females and untreated males were collected; N = 4 except for 500 mg/L treatments, in which N = 3 adult females. 80 embryos/frog/concentration were evaluated.

³ Not performed due to lack of normal appearing embryos.

* Statistically different than the control (Dunnett's Test, P > 0.05).

Table 10. Effect of Ethylene Glycol Monomethyl Ether (EGME) on Reproductive Endpoints in *X. laevis*

Concentration ¹ (mg/L)	Female Parameters					Male Parameters				Breeding	
	Ovary Weight ² (%)	Egg Mass		Testes Weight ² (%)	Sperm Count (x10 ⁶)	Sperm Abnormal (%)	Amplexus ⁴ (%) ⁵	Fertilization (%) ⁵	Embryo Viability at 96 h ⁶ (%) ⁵		
		< Stage ³ (%)	≥ Stage ³ (%)							Necrotic (%)	Total
0.0	0.14 (0.03)	13.8 (436.0)	76.8 (5.3)	0.08 (0.02)	2.4 (0.3)	0.2 (0.3)	100.0/100.0	89.9/90.1 (1.8)/(5.2)	95.6/92.1 (3.2)/(4.2)		
0.1	0.13 (0.02)	3215.0 (526.0)	75.2 (7.4)	0.07 (0.01)	2.5 (0.4)	0.5 (0.4)	100.0/100.0	88.2/92.3 (2.4)/(4.5)	92.8/95.2 (4.9)/(5.1)		
0.5	0.09 (0.02)	1959.0* (452.0)	54.3* (9.1)	0.04* (0.02)	2.1 (0.2)	4.3* (1.1)	100.0/100.0	79.3/93.5 (8.8)/(4.6)	79.3*/98.1 (7.8)/(3.8)		
0.75	0.05* (0.01)	860.0* (196.0)	15.9* (2.3)	0.02* (0.01)	1.6* (0.1)	31.3* (5.6)	50.0/50.0	59.9*/86.2 (5.8)/(5.9)	0.0*/89.2 (0.0)/(6.3)		
1.0	0.04* (0.02)	532.0* (159.0)	2.2* (0.8)	0.02* (0.01)	0.7* (0.09)	69.3* (10.2)	50.0/50.0	31.3*/73.2* (9.3)/(8.9)	0.0*/82.5* (0.0)/(7.9)		
5.0	0.01* (0.01)	0.0* (0.0)	--- (---)	0.01* (0.01)	0.03* (0.01)	100.0* (0.0)	0.0/0.0	---/--- (---)/(---)	---/0.0 (---)/(0.0)		

¹ Concentration (expressed as B) in FETAX Solution. Adults were exposed to B for 30 d prior to evaluation. N = 4 for reproductive endpoint evaluation and 4 for breeding response.

² Expressed as % of body weight.

³ Based on Dumont stages.²⁸

⁴ Breeding response.

⁵ Presented as treated females bred with untreated males/treated males bred with untreated females.

⁶ FETAX assessment culturing embryos in FETAX Solution alone.

* Statistically different than the control (Dunnnett's Test, P > 0.05).

Table 11. Effect of Maternal Ethylene Glycol Monomethyl Ether (EGME) Exposure Prior to Breeding on Early Embryo-Larval Development in *X. laevis*

Concentration ¹ (mg/L)	Mortality ² (%)	Malformation ² (%)	Type of Terata
0	3.8 (1.2)	0.4 (0.2)	Gut mal-development
0.1	1.0 (0.3)	6.2 (0.9)	Gut mal-development
0.5	2.2 (0.6)	18.5* (3.4)	Gut mal-development, pericardial edema, mal-developed aorta, notochord kinking
0.75	23.5* (4.3)	76.5* (10.2)	Gut mal-development, pericardial edema, mal-developed aorta, notochord kinking, hydroencephaly
1.0	36.3* (4.9)	100.0* (0.0)	Gut mal-development, pericardial edema, mal-developed aorta, notochord kinking, hydroencephaly, visceral hemorrhage
5.0	---	---	---
	(---)	(---)	

¹ Concentration of ethylene glycol monomethyl ether (expressed as EGME) exposed to adult females via culture water for 30 d.

² Based on 96-h developmental studies in FETAX Solution. Embryos from successfully bred treated females and untreated males were collected; N = 4 except for 500 mg/L treatments, in which N = 3 adult females. 80 embryos/frog/concentration were evaluated.

* Statistically different than the control (Dunnett's Test, P > 0.05).

Table 12. Effect of Boric Acid, Cadmium, and Ethylene Glycol Monomethyl Ether (EGME) Exposure on Progesterone Responsiveness in *X. laevis* Oocytes¹

Test Material	Exposure Concentration² (mg/L)	Progesterone Responsive (%)
Control	0.0	98.2 (3.1)
Boric Acid	100.0	92.8 (10.2)
Cadmium	1.0	89.8 (15.8)
EGME	0.5	23.5 ³ (11.5)

¹ Responsiveness indicated by maturation of normal-appearing stage VI oocytes to undergo germinal vessicle breakdown over a 24-h period when cultured with 1 μ M progesterone *in vitro*; n = 4 females per test material.

² Expressed as total B, total Cd, and EGME.

³ Statistically less than the control (Dunnett's Test, P > 0.05).

APPENDIX II:
FIGURES

FIGURES

LEGENDS

Figure 1. Effect of Maternal Cadmium Treatment on *Xenopus* Oocyte Development in Frogs Not Stimulated with Human Chorionic Gonadotropin. Mean percentage of oocytes present at each stage of oogenesis. Error bars indicate standard deviation. Number of frogs: Control, 7; 0.5 mg, 7; 3.0 mg, 7; 5.0 mg, 14.

Figure 2. Effect of Maternal Cadmium Treatment on *Xenopus* Oocyte Development in Frogs Not Stimulated with Human Chorionic Gonadotropin. Mean number of oocytes present at each stage of oogenesis. Error bars indicate standard deviation. Number of frogs: Control, 7; 0.5 mg, 7; 3.0 mg, 7; 5.0 mg, 14.

Figure 3. Effect of Maternal Cadmium Treatment on *Xenopus* Oocyte Development in Frogs Stimulated with Human Chorionic Gonadotropin. Mean percentage of oocytes present at each stage of oogenesis. Error bars indicate standard deviation. . Number of frogs: Control, 3; 0.5 mg, 7; 3.0 mg, 7; 5.0 mg, 14.

Figure 4. Effect of Maternal Cadmium Treatment on *Xenopus* Oocyte Development in Frogs Stimulated with Human Chorionic Gonadotropin. Mean number of oocytes present at each stage of oogenesis. Error bars indicate standard deviation. Number of frogs: Control, 3; 0.5 mg, 8; 0.75 mg, 6; 1.0 mg, 8; 5.0 mg, 14.

Figure 5. Effects of Maternal Cadmium Treatment on Frogs Not Stimulated with Human Chorionic Gonadotropin. Oocytes from (A) control female in which stage II-VI oocytes are clearly visible, (B) from females exposed at 0.5 mg/kg (noticeable change in morphology), (C) from female exposed at 3.0 mg/kg (majority of oocytes are atretic and completely degenerated).

Figure 6. Percent Incidence of Malformations in Tadpoles after Maternal Exposure to Cd. There were no facial malformations in the 0.5 mg treatment group.

Figure 7. Effects of Transgenerational Cadmium Exposure on *Xenopus* Embryo Development. The adult frogs were stimulated with human chorionic gonadotropin prior to the start of exposure. After the exposure period exposed females frogs were mated to control males and the resulting offspring were cultured in FETAX solution for four days. (A) Stage 46 tadpole from control female. (B) and (C) Stage 46 tadpoles from stimulated female exposed to 0.75 mg/kg cadmium.

Figure 8. Effects of Transgenerational Cadmium Exposure on *Xenopus* Embryo Development. The adult frogs were stimulated with human chorionic gonadotropin prior to the start of exposure. After the exposure period exposed females frogs were mated to control males and the resulting offspring were cultured in FETAX solution for four days. (A) Stage 46 tadpole from control female. (B) Stage 46 tadpole from stimulated female exposed to 0.75 mg/kg cadmium.

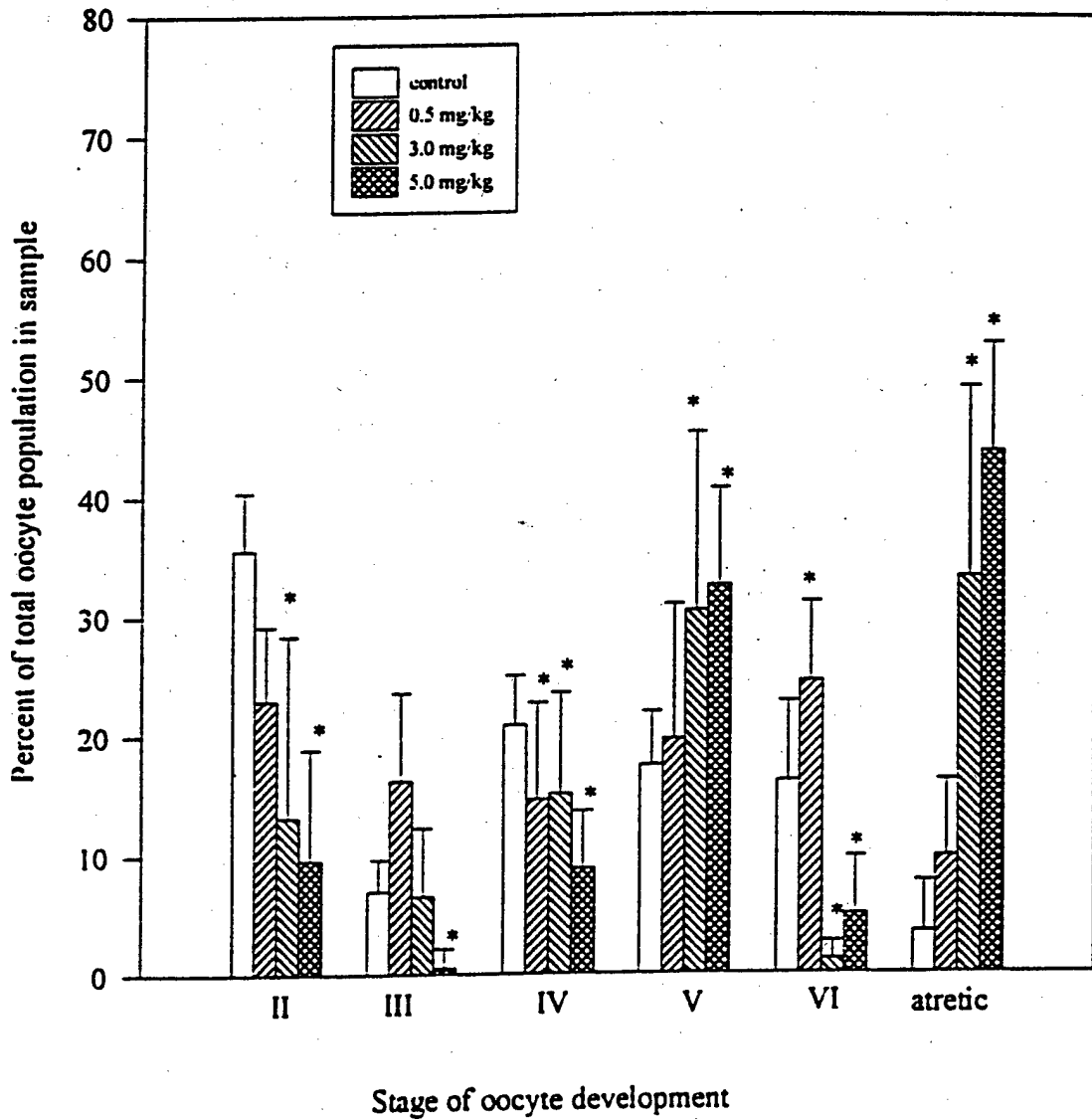


Figure 1. Mean percentage of oocytes present at each stage of oogenesis in non-stimulated frogs. Error bars indicate standard deviation. (* denotes $p < 0.05$)

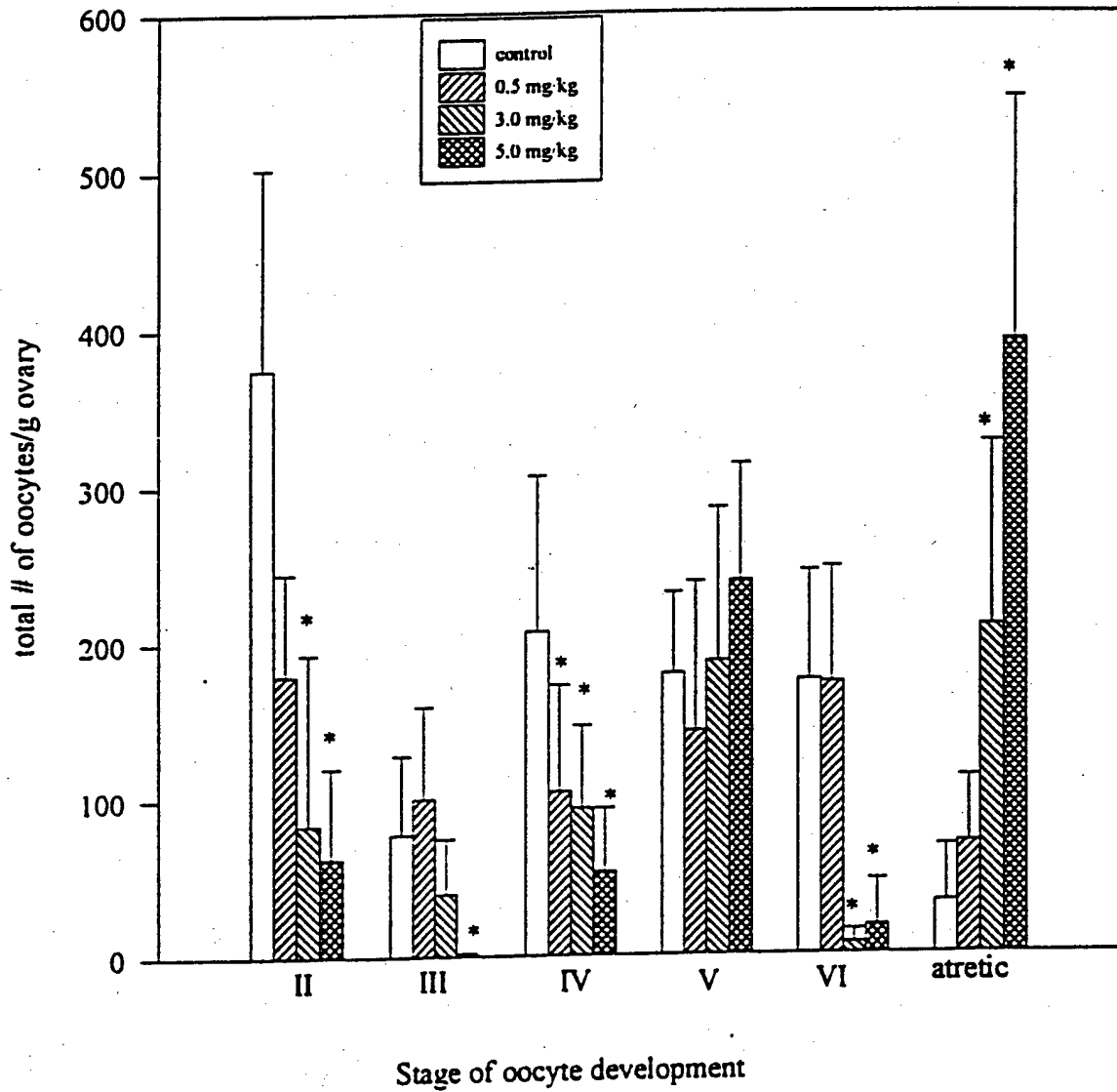


Figure 2. Mean number of total oocytes/g ovary present at each stage of oogenesis in hCG-non-stimulated frogs. Error bars represent standard deviation. (* denotes $p < 0.05$)

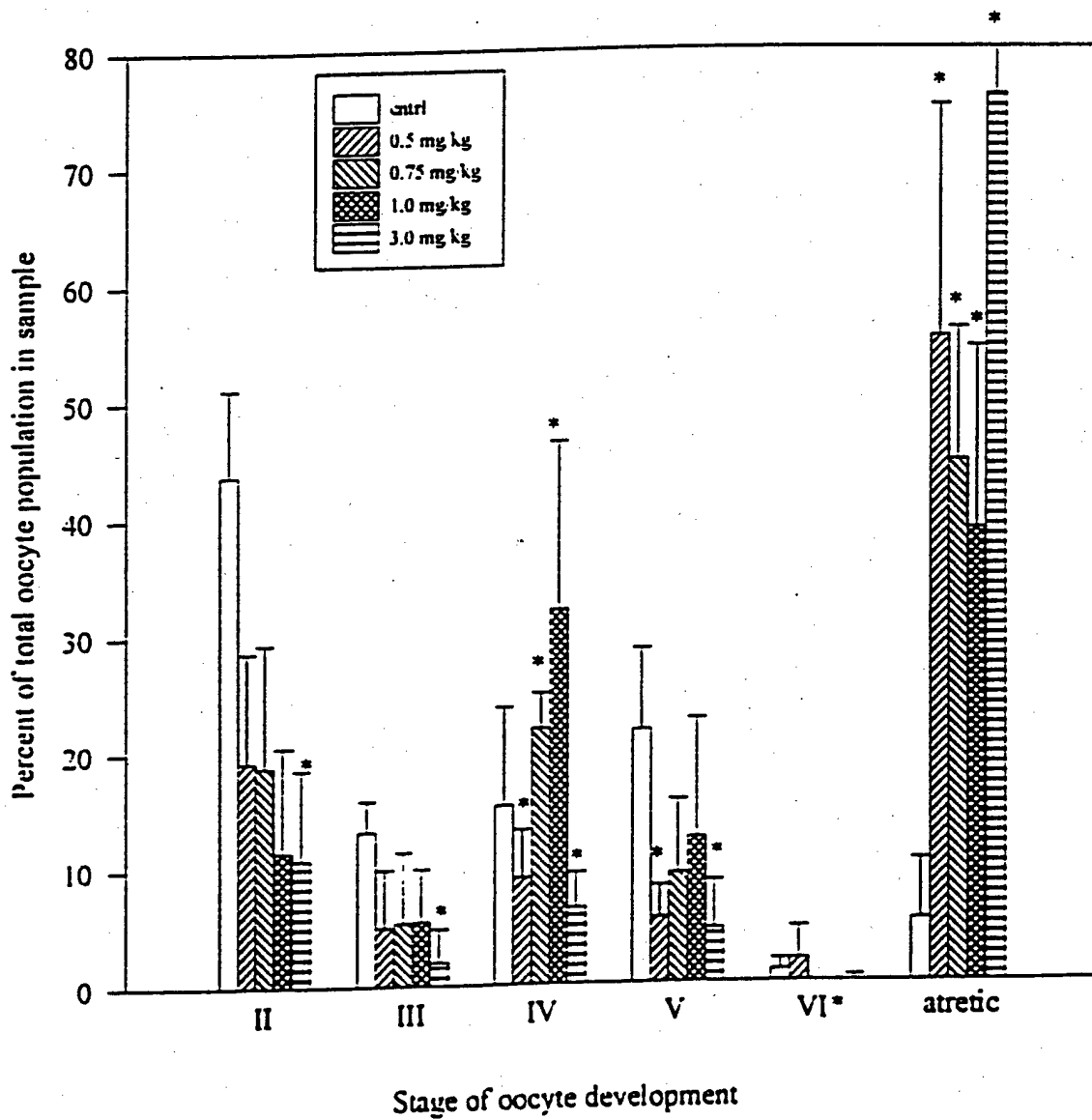


Figure 3. Mean percentage of oocytes present at each stage of oogenesis in hCG-stimulated frogs. Error bars represent standard deviation. (* denotes $p < 0.05$)

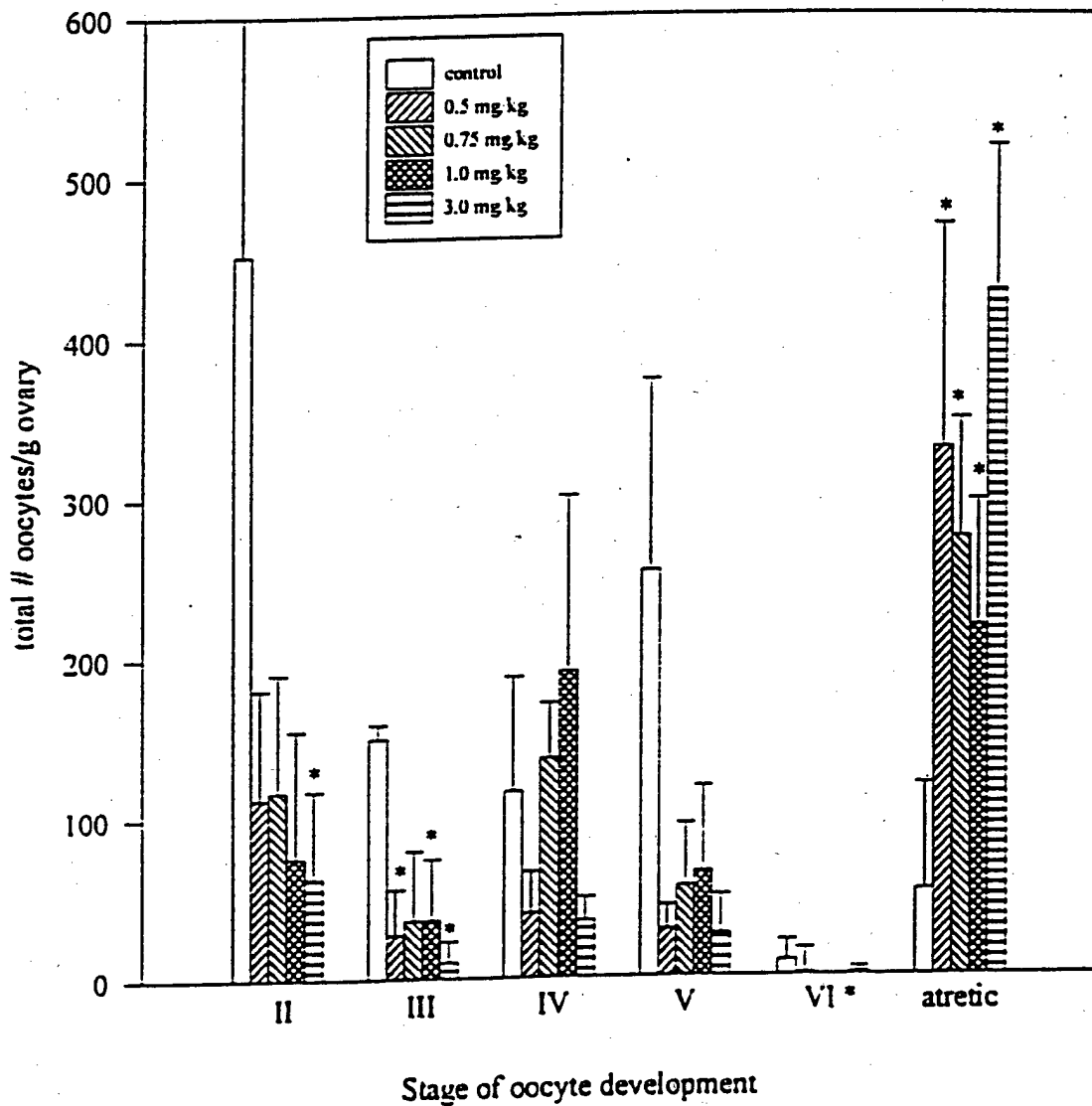


Figure 4. Mean number of total oocytes/g ovary present at each stage of oogenesis in hCG-stimulated frogs. Errors bars represent standard deviation. (* denotes $p < 0.05$)

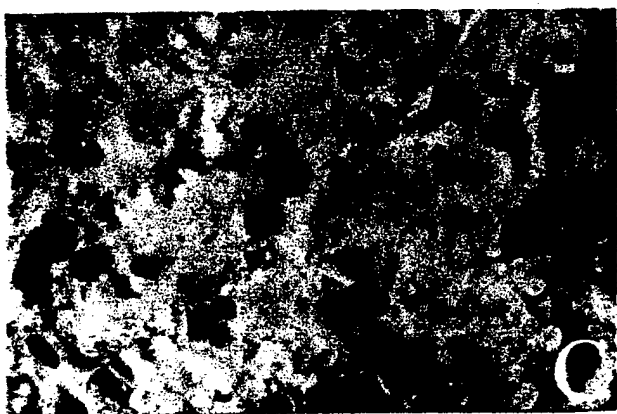
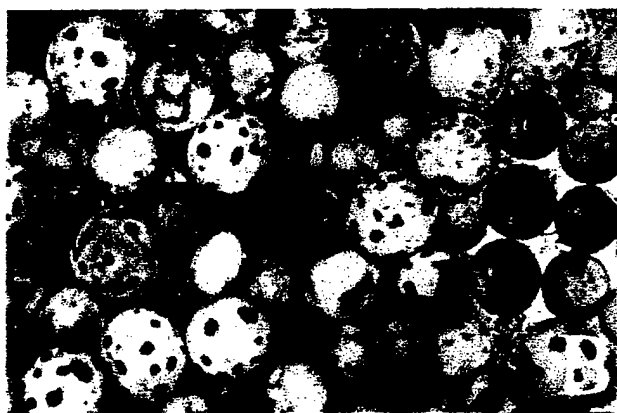
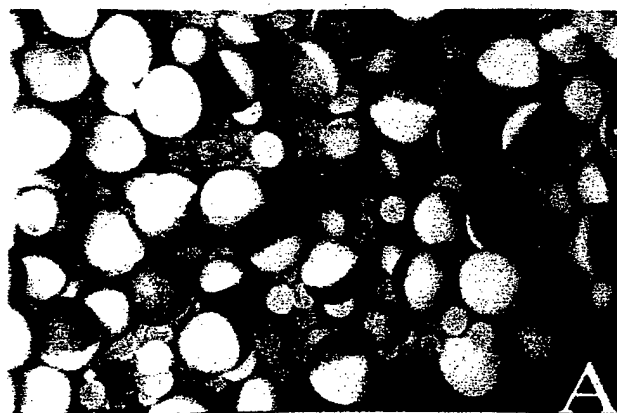


Figure 5. Oocytes from control female (A). Stage II oocytes appear opaque, stage IV-VI oocytes have distinct animal (dark) and vegetal (light) hemisphere. (B) oocytes from female exposed at 0.5 mg/kg. Morphology of oocytes is different from controls. Numerous oocytes are speckled or mottled and viability is questionable. (C) oocytes from female exposed at 3.0 mg/kg. Completely degenerated oocyte follicles appear as darkly pigmented spheres.

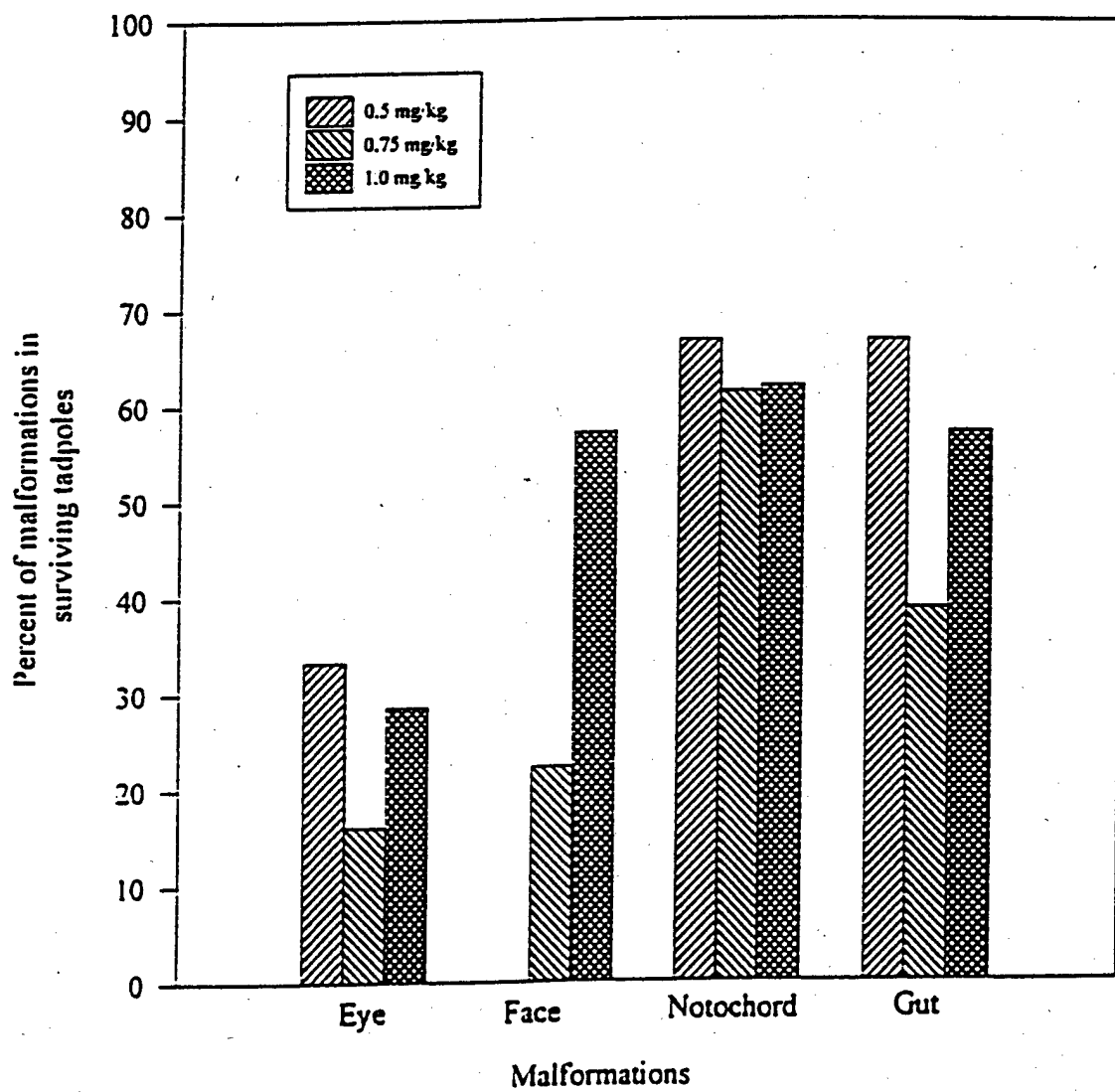


Figure 6 Incidence of malformations in tadpoles after maternal exposure to Cd.



Figure 7. Notochord malformations. (A) embryo from control female. (B) and (C) embryos from female exposed at 0.75 mg/kg.

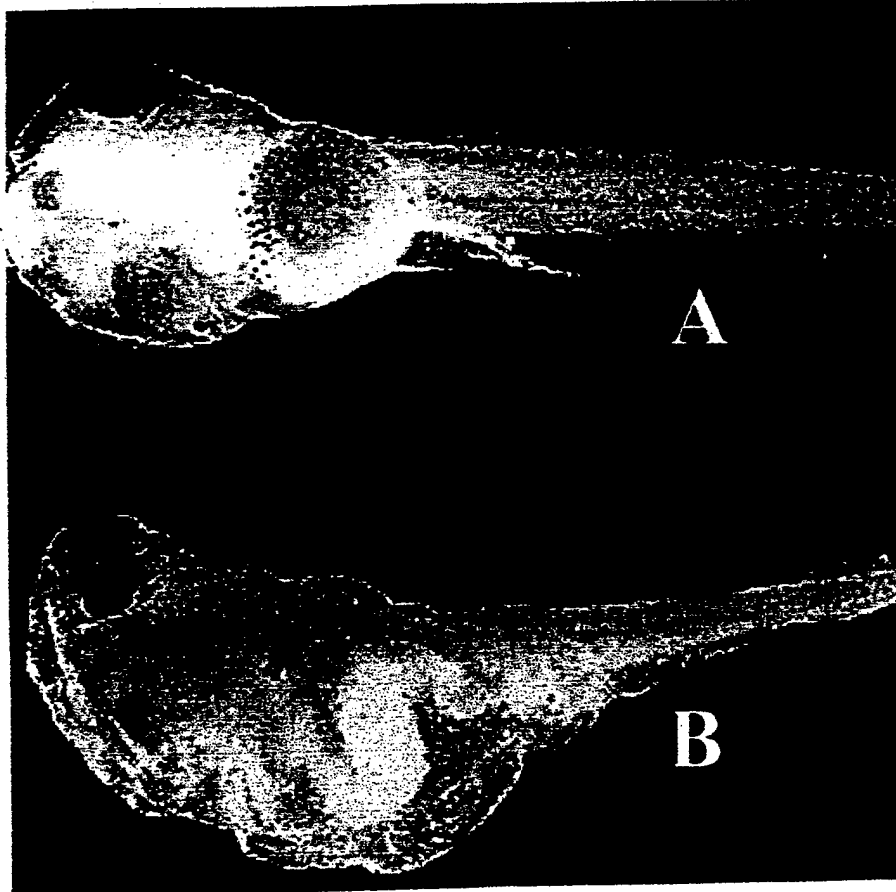


Figure 8. Gut malformations. (A) embryo from control female. (B) embryo from female exposed at 0.75 mg/kg.

**APPENDIX III:
JOURNAL ARTICLE**

Evaluation of a Reproductive Toxicity Assay Using *Xenopus laevis*: Boric Acid, Cadmium, and Ethylene Glycol Monomethyl Ether

Short title: *Xenopus* Reproductive Toxicity Assay

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Abstract

Cadmium (Cd), boric acid (B), and ethylene glycol monomethyl ether (EGME) were evaluated for reproductive and developmental toxicity in *X. laevis*. Eight reproductively mature adult male and eight superovulated female *X. laevis* were exposed to at least five separate sublethal concentrations of each material via the culture water for a period of 30 d. Four respective pairs were mated and the offspring evaluated for developmental effects; an evaluation of reproductive status was performed on the remaining four specimens. Ovary pathology, oocyte count, oocyte maturity and maturation capacity (germinal vesicle breakdown [GVBD]), and necrosis were evaluated in the female, while testis pathology, sperm count, dysmorphology, and motility were studied in the male. Based on this assessment, each test material exerted reproductive toxicity in *X. laevis*, but with varying potencies. Adult female exposure to Cd and EGME, particularly, and, to a lesser extent, B, resulted in transgenerational toxicity to the developing progeny. Further, this model appears to be a useful tool in the initial assessment and prioritization of potential reproductive toxicants for further testing.

Key words: *Xenopus*, reproductive toxicity assay, cadmium, boric acid, ethylene glycol monomethyl ether, transgenerational toxicity

Introduction

Although the use of *Xenopus laevis* as a model system for evaluating reproductive and developmental toxicity is currently being assessed by the Endocrine Disruptor Screening and Testing Advisory Council (EDSTAC) as a Tier 2 test, standardized and validated methods of evaluating reproductive toxicity in amphibians have not yet been fully developed. Because standard mammalian reproductive toxicity models rely on either gonadal or post-fertilization effects, a toxicity model providing information on the effects of potential toxicants on reproductive performance, as well as gametogenesis, is warranted. Amphibians represent a suitable model for simultaneously monitoring reproductive performance and gametogenesis. *X. laevis* also serves as an excellent model to evaluate early embryonic development.¹⁻⁵ Collection of simultaneous information on the effects on gametogenesis and reproductive outcome not only provides hazard assessment information, but also provides mechanistic clues concerning the modes of action of reproductive toxicants.

Therefore, a reproductive toxicity assay battery was developed using *X. laevis* focusing on 1) gametogenesis, 2) reproductive performance, and 3) embryonic development. In this report, development of this reproductive toxicity test battery with boric acid,⁶⁻¹³ cadmium,¹⁴⁻²⁰ and ethylene glycol monomethyl ether,²¹⁻²³ three toxicants shown to possess varying degrees of reproductive toxicity in mammals, is described. Prior to initiating reproductive toxicity studies, an evaluation of toxicant delivery was performed to determine which route was most effective, as well as relevant.

Gametogenesis endpoints monitored in the *X. laevis* battery included ovary/testes pathology, oocyte and sperm counts, oocyte maturity and maturation capacity (germinal vesicle breakdown [GVBD]), oocyte necrosis and viability, and sperm dysmorphology. Breeding success (amplexus), fertilization, and 4-d embryo-larval viability (mortality and teratogenesis) were also evaluated. Based on the present studies, each of the three test materials displayed varying degrees of reproductive toxicity in the *X. laevis* system.

Overall, these results suggest that this model will be a useful tool in the initial assessment and prioritization of potential toxicants for further reproductive toxicity testing.

Materials and Methods

Chemicals and Biochemicals

Boric acid (99%, expressed as boron, B), cadmium chloride (>99%, expressed as Cd), ethylene glycol monomethyl ether (99%, expressed as EGME), and human chorionic gonadotropin (hCG) were obtained from the Sigma Chemical Company (St. Louis, MO). FETAX Solution salts were purchased from Fisher Scientific (Houston, TX). All other reagents used were obtained from the Aldrich Chemical Company (Milwaukee, WI). FETAX Solution (amphibian culture media) consisted of 625 mg NaCl, 96 mg NaHCO₃, 30 mg KCl, 15 mg CaCl₂, 60 mg CaSO₄•2H₂O, and 75 mg MgSO₄ per liter of deionized water.²⁴⁻²⁶

Adult Care and Culture

For the route of exposure studies, one hundred and twenty adult *X. laevis* (60 female and 60 male, *Xenopus* I, Dexter, MI) were maintained in groups of eight (by sex) in polyethylene tubs and were fed ground beef liver *ad libitum*, as described previously.²⁵⁻²⁶ Following 14 d of acclimation, four female and four male frogs were administered either boric acid, Cd, or EGME as described in the following sections.

For the reproductive toxicity studies, 96 newly acquired adult *X. laevis* (48 female and 48 male, *Xenopus* I, Ann Arbor, MI) were obtained for evaluation with each test material. The adults were fed, housed, and acclimated as described above.

Route of Administration Evaluation

In an effort to determine the most efficacious route of test material administration, eight adult *X. laevis* (four male and four female) were administered either boric acid, CdCl₂, or EGME via the culture water,

food, or by i.p. or s.c. injections. Efficacy of the delivery method for the purposes of the present study was determined not only by which route delivered the most toxicant to the reproductive organs, but also by ease of administration and the environmental relevance of the route. For exposure via the culture water, 100 mg B/L, 2.5 mg Cd/L, and 1.0 mg EGME/L were provided via the culture water (FETAX Solution). Culture water was renewed every 48 h for a total exposure period of 30 d. Enteral toxicant administration was provided using B-, Cd- or EGME-enriched beef liver. The total B, Cd, or EGME administered was 10 mg/Kg/day, 2.5 mg/Kg/day, or 1.0 mg/Kg/day, respectively, for 30 d. I.p. or s.c. injections of 100 mg B/Kg/day, 2.5 mg Cd/Kg/day, or 1.0 mg EGME/Kg/day for five concurrent days were performed. For the s.c. route, the injection was made directly into the dorsal lymph sac. At the conclusion of the administration studies and immediately prior to euthanasia, the frogs were weighed and anesthetized with 1% w/v 3-aminobenzoic acid, ethyl ester (MS-222). A 10% solution (w/v) of MS-222 at a dose of 0.2 mL per 50 g of body weight was injected into the dorsal lymph sac of the animal to be euthanized.²⁶ Gonadal and liver tissue from each specimen was collected and analyzed for each respective test material. Livers were collected for gross pathology to determine systemic toxicity of each compound.

Reproductive Toxicity Evaluation

Female Assessment

Exposure, Sample Collection and Breeding

Healthy female frogs were superovulated and bred by injecting 750 IU of human chorionic gonadotropin (hCG) into the dorsal lymph sac s.c.²⁵⁻²⁷ Within 3-4 hours post injection, ovulation commenced. For breeding, females were paired with males injected with ~500 IU hCG s.c. Each mating pair was placed in a polyethylene breeding chamber and allowed to breed overnight. In order to ensure that each female fully discharged the majority of mature oocytes, each female was squeezed gently along the flanks at the

anterior portion of the ovary with posterior movement down the oviduct so as to strip the ovary of oocytes not yet released. Each female was then placed into her respective treatment for 30 d, at which time a thorough evaluation of reproductive status was performed. Four of the eight females were paired with unexposed males to evaluate breeding response as previously described. The females were weighed and anesthetized with 1% w/v MS-222. The ovaries, oviducts and livers were surgically removed prior to euthanasia. A 10% solution (w/v) of MS-222 at a dosage level of 0.2 mL per 50 g of body weight was injected into the dorsal lymph sac of the animal to be euthanized. The weight of each organ was determined, and the ovary and liver were further processed for pathological evaluation.

Gametogenesis

Oocyte staging was performed in accordance with the method of Dumont,²⁸ in which stages are based on maturity and numbered from I to VI, with I representing the most immature oocytes and VI representing fully mature, banded oocytes. A thorough count of all oocytes was then performed. Once this information was collected, this process was repeated twice to verify the data collected. The number of necrotic oocytes was also determined.

***In Vitro* Oocyte Maturation - GVBD Assay**

Ovaries were excised and placed in sterile, chilled Ca^{2+} /phenol red-free Hanks balanced salts solution, pH 7.6, and diluted to 230 milliosmoles (mOsm) with distilled water (Hanks O) in accordance with the methods of Pickford and Morris.^{28,29} Ovarian tissue was then cut into strips, rinsed, and incubated overnight at 4°C in Hanks O. Tissue was then digested for 75 min. in a 0.2% solution of collagenase D in Hanks O until all of the follicle cell layer was removed from the oocytes. After sufficient digestion, the oocytes were rinsed again in fresh Hanks O and transferred to a disposable 100-mm Petri dish containing modified defined nutrient oocyte medium (mDNOM, pH 7.6).^{28,29}

Large, banded, pre-ovulatory stage VI oocytes were selected by hand using a sterile Pasteur pipette under a dissecting microscope and plated 20/well in sterile 36-well Pyrex glass culture plates in 2

mL mDNOM. One μM progesterone was added and the plates were incubated in a shaker at room temperature (20-23°C) for 24 hr. The medium was then aspirated and the oocytes were fixed in 5% (w/v) trichloroacetic acid. Maturation was visible externally as a white "Roux" spot that indicates where the spindle has anchored to the plasma membrane at the animal pole of the oocyte. GVBD was verified by cracking open representative samples from the fixed oocyte. The maturational response of 20 oocytes in each well was expressed as the percentage exhibiting GVBD, and the mean maturational response for each treatment combination represents a minimum of four replicate wells.

Male Assessment

Breeding and Sample Collection

Following 30 d of exposure, four male adults were collected and used to breed with respective females. The remaining four adult males were also anesthetized at 30 d of exposure. At this time the testes and liver were removed and the specimen euthanized. One testis was used exclusively for determining sperm counts, whereas the other, which was sectioned rather than minced/homogenized, was used to determine rates of dysmorphology and for histopathological examination.³⁰ The frogs were anesthetized as described previously for the females. The testes were removed and any remaining fat was trimmed from the testis. Each testis was weighed and the information recorded.

Sperm Count

For the total sperm count, saline-merthiolate-Triton (SMT) [1 mL/10 mg tissue] [0.9% (w/v) NaCl, 0.01% (w/v) merthiolate, 0.05% (v/v) Triton X-100] was used to maintain the tissues during homogenization.³⁰ Testes were placed in a clean scintillation vial with SMT, minced with scissors, and homogenized (Powergen® 125, Fisher Scientific, Houston, TX) for two min. A sample was then placed into a hemacytometer and the spermatids counted. At least three chambers were counted for each sample. If the totals were not within 10%, the samples were recounted.

Dysmorphology Assessment

Dysmorphology was assessed by fixing the testis in 10% (w/v) formalin, pH = 8.0. The preserved sperm cells were then surveyed. The total number of abnormal sperm was counted and the types of abnormalities recorded. Several testes were surveyed per testis to ensure that the rates fell within 10% of one another. Sections were also evaluated for overt pathology.

Data Analysis

Reproductive status, including ovary and testis weights and pathology, total egg count, oocyte necrosis, oocyte stage distribution, maturation capacity, sperm counts and sperm dysmorphology rates were determined for each adult. Breeding success, fertilization rates, and embryonic viability were also determined. Comparisons of reproductive fitness evaluations were performed using ANOVA (Dunnett's Test, $P < 0.05$).

Chemical Analysis

B concentrations were measured in the biological samples using inductively coupled plasma-mass spectrometry (ICP-MS) analysis. Cd and EGME were determined by graphite furnace-atomic absorption spectroscopy (AAS-GF) and mass selective gas chromatography (GC-MS), respectively. Practical quantitation limits (method detection limit * dilution factor) for B, Cd, and EGME were 10.0 $\mu\text{g}/\text{Kg}$, 17.5 $\mu\text{g}/\text{Kg}$, and 50.0 $\mu\text{g}/\text{Kg}$, respectively, for the biological samples.

Results

Route of Administration

Results from the route of administration studies are presented in Table 1. With each of the three test materials, the parenteral routes, specifically s.c. injection into the dorsal lymph sac, was the most effective in terms of the amount accumulated in the gonads. For each toxicant, however, Similar trends of accumulation were observed in the gonads via the enteral and parenteral routes. Because each toxicant accumulated when administered via the culture water, exposure via the culture water was selected for the reproductive toxicity evaluation since it represented an environmentally realistic route of exposure.

Reproductive Toxicity Evaluation

Boric Acid

Female Assessment

Although some B accumulation was found in the livers of females (control = $43.2 \pm 17.2 \mu\text{g B/Kg}$; 1,000 mg B/L treatment = $108.0 \pm 23.2 \mu\text{g B/Kg}$) exposed to 1,000.0 mg B/L, no overt pathology was noted. The effects of 30-d B exposure via culture water on female reproductive endpoints are provided in Table 2. Results indicated that a significant reduction (Dunnett's Test, $P < 0.05$) in ovary weight occurred in specimens exposed to 500.0 mg B/L. General necrosis and histopathology of the ovary was observed at 1,000.0 mg B/L. A significant reduction (Dunnett's Test, $P < 0.05$) in the total number of oocytes was found in females exposed to 500.0 mg B/L for 30 d via the culture water. Increased rates of oocyte necrosis were noted in the 50.0 mg B/L treatment, with increasing necrosis occurring with increasing B concentration. Oocytes collected from females exposed to 1,000.0 mg B/L were all

necrotic. In addition, the proportion of oocytes (> stage 3) decreased in a concentration-dependant manner, with a significant effect (Dunnett's Test, $P < 0.05$) noted at 50 mg B/L.

Successful breeding responses as determined by amplexus occurred with each of the females exposed to B concentrations ranging from 0.0 to 100.0 mg/L. Three of the four females (75%) exposed to 500.0 mg B/L for 30 d via the culture media successfully bred. None of the four females exposed to 1,000.0 mg B/L initiated breeding activity. Interestingly, no effect on fertilization rates was observed with females exposed to B concentrations ranging from 0.0 to 500.0 mg/L for 30 d in the culture media; however, a significant reduction (Dunnett's Test, $P < 0.05$) in 96-h embryo viability was detected at both the 100.0 and 500.0 mg B/L treatments.

The effect of maternal B exposure on early embryo-larval development is provided in Table 3. Although no statistically significant (Dunnett's Test, $P < 0.05$) increases in mortality were detected, a significant increase (Dunnett's Test, $P < 0.05$) in abnormal development was observed in fertilized embryos collected from females exposed to 100.0 and 500.0 mg B/L for 30 d and cultured in FETAX Solution for 4 d. Female exposure to 500.0 mg B/L for 30 d induced abnormal development of the craniofacial region, gut, kinking of the notochord, and microencephaly.

Male Assessment

The effect of boric acid on male reproductive endpoints and reproductive performance is presented in Table 2. As with the female *X. laevis* exposed to 1,000.0 mg B/L, some accumulation of B was noted (control = 31.5 ± 15.2 $\mu\text{g B/L}$; 1,000.0 mg B/L = 136.3 ± 29.4 $\mu\text{g B/L}$); however, no overt pathological effects were observed in the male specimens. Exposure to 50.0 mg B/L for 30 d significantly reduced (Dunnett's Test, $P < 0.05$) testis weight, although overt pathology was only noted in males exposed to 1,000.0 mg B/L. The sperm count was significantly reduced and the rate of sperm dysmorphology increased at a concentration of 500.0 mg B/L. The rate of sperm dysmorphology in males exposed to 1,000.0 mg B/L for 30 d was $18.3 \pm 1.2\%$. No effect on breeding response was noted except at 1,000.0

mg B/L, at which level amplexus was not induced in any of the four males bred. In addition, no significant reduction (Dunnett's Test, $P < 0.05$) in fertilization rates or embryonic viability at 96-h was observed.

Cadmium

Female Assessment

Cd accumulation in the liver of females exposed to 10.0 mg Cd/L was appreciable (control = $<30.0 \mu\text{g Cd/Kg}$; 10 mg Cd/L treatment = $192.3 \pm 38.6 \mu\text{g Cd/L}$), and moderate pathological effects, including decreased weight and discoloration, were noted; however, no liver pathology was observed in animals exposed to concentrations $<10.0 \text{ mg Cd/L}$. The effects associated with 30-d Cd exposure via the culture water on female reproductive endpoints are presented in Table 4. A significant (Dunnett's Test, $P < 0.05$) reduction in ovary weight was observed in females exposed to 5.0 mg Cd/L. Overt pathology was noted in ovaries examined from females exposed to either 5.0 mg Cd/L or, to a greater extent, 10.0 mg Cd/L. A significant reduction (Dunnett's Test, $P < 0.05$) in total oocytes was found at 2.5 mg Cd/L. A significant increase (Dunnett's Test, $P < 0.05$) in the incidence of necrotic oocytes was observed in females exposed to 1.0 mg Cd/L in the culture water for 30 d. All oocytes collected from females exposed to $\geq 5.0 \text{ mg Cd/L}$ in the culture water for 30 d were necrotic. A decrease in the proportion of $>$ stage III oocytes was observed in females exposed to 1.0 mg Cd/L, but more notably in females treated with 2.5 mg Cd/L.

Breeding response was not affected in females exposed to either 0.5 or 1.0 mg Cd/L for 30 d. Half of the females exposed to 2.5 mg Cd/L bred, while none of the females treated with either 5.0 or 10.0 mg Cd/L bred with healthy untreated males. A significant reduction (Dunnett's Test, $P < 0.05$) in the fertilization rate was noted in oocytes collected from females exposed to 2.5 mg Cd/L for 30 d. A reduction in 96-h embryo-larval viability was observed in embryos obtained from females exposed to

1.0 mg Cd/L for 30 d. A concentration-dependent decrease in embryo-larval viability was observed, although no embryos were available to evaluate the 5.0 and 10.0 mg Cd/L treatments.

The specific effects of Cd exposure to adult female *X. laevis* on embryo-larval development and teratogenesis are given in Table 5. The effects of maternal Cd exposure prior to breeding were predominantly embryolethal; however, malformations induced in 4-d larvae from females exposed to 2.5 mg Cd/L for 30 d prior to breeding included visceral edema, skeletal kinking of the notochord, craniofacial defects, ruptured pigmented retina, and microencephaly.

Male Assessment

As with the females, Cd accumulation in the liver of males exposed to 10.0 mg Cd/L was appreciable (control = $<30.0 \mu\text{g Cd/Kg}$; 10 mg Cd/L treatment = $139.5 \pm 21.6 \mu\text{g Cd/L}$), and moderate pathological effects, including decreased weight and discoloration, were noted; however, no liver pathology was observed in animals exposed to $<10.0 \text{ mg Cd/L}$. The effects of 30-d Cd exposure via the culture water on selected male gametogenesis endpoints and reproductive performance are provided in Table 4. A significant reduction (Dunnett's Test, $P < 0.05$) in testis weight and sperm count was observed in males treated with 2.5 and 5.0 mg Cd/L, respectively. An increase in the proportion of abnormal sperm was detected in the 1.0 mg Cd/L treatment.

A slight reduction in breeding response (three of four pairs successfully bred) was noted in males exposed to 2.5 mg Cd/L. No breeding response was observed in the 10.0 mg Cd/L treatment. A reduction in fertilization rates was seen only in the 10.0 mg Cd/L treatment (Dunnett's Test, $P < 0.05$). A slight reduction in embryo-larval viability was noted in oocytes fertilized by males treated with 1.0 – 5.0 mg Cd/L, whereas a marked decrease (Dunnett's Test, $P < 0.05$) in viability was noted in the 10.0 mg Cd/L treatment.

Ethylene Glycol Monomethyl Ether

Female Assessment

Although some accumulation of EGME (control = $<50 \mu\text{g EGME/Kg}$; 5.0 $\mu\text{g EGME/L}$ treatment = $158.3 \pm 58.2 \mu\text{g EGME/Kg}$) was noted in liver tissue from females exposed to 5.0 mg EGME/L and a slight increase in liver weight was noted, no pathology was detected. The effects of 30-d EGME exposure via culture water on female reproductive endpoints are provided in Table 6. Results indicated that a significant reduction (Dunnett's Test, $P < 0.05$) in ovary weight occurred in specimens exposed to 0.75 mg EGME/L. General necrosis and histopathology of the ovary was observed at 1.0 mg EGME/L. A significant reduction (Dunnett's Test, $P < 0.05$) in the number of oocytes was found in females exposed to 0.5 mg EGME/L for 30 d via the culture water. An increased rate of oocyte necrosis was noted in the 0.5 mg EGME/L treatment, with increasing necrosis occurring with increasing EGME concentration. Oocytes collected from females exposed to 5.0 mg EGME/L were all necrotic. In addition, the proportion of oocytes ($>$ stage 3) decreased in a concentration-dependant manner, with a significant effect (Dunnett's Test, $P < 0.05$) noted at 0.5 mg EGME/L.

Successful breeding responses as determined by amplexus occurred with each of the females exposed to EGME concentrations ranging from 0.0 to 0.5 mg/L. Two of the four females (50%) exposed to either 0.75 or 1.0 mg EGME/L for 30 d via the culture media successfully amplexed. None of the four females exposed to 5.0 mg EGME/L initiated breeding activity. No effect on fertilization rates was observed with females exposed to EGME concentrations ranging from 0.0 to 0.5 mg/L for 30 d in the culture media; however, a significant reduction (Dunnett's Test, $P < 0.05$) in fertilization rates was found in the 0.75 mg EGME/L treatment. A significant reduction (Dunnett's Test, $P < 0.05$) in 96-h embryo viability was detected at 0.5 mg EGME/L. Embryos from females treated with ≥ 7.5 mg EGME/L were completely inviable.

The effect of maternal EGME exposure on early embryo-larval development is provided in Table 3. A statistically significant (Dunnett's Test, $P < 0.05$) increase in embryo lethality was detected in 0.75 mg EGME treatment. In addition, a significant increase (Dunnett's Test, $P < 0.05$) in abnormal development was observed in fertilized embryos collected from females exposed to 0.5 mg EGME/L for 30 d and cultured in FETAX Solution for 4 d. Maternal exposure to >1.0 mg EGME/L for 30 d induced mal-development of the gut, pericardial edema, mal-development of the aorta, notochord kinking, hydroencephaly, and hemorrhage.

Male Assessment

Unlike females exposed to 5.0 mg EGME/L, males exposed to this concentration for 30 d more significantly accumulated EGME (control = <50 μg EGME/Kg; 5.0 mg EGME/L treatment = 304.9 ± 72.3 μg EGME/Kg) and exhibited significant liver necrosis, characterized by zonal necrosis. No adverse effects on the liver were noted in concentrations <2.5 mg EGME/L. The effect of EGME on male reproductive endpoints and reproductive performance is presented in Table 7. Exposure to 0.5 mg EGME/L for 30 d significantly reduced (Dunnett's Test, $P < 0.05$) testis weight, although overt pathology was only noted in males exposed to 1.0 mg EGME/L. The sperm count was significantly reduced and the rate of sperm dysmorphology increased (Dunnett's Test, $P < 0.05$ for both) at concentrations of 0.5 and 0.75 mg EGME/L, respectively. The rate of sperm dysmorphology in males exposed to 5.0 mg EGME/L for 30 d was $100.0 \pm 0.0\%$. No effect on breeding response was noted in treatments ranging from 0.0 to 0.5 mg EGME/L. At 5.0 mg EGME/L amplexus was not induced in any of the four males bred. In addition, a significant reduction (Dunnett's Test, $P < 0.05$) in fertilization rates and embryonic viability at 96-h were observed in the 1.0 mg EGME/L treatment.

***In Vitro* Oocyte Maturation (GVBD)**

In an effort to determine if the increased proportion of immature oocytes found in toxicant-exposed females was the result of the lack of hormonal stimulation or the inability to be stimulated to mature, normal-appearing stage VI oocytes were removed from four females within the 100.0 mg B/L, 1.0 mg Cd/L, and 0.5 mg EGME/L treatments and co-cultured with 1 μ M progesterone. Results from these studies are given in Table 8. The majority of stage VI oocytes from the control females successfully completed GVBD when cultured with 1 μ M progesterone. However, a significant proportion (Dunnett's Test, $P < 0.05$) of the stage VI oocytes from the EGME-treated group did not undergo GVBD, indicating that they were not progesterone responsive. Cd or B-treated females were similarly responsive to progesterone as to control oocytes in terms of successfully completing GVBD.

Discussion

Results from the present study clearly demonstrate the reproductive and developmental toxicity of boric acid, Cd, and EGME in *X. laevis*. Based on these studies, it appears that both male and female gonads and gametogenesis were affected by the selected toxicants and that accumulation of each toxicant occurred in varying levels in gonadal tissue. Results from these studies indicate that boric acid is capable of affecting both male and female gametogenesis and reproduction, but only at high concentrations (100.0 mg B/L/d for 30 d). Although effects on gametogenesis and the gonads were observed in the 50.0-250.0 mg B/L/d for 30 d range, impact on reproductive performance was not noted until a concentration of 1,000.0 mg B/L/d was reached. In addition, a 20.8% reduction in sperm count was detected in males exposed to 50.0 mg B/L/d, although little effect on breeding response or success (fertilization and embryonic viability) was noted. The fertilization rate of oocytes from females exposed to 500.0 mg B/L/d was not reduced; however, the 96-h viability of the fertilized oocytes was significantly lowered. Malformations induced in progeny by maternal exposure to 500.0 mg B/L/d included gut mal-development, craniofacial defects, kinking of the notochord and microencephaly. Interestingly, similar effects have been induced in normal-appearing *X. laevis* embryos from unexposed mothers exposed to 500.0 mg B/L in the FETAX model.³¹ These results suggest that similar modes of action on the developing embryo are occurring, regardless of whether exposure is provided through the mother to the oocyte or directly to the developing embryo following fertilization. The teratogenic effects noted in studies applying post-fertilization exposure are significantly more dramatic in terms of the incidence of malformation and the severity of effects.³¹

Effects on the testis have been observed in both subchronic and chronic studies in rats and mice exposed to B.³² After 14 d of treatment, doses of 93 mg B/Kg/d caused a reduction in spermiation.⁶ Testicular atrophy, degeneration of seminiferous tubules, reduced sperm count, and a reduction in fertility was observed in a three-generation study of boric acid toxicity in rats at 58.5 mg B/kg.⁷ In male

rats fed borax for either 30 or 60 d at 100.0 or 200.0 mg B/kg/d (NOAEL, 50 mg B/kg/d), testis weight was reduced, testicular germ cells were depleted, selected testicular enzymes were affected, and fertility was reduced.⁸

In a continuous breeding study of boric acid in mice, a dose-related effect on the testis (testicular atrophy and effects on sperm, motility, morphology, and concentration) was noted; fertility was partially reduced at 111 mg B/kg/d, and totally reduced at 221 mg B/Kg/d.⁹ In rats, at non-maternally toxic doses, there was a reduction in fetal body weight and some skeletal anomalies.³⁴ With the exception of shortened 13th rib, these malformations had reversed by postnatal day 21 at 13.7 and 28.6 mg B/kg/d in a study designed to look at postnatal recovery.¹⁰ At higher, maternally toxic doses, other indications of developmental effects were observed, including resorptions and visceral malformations; however, these may have been secondary to the maternal toxicity.^{11,12} In mice, the key developmental effects observed were similar to those seen in rats, including reduction in fetal body weight at the mid-dose and an increase in skeletal variations, malformations, and resorptions at the highest, more maternally toxic dose.¹²

In rabbits, developmental effects were seen only in the high dose, where the majority of the embryos were resorbed and malformations were primarily visceral (major heart and/or great vessel defects).¹² Some skeletal variations and malformations were observed at high doses, similar to rats;¹³ however, these effects may have been secondary to the maternal toxicity. A small, insignificant decrease in fetal body weight was also observed. The NOAEL for both maternal and developmental toxicity in the rabbit was 21.8 mg B/kg/d.¹³

Similar to boric acid, but at much lower concentrations (2.5 mg/L/d), Cd exposure to female *Xenopus* for 30 d via the culture water appreciably affected ovary weight, total oocyte counts, oocyte maturity and necrosis. Greater concentrations rendered all of the oocytes necrotic. Reproductive effects in males were also observed at ≥ 2.5 mg Cd/L/d, including reduced testis weight, decreased sperm count,

and increased proportion of abnormal sperm cells. Effects on breeding success, rate of fertilization, and 96-h embryo viability were substantially more marked in treated females than in treated males. The effect of maternal Cd exposure on embryo-larval development was primarily embryolethal, although terata, gut mal-development, visceral edema, skeletal kinking of the notochord, craniofacial abnormalities, ruptured pigmented retina, and microencephaly were induced in the 2.5 mg/L/d treatment.

Cadmium has been shown to accumulate in vertebrates, with slow elimination by the kidneys.¹⁴ A variety of mammalian models have examined the reproductive toxicity of Cd to adults and fetuses.¹⁷ Reproductive tissues such as the gonads and uterus readily accumulate Cd.¹⁸ Acute effects of Cd in mammals include testicular necrosis, ovarian hemorrhaging, and delayed embryo implantation.^{19,20} An examination of oogenesis in mice indicated an increase in chromosomal anomalies in the ovulated oocytes and fewer oocytes ovulated, and showed an increase in necrotic oocytes.²⁰

Reproductive endocrine function was disrupted in adult female Atlantic croaker (*Micropogonias undulatus*) after Cd exposure³³ and Cd altered vitellogenin metabolism in flounder (*Platichthys flesus*).³⁴ In *Medaka*, early stages of development were the most sensitive and fertilized eggs accumulated most of the Cd in the chorion.³⁵ Continuous exposure of rainbow (*O. mykiss*) and brown (*S. trutta*) trout to Cd for 30-d did not affect the survival and growth of the adults, although eggs from *O. mykiss* failed to develop to the fry stage at ca. 2 µg/L, while oogenesis in *S. trutta* was delayed at ca. 9 µg/L. Pramoda and Saidapur³⁶ found that the weight of ovaries and oviducts of CdCl₂-exposed Indian bullfrogs (*Rana tigrina*) was decreased and that the vitellogenic growth of oocytes was impaired.

Teratogenic activity of Cd has also been demonstrated.^{15,16} Malformations induced in mammalian models included craniofacial and palate defects, eye defects, hydroencephaly, and various limb malformations.³⁷ Interestingly, Weir et al.³⁸ found that perfusing human placenta with low concentrations of Cd caused a change in oxygen tension and markedly reduced glucose and amino acid utilization, as well as reducing the synthesis and release of hCG. Because gonadotropin activity in the

frog is responsible for ovulation and breeding, it is possible that these endocrine-disrupting effects are responsible for the reproductive toxicity associated with Cd exposure in *Xenopus*.

In female *Xenopus*, EGME significantly reduced ovary and oviduct weight and increased the rate of oocyte necrosis at a concentration of 0.5 mg/L. Decreased maturation of normal-appearing oocytes was noted at an EGME concentration of 0.75 mg/L. Normal-appearing oocytes collected from adults exposed to 1 or 5 mg/L EGME were either partially responsive or completely unresponsive to progesterone, respectively, when cultured *in vitro*. Reduced testis weight and atrophy were noted in males at 0.5 mg/L. Reduced sperm counts and increased dysmorphogenesis rates were detected at 1.0 mg/L. Females exposed to 1.0 mg EGME/L/d for 30 d in the culture water had a much greater proportion of immature oocytes in stages I and II than control females, which had a greater proportion of mature oocytes in stages V and VI (mature).

Although the reproductive toxicity of EGME is not as well understood as for boric acid and Cd, sufficient literature exists to classify EGME as a reproductive toxicant and suspect developmental toxicant, depending on the dose and route of administration. EGME has been shown to reduce fertility indices, reduce epididymis, prostate and seminal vesicle weights, and increase the length of the estrous cycle in Swiss CD-1 mice.²¹ Nagano et al.²² also reported that EGME induced testicular atrophy in mice. Characteristic malformations induced in mammals by exposure to EGME include skeletal defects, cardiovascular defects, vertebral anomalies, exencephaly, and limb (paw) defects.²³ These defects were similar in type to the abnormalities induced in *Xenopus* larvae from females exposed to EGME.

Co-culture of progesterone with stage VI oocytes *in vitro* induced successful GVBD from control females, but not in the EGME-treated females. A significant reduction in the number of stage VI oocytes that successfully completed GVBD following progesterone stimulation *in vitro* from Cd-treated females was also observed when compared to the controls. Adult females exposed to reproductively toxic levels of B had no appreciable effect on progesterone-induced GVBD *in vitro*. Interestingly, however, chronic B deficiency substantially reduced oocyte progesterone responsiveness.³⁰

In the present study, incomplete maturation of "normal appearing" EGME-treated oocytes was marked by size and incomplete GVBD. Normal GVBD was observed in oocytes from control females. Unlike the oocytes from control females, addition of progesterone *in vitro* did not significantly stimulate GVBD in oocytes from EGME-treated females. The inability of the oocytes which were "normal appearing" to mature in the presence of progesterone *in vitro* suggests that EGME or Cd may disrupt the maturation process at either the progesterone receptor level, or possibly in the cascade of secondary and tertiary messenger events responsible for inducing the maturation of the oocyte.

Under natural conditions, gonadotropins released from the pituitary gland of female *X. laevis* trigger ovulation and the egg laying response. Maturation of the oocyte is triggered by the binding of progesterone produced by the follicle in response to gonadotropin stimulation. The progesterone receptor in the *X. laevis* oocyte is associated with the plasma membrane, rather than existing as an intracellular receptor, which is common for steroid hormones.³⁹⁻⁴¹ The morphological effects associated with progesterone binding include the breakdown of the germinal vesicle and formation of the maturation spindle. A cascade of intracellular events associated with second messenger activity occurs shortly after progesterone binding, marked by a transient increase in free Ca^{+2} , decreased levels of cAMP and adenylate cyclase activity, membrane depolarization, rise in intracellular pH, and increase in protein synthesis and non-ribosomal protein phosphorylation immediately prior to germinal vesicle breakdown.

A maturation-promoting factor (MPF) has been identified as the link between the aforementioned events and progesterone binding.⁴⁰ MPF is formed from a pool of pro-MPF immediately prior to germinal vesicle breakdown, but the protein responsible for this activation process has yet to be identified. However, purified MPF has been shown to induce normal maturation without signal transduction when injected into immature *X. laevis* oocytes.⁴¹ Similar experiments to identify the responsiveness of the oocytes from the EGME-exposed females to purified MPF are currently being performed, along with an evaluation of expression and structural integrity of the oocyte progesterone receptor. Preliminary findings indicate that progesterone levels in the adult females exposed to either

EGME (>1.0 mg/L/d) are similar to control animals, and that administration of human chorionic gonadotropin (hCG) stimulates the ovulatory response and the production of progesterone. Thus, it is unlikely that the effects induced on oocyte maturation by EGME are the result of decreased hormone synthesis or effects on regulatory feedback loops, which are also being studied.

A greater proportion of previtellogenic oocytes was noted with exposure to high concentrations of each toxicant, in which greater proportions of immature oocytes were found. Since the process of vitellogenesis does not begin until the oocyte is ca. 400 μm (stage 3), the greater proportion of previtellogenic oocytes in the toxicant-treated females is most likely a secondary effect of the inability of the oocytes to mature. However, the effect of these test materials, particularly at lower concentrations, on the fecundity of the oocyte membrane vitellogenin receptor responsible for binding vitellogenin and yolk platelet incorporation in the oocyte will require further evaluation.

Data regarding the potential effects of endocrine-disrupting contaminants (EDCs) on reproductive function in amphibians are particularly lacking. On a larger scale, female reproductive function in amphibians could be affected by EDCs at a number of target sites, including the brain, pituitary, gonad, liver, and oviduct. Gonadal effects of EDCs have considerable potential to impair the reproduction of female amphibians and have been reported in other lower vertebrate wildlife groups. Female juvenile alligators from pesticide-contaminated Lake Apopka, Florida, exhibit a number of ovarian abnormalities, including polynuclear oocytes and polyovular follicles,⁴² suppressed synthesis of 17β -estradiol (E_2), and reduced aromatase activity *in vitro*.^{43,44} Although these organizational alterations in the structure and/or function of reproductive tissues may have the greatest potential impact on the reproductive fitness of a population,⁴⁵ activational effects of EDCs such as the modulation of endocrine signaling in the adult gonad may also significantly impair reproduction. Polyaromatic hydrocarbons (PAHs) have been shown to impair various aspects of ovarian function in adult fish. PAHs inhibited oocyte growth, caused increased follicular atresia, and prevented final oocyte maturation in the Atlantic

croaker.⁴⁶ Furthermore, the organochlorine compounds kepone and o,p'-DDD inhibited *in vitro* final maturation of Atlantic croaker oocytes, which is induced by the steroid 17 α ,20 β ,21-trihydroxy-4-pregnen-3-one (20 β -S).⁴⁷ In light of the current study, Pickford and Morris⁴⁸ found that although a series of natural and synthetic estrogens did not alter progesterone-induced oocyte maturation, the proestrogenic pesticide methoxychlor was a surprisingly potent inhibitor of progesterone-induced oocyte maturation. This inhibitory activity was specific to methoxychlor, rather than to estrogenic contaminants or its metabolites, and was not antagonized by the estrogen receptor antagonist ICI 162,780, suggesting that this activity is not estrogenic. Further, the inhibitory activity of methoxychlor was found to be dose dependent, reversible, and early acting.

Results from these studies demonstrate the importance of concurrently monitoring effects on the reproductive organs, gametogenesis, reproductive performance, and developmental fitness. Although potentially toxic effects on the gonad or gametogenesis in males did not necessarily translate to effects on either reproductive performance or developmental fitness, adverse transgenerational effects of B and Cu deficiency in adult female *X. laevis* have also been detected in developing offspring.²⁷ The present work demonstrated that transgenerational effects of adult toxicant exposure can be monitored with the *X. laevis* reproductive toxicity battery. These studies clearly demonstrate transgenerational effects associated with adult *X. laevis* female exposure to Cd and EGME. These transgenerational effects were also observed with female exposure to B, but not to the same extent as noted with EGME and Cd. The *X. laevis* reproductive toxicity assay battery appears to be capable of monitoring reproductive toxicity, providing potential connective and mechanistic information concerning effects at the gonad or gamete level through embryo-larval development. The use of the progesterone-responsiveness assay with immature oocytes may also provide links between gamete production and endocrine disruption.

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Table 1. Effect of Route of Exposure/Administration on Boric Acid, Cadmium, or Ethylene Glycol Monomethyl Ether (EGME) Accumulation in *Xenopus* Gonadal Tissue¹

Route	B ²		Cd ²		EGME ²	
	Testes	Ovaries	Testes	Ovaries	Testes	Ovaries
Control	320.0 (52.3)	94.6 (19.6)	<30.0 ³ (0.0)	<30.0 ³ (0.0)	<50.0 ³ (0.0)	<50.0 ³ (0.0)
Culture Water ⁴	405.0 (44.0)	178.0 (76.9)	60.0 (8.0)	256.8 (3.0)	326.5 (73.2)	549.3 (31.5)
Food ⁵	633.2 (52.2)	191.3 (31.3)	118.5 (9.1)	496.2 (230.0)	273.6 (19.5)	466.7 (43.2)
i.p. ⁶	580.6 (60.2)	309.2 (117.0)	3823.0 (306.0)	5851.7 (468.0)	1374.2 (99.8)	1217.6 (100.2)
s.c. ⁶	792.0 (79.4)	451.2 (90.2)	4530.0 (270.0)	6461.7 (517.0)	2463.5 (117.3)	2991.4 (129.5)

¹ Total B (ICP-MS), Cd (AAS-GF), and EGME (GC-MS). N = 4 frogs per dose regimen/toxicant.

² Expressed as µg/Kg with SE in parenthesis.

³ Practical Quantitation Limit (detection limit * dilution factor).

⁴ 100 mg B/L, 2.5 mg Cd/L, or 1.0 mg EGME/L exposure provided via culture water (FETAX Solution). 48-h renewal for 30 days.

⁵ Administered via B-, Cd-, or EGME-enriched liver. Total B administered = 10 mg/Kg/day for 30 days. Total Cd administered = 2.5 mg/Kg/day. Total EGME administered = 1.0 mg/Kg/day.

⁶ 100 mg B/Kg/day, 2.5 mg Cd/Kg/day, or 1.0 mg EGME/Kg/day administered daily for 5 days.

Table 2. Effect of Boric Acid on Reproductive Endpoints in *X. laevis*

Concentration ¹ (mg/L)	Female Parameters				Male Parameters				Breeding	
	Ovary Weight ² (%)	Egg Mass		Testes Weight ² (%)	Sperm		Amplexus ⁴ (%) ⁵	Fertilization (%) ⁵	Embryo Viability at 96 h (%) ⁵	
		< Stage ³ 3 (%)	≥ Stage ³ 3 (%)		Count (x10 ⁶)	Abnormal (%)				
0.0	0.11 (0.03)	12.5 (4.6)	82.1 (5.3)	0.05 (0.01)	2.4 (0.2)	0.2 (0.1)	100.0/100.0	86.3/87.4 (7.2)/(6.3)	97.1/95.2 (4.2)/(5.6)	
1.0	0.09 (0.02)	8.2 (2.3)	78.6 (7.3)	0.04 (0.01)	2.2 (0.4)	0.5 (0.3)	100.0/100.0	84.7/89.2 (8.3)/(5.9)	91.9/90.3 (3.8)/(4.2)	
10.0	0.08 (0.01)	21.1 (5.6)	69.3 (7.8)	0.06 (0.02)	2.5 (0.3)	0.8 (0.4)	100.0/100.0	86.3/86.2 (5.7)/(9.2)	94.2/92.3 (5.6)/(4.6)	
50.0	0.09 (0.02)	25.2* (4.7)	58.3* (9.9)	0.03* (0.01)	1.9 (0.1)	1.2 (0.3)	100.0/100.0	82.2/87.1 (6.4)/(7.6)	89.3/90.1 (8.3)/(5.6)	
100.0	0.10 (0.03)	41.2* (6.9)	30.6* (5.8)	0.02* (0.01)	2.0 (0.3)	3.2 (0.9)	100.0/100.0	81.8/83.4 (9.2)/(8.2)	79.2*/86.1 (4.9)/(9.3)	
500.0	0.05* (0.02)	31.3* (7.8)	16.0* (4.9)	0.02* (0.02)	1.5* (0.2)	12.6* (0.5)	75.0/100.0	83.2/88.1 (7.3)/(5.3)	58.7*/92.3 (8.7)/(6.1)	
1000.0	0.03* (0.01)	0.0* (---)	0.0* (---)	0.01* (0.01)	0.9* (0.1)	18.3* (1.2)	0.0/0.0	---/--- (---)/(---)	---/--- (---)/(---)	

¹ Concentration (expressed as B) in FETAX Solution. Adults were exposed to B for 30 d prior to evaluation. N = 4 for reproductive endpoint evaluation and 4 for breeding response.

² Expressed as % of body weight.

³ Based on Dumont stages.²⁸

⁴ Breeding response.

⁵ Presented as treated females bred with untreated males/treated males bred with untreated females.

⁶ FETAX assessment culturing embryos in FETAX Solution alone.

* Statistically different than the control (Dunnnett's Test, P > 0.05)

Table 3. Effect of Boric Acid on Embryo-Larval Development in *X. laevis*

Concentration ¹ (mg/L)	Mortality ² (%)	Malformation ² (%)	Type of Terata
0	1.2 (0.4)	2.5 (0.3)	Gut mal-development
1.0	1.6 (0.7)	6.5 (0.7)	Gut mal-development
10.0	5.4 (0.9)	2.4 (1.1)	Gut mal-development
50.0	3.7 (0.8)	7.0 (2.4)	Gut mal-development
100.0	5.2 (1.1)	16.6* (3.4)	Gut mal-development, craniofacial defects
500.0	7.5 (1.3)	33.8* (4.6)	Gut mal-development, craniofacial defects, kinking of the notochord, microencephaly
1000.0	--- ³	--- ³	---

¹ Concentration of boric acid (expressed as B) exposed to adult females via culture water for 30 d.

² Based on 96-h developmental studies in FETAX Solution. Embryos from successfully bred treated females and untreated males were collected; N = 4 except for 500 mg/L treatments, in which N = 3 adult females. 80 embryos/frog/concentration were evaluated.

³ Not performed due to lack of normal appearing embryos.

* Statistically different than the control (Dunnett's Test, P > 0.05).

Table 4. Effect of Cadmium on Reproductive Endpoints in *X. laevis*

Concentration ¹ (mg/L)	Female Parameters				Male Parameters				Breeding	
	Ovary Weight ² (%)	Egg Mass		Testes Weight ² (%)	Sperm		Amplexus ⁴ (%) ⁵	Fertilization (%) ⁵	Embryo Viability at 96 h ⁶ (%) ⁵	
		< Stage ³ 3 (%)	≥ Stage ³ 3 (%)		Count (x10 ⁶)	Abnormal (%)				
0.0	0.13 (0.04)	19.4 (3.2)	77.0 (5.9)	0.07 (0.02)	2.3 (0.3)	0.4 (0.1)	100.0/100.0	89.4/89.8 (6.2)/(2.1)	94.6/91.2 (3.9)/(7.3)	
0.5	0.11 (0.02)	2654.0 (336.0)	61.3 (7.1)	0.08 (0.01)	2.5 (0.4)	6.1 (4.1)	100.0/100.0	91.2/95.2 (9.5)/(3.8)	88.6/92.3 (8.3)/(5.3)	
1.0	0.11 (0.03)	1923.0 (492.0)	32.6* (8.3)	0.06 (0.03)	2.1 (0.2)	15.4 (6.9)	100.0/100.0	86.3/90.3 (10.2)/(3.1)	65.8*/87.2 (9.3)/(4.6)	
2.5	0.08 (0.02)	1231.0* (562.0)	5.0* (3.2)	0.02* (0.02)	1.9 (0.4)	39.6 (3.9)	50.0/75.0	51.6*/85.2 (19.3)/(8.3)	11.7*/85.2 (0.9)/(5.2)	
5.0	0.06* (0.02)	394.0* (196.0)	0.0* (---)	0.03* (0.01)	1.5* (0.3)	52.8 (5.6)	0.0/50.0	---/83.2 (---)/(7.2)	---/84.2 (---)/(7.8)	
10.0	0.02* (0.01)	152.0* (36.0)	0.0* (---)	0.01* (0.01)	0.3* (0.1)	98.4 (1.6)	0.0/0.0	---/9.5* (---)/(10.7)	---/18.6* (---)/(5.9)	

¹ Concentration (expressed as B) in FETAX Solution. Adults were exposed to B for 30 d prior to evaluation. N = 4 for reproductive endpoint evaluation and 4 for breeding response.

² Expressed as % of body weight.

³ Based on Dumont stages.²⁸

⁴ Breeding response.

⁵ Presented as treated females bred with untreated males/treated males bred with untreated females.

⁶ FETAX assessment culturing embryos in FETAX Solution alone.

* Statistically different than the control (Dunnett's Test, P > 0.05).

Table 5. Effect of Maternal Cadmium Exposure Prior to Breeding on Early Embryo-Larval Development in *X. laevis*

Concentration ¹ (mg/L)	Mortality ² (%)	Malformation ² (%)	Type of Terata
0	3.8	5.2	Gut mal-development
0.5	5.2 (1.4)	6.2 (1.9)	Gut mal-development, visceral edema
1.0	22.5* (3.7)	11.7* (2.6)	Gut mal-development, visceral edema, skeletal kinking
2.5	62.5* (4.7)	25.8* (3.5)	Gut mal-development, visceral edema, skeletal kinking, craniofacial defects, ruptured pigmented retina, microencephaly
5.0	---	---	---
10.0	---	---	---

¹ Concentration of Cadmium (expressed as Cd) exposed to adult females via culture water for 30 d.

² Based on 96-h developmental studies in FETAX Solution. Embryos from successfully bred treated females and untreated males were collected; N = 4 except for 500 mg/L treatments, in which N = 3 adult females. 80 embryos/frog/concentration were evaluated.

³ Not performed due to lack of normal appearing embryos.

* Statistically different than the control (Dunnett's Test, P > 0.05).

Table 6. Effect of Ethylene Glycol Monomethyl Ether (EGME) on Reproductive Endpoints in *X. laevis*

Concentration ¹ (mg/L)	Female Parameters					Male Parameters				Breeding	
	Ovary Weight ² (%)	Egg Mass		Testes Weight ² (%)	Sperm Count (x10 ⁵)	Abnormal (%)	Amplexus ⁴ (%) ⁵	Fertilization (%) ⁵	Embryo Viability at 96 h ⁶ (%) ⁵		
		< Stage ³ (%)	≥ Stage ³ (%)							Necrotic (%)	
0.0	0.14 (0.03)	13.8 (5.8)	76.8 (5.3)	0.08 (0.02)	2.4 (0.3)	0.2 (0.3)	100.0/100.0	89.9/90.1 (1.8)/(5.2)	95.6/92.1 (3.2)/(4.2)		
0.1	0.13 (0.02)	15.9 (4.3)	75.2 (7.4)	0.07 (0.01)	2.5 (0.4)	0.5 (0.4)	100.0/100.0	88.2/92.3 (2.4)/(4.5)	92.8/95.2 (4.9)/(5.1)		
0.5	0.09 (0.02)	21.4 (8.6)	54.3* (9.1)	0.04* (0.02)	2.1 (0.2)	4.3* (1.1)	100.0/100.0	79.3/93.5 (8.8)/(4.6)	79.3*/98.1 (7.8)/(3.8)		
0.75	0.05* (0.01)	34.9* (11.5)	15.9* (2.3)	0.02* (0.01)	1.6* (0.1)	31.3* (5.6)	50.0/50.0	59.9*/86.2 (5.8)/(5.9)	0.0*/89.2 (0.0)/(6.3)		
1.0	0.04* (0.02)	19.3* (8.9)	2.2* (0.8)	0.02* (0.01)	0.7* (0.09)	69.3* (10.2)	50.0/50.0	31.3*/73.2* (9.3)/(8.9)	0.0*/82.5* (0.0)/(7.9)		
5.0	0.01* (0.01)	---	---	0.01* (0.01)	0.03* (0.01)	100.0* (0.0)	0.0/0.0	---/--- (---)/(---)	---/0.0 (---)/(0.0)		

¹ Concentration (expressed as B) in FETAX Solution. Adults were exposed to B for 30 d prior to evaluation. N = 4 for reproductive endpoint evaluation and 4 for breeding response.

² Expressed as % of body weight.

³ Based on Dumont stages.²⁸

⁴ Breeding response.

⁵ Presented as treated females bred with untreated males/treated males bred with untreated females.

⁶ FETAX assessment culturing embryos in FETAX Solution alone.

* Statistically different than the control (Dunnett's Test, P > 0.05).

Table 7. Effect of Maternal Ethylene Glycol Monomethyl Ether (EGME) Exposure Prior to Breeding on Early Embryo-Larval Development in *X. laevis*

Concentration ¹ (mg/L)	Mortality ² (%)	Malformation ² (%)	Type of Terata
0	3.8 (1.2)	0.4 (0.2)	Gut mal-development
0.1	1.0 (0.3)	6.2 (0.9)	Gut mal-development
0.5	2.2 (0.6)	18.5* (3.4)	Gut mal-development, pericardial edema, mal-developed aorta, notochord kinking
0.75	23.5* (4.3)	76.5* (10.2)	Gut mal-development, pericardial edema, mal-developed aorta, notochord kinking, hydroencephaly
1.0	36.3* (4.9)	100.0* (0.0)	Gut mal-development, pericardial edema, mal-developed aorta, notochord kinking, hydroencephaly, visceral hemorrhage
5.0	---	---	---
	(---)	(---)	

¹ Concentration of ethylene glycol monomethyl ether (expressed as EGME) exposed to adult females via culture water for 30 d.

² Based on 96-h developmental studies in FETAX Solution. Embryos from successfully bred treated females and untreated males were collected; N = 4 except for 500 mg/L treatments, in which N = 3 adult females. 80 embryos/frog/concentration were evaluated.

* Statistically different than the control (Dunnett's Test, P > 0.05).

Table 8. Effect of Boric Acid, Cadmium, and Ethylene Glycol Monomethyl Ether (EGME) exposure on Progesterone responsiveness in *X. laevis* Oocytes¹

Test Material	Exposure Concentration ² (mg/L)	Progesterone Responsive (%)
Control	0.0	98.2 (3.1)
Boric Acid	100.0	92.8 (10.2)
Cadmium	1.0	89.8 (15.8)
EGME	0.5	23.5 ³ (11.5)

¹ Responsiveness indicated by maturation of normal-appearing stage VI oocytes to undergo germinal vesicle breakdown over a 24-h period when cultured with 1 μ M progesterone *in vitro*; n = 4 females per test material.

² Expressed as total B, total Cd, and EGME.

³ Statistically less than the control (Dunnett's Test, P > 0.05).