Effect of Methoxychlor on Various Life Stages of *Xenopus laevis*

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INTRODUCTION

In spite of increasing alarm over global population declines in amphibian species over the last 20 years, only recently has evidence of adverse effects due to exposure to a variety of different physical and chemical stressors been provided (Sparling et al., 2000). It is likely that several of these factors are working together as multiple stressors. In some cases, amphibians also provide a critical link to identifying potential hazards for other animals, including humans (Birge, 2000; Burkhart et al., 1998; Fort et al. 1999a, 1999b, in press a, in press b; Fort and McLaughlin, 2003). Thus, amphibians are sentinels, making them suitable receptors for assessing ecological health.

Many hypotheses have been provided to explain the global declines of amphibian populations; however, few studies have provided the clear cause–effect results required to demonstrate effects at the local population level. This deficiency does not diminish the efforts of the investigators providing these hypotheses, but is rather a tribute to the complexity involved in establishing cause–effect relationships at the local population level. Potential causes of declines in amphibian species include habitat destruction, predation, competition by introduced species (Wake, 1991), increased ultraviolet (uv) radiation resulting from atmospheric ozone depletion (Blustein et al., 1994), and particular vulnerability to waterborne environmental contaminants due to their largely aquatic life histories and their highly permeable skin (Boyer and Grue, 1995). Newer reports (Fort et al., 2000; 2001b) have implicated the importance of thyroid dysfunction as a mediating factor in amphibian maldevelopment and population decline.

The diversity in the life history traits of most amphibian species compared to other vertebrates makes them a unique target in the environment. Many amphibians utilize a water- and terrestrial-based environment for breeding and larval development and the adult life phase, respectively. These history traits effectively increase the number of potential exposure routes, and thus the probability of being exposed during the life of the amphibian. Early embryo-larval development has typically been considered the most sensitive stage of the vertebrate life cycle. For example, in most fish species the embryo-larval stage of development is typically considered the most sensitive to chemical exposure (Loewengart, 2001; Nguyen and Janssen, 2002, 2001;
et al., 2003; Fort and McLaughlin, 2003), advanced development including metamorphosis (Carr et al., 2003; Fort et al., 2000, 2001b; Fort and Stover 1997), and sexual maturation (Hayes et al., 2002; Kloas et al., 1999; Pickford and Morris, 2003; Tavera-Mendoza et al., 2002a, 2002b). Collection of concurrent information on the effects on the sensitivity of various developmental stages throughout the life cycle not only provides valuable hazard assessment information, but also provides mechanistic clues concerning the modes of action of developmental and reproductive toxicants. In this report, we describe the developmental and reproductive effects of the estrogenic organochlorine pesticide methoxychlor at various life stages of the South African clawed frog, *Xenopus laevis*, in an effort to determine stage-specific sensitivity.

**MATERIALS AND METHODS**

**Materials.** Adult male and female *X. laevis* were acquired from Xenopus 1 (Dexter, MI). Chemicals and biochemicals used in the culture of *X. laevis* in this study were purchased from either the Sigma Chemical Company (St. Louis, MO) or Fisher Scientific (Houston, TX). Methoxychlor (99% purity) was purchased from Chem Service (West Chester, PA). Methoxychlor was dissolved in DMSO (99.5% purity; Sigma Chemical Co.) to prepare the appropriate stock solution. The final concentration of DMSO in each of the tests performed was held constant through the test series and did not exceed a final concentration of 0.5 % (v/v). Human chorionic gonadotropin (hCG) used to induce breeding in *X. laevis* was dissolved in 0.9% (w/v) saline to produce a 1,000 IU/ml solution.

**Methods.** The experimental design used in this study is summarized in Table 1.

**Developmental Exposure Studies**

**Experimental Animals**

*Xenopus* adult care, breeding, and embryo collection were performed generally as described in ASTM E1439–98 (ASTM, 1998). Adult *X. laevis* were fed a Salmon Chow diet (Rangen Company, Buhl, ID) daily *ad libitum*. Larvae used in

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**TABLE 1**

Summary of Experimental Design

<table>
<thead>
<tr>
<th>Exposure Study</th>
<th>Exposure stages*</th>
<th>Exposure description</th>
<th>Developmental/reproductive significance</th>
<th>Endpoints measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8–46</td>
<td>FETAX</td>
<td>Early embryo-larval development including major stages of organogenesis</td>
<td>Mortality, malformation, and growth</td>
</tr>
<tr>
<td>2</td>
<td>8–54</td>
<td>Hind limb</td>
<td>Completion of hind limb development; marks initiation of premetamorphosis</td>
<td>Mortality and limb malformation</td>
</tr>
<tr>
<td>3</td>
<td>58–66</td>
<td>Metamorphic climax</td>
<td>Metamorphic climax; marks climax of larval-to-frog transition; induced by thyroxin surge at onset of climax</td>
<td>Rate and extent of tail resorption</td>
</tr>
<tr>
<td>4</td>
<td>30-day adult exposure</td>
<td>Adult reproduction</td>
<td>Female and male reproductive assessment</td>
<td>Female: ovary weight; gross pathology; total oocyte count; oocyte necrosis; and oocyte stage distribution and maturation capacity (undergo GVBD) Male: testes weight and sperm counts Breeding success: induction of breeding response (ampelxus), fertilization, and early embryo-larval viability (FETAX format-no exposure)</td>
</tr>
</tbody>
</table>

*Based on Nieuwkoop and Faber (1994).
the metamorphic climax assay were raised in 70-liter aquaria at a density of 1 organism per liter of dechlorinated tap water. At 5 days, larvae used in the limb development and metamorphic climax assays were fed the supernatant of ground Salmon Starter diet (Silver Cup tadpole starter, Xenopus 1, Dexter, MI) slurry prepared by blending ca. 6 g diet per liter of FETAX solution, a defined reconstituted water medium suitable for the short-term culture of developing Xenopus embryos (Dawson and Bantle, 1987). Larvae were fed ca. 2 ml of the slurry/organism twice per day through metamorphosis.

Exposure Study 1—FETAX
Assay methodology. Experiments were performed in general accordance with ASTM E1439-98 (ASTM, 1998) with the following modifications. Groups of 20 embryos were placed in covered 100 mm Petri dishes (Fisher Scientific) with varying concentrations of methoxychlor. The methoxychlor stock solution was diluted in appropriate volumes of FETAX solution to prepare the appropriate test dilutions. Eight to ten concentrations were tested in duplicate, and four separate dishes of 20 embryos each were exposed to FETAX Solution and were designated as FETAX Solution controls. For the definitive tests, the following test concentrations were used: 0.001, 0.005, 0.1, 0.25, 0.5, 0.75, and 1.0 mg/l and 0.5, 0.6, 0.65, 0.7, 0.75, 0.8, 0.85, 0.9, 0.95, and 1.0 mg/l, respectively. A 0.5% (v/v) DMSO control was tested concurrently with each test. The DMSO concentration in all test vessels, with the exception of the FETAX solution controls, was normalized to 0.5% (v/v). In addition, two dishes of 20 embryos were exposed to either 5.5 mg/l 6-aminothiopurinamide (6-AN) (approximate 4-d EC50 [malformation]) or 2500 mg/l 6-AN (approximate 4-day LC50) (ASTM, 1998). Each treatment vessel contained a total of 25 ml of solution.

One range-finding and two definitive concentration-response experiments were conducted. The pH of each of the stock solutions was adjusted to 7.0. Embryos were cultured at 23°C ± 1.0°C. All solutions were changed every 24 h of the 4-day test, dead embryos removed, and fresh solutions added. After 96 hours of exposure (NF stage 46 embryos) (Nieuwkoop and Faber, 1948, 1998), living larvae were fixed in 3.0% (w/v) formalin (pH 7.0), and the number of malformed embryos was ascertained using a dissecting microscope.

Exposure Study 2—Hind Limb Development
Assay methodology. Experiments designed to evaluate hind limb development were conducted for approximately 30 days from NF stage 8 through NF stage 54. Ten blastulae-stage embryos were placed in each of four replicate 12-liter aquaria (40 organisms per concentration) containing six concentrations of methoxychlor; 0.0001, 0.001, 0.005, 0.01, 0.05, and 0.1 mg/l. The methoxychlor stock solution was diluted in appropriate volumes of dechlorinated tap water to prepare the appropriate test dilutions. Because of the intensive water requirements associated with each test, the DMSO concentration in all test vessels, with the exception of the FETAX solution controls, was normalized to 0.5% (v/v). In addition, two dishes of 20 embryos were exposed to either 5.5 mg/l 6-aminothiopurinamide (6-AN) (approximate 4-d EC50 [malformation]) or 2500 mg/l 6-AN (approximate 4-day LC50) (ASTM, 1998). Each treatment vessel contained a total of 25 ml of solution.

All solutions were administered through a flow-through delivery system to provide a complete volume replacement every 24 h during the test period. Staging was performed daily, and dead organisms were removed and the numbers recorded. Tests were terminated once 80% of the control larvae reached NF stage 54. At the completion of the exposure, larvae were fixed in 3% formalin, pH 7.0, and the gross effects on limb development, including the status of differentiation of the hind limbs were noted in each of the individual specimens. Limb differentiation and defect assessment was aided by the use of a dissecting microscope. Limb malformations were scored as being distinct from developmental delay in differentiation and included gross morphological examination of elongation of the long bones (femur and tibia-fibula), and metatarsal and digit formation.

In an effort to evaluate if the effects on developmental rate were the result of disruption of thyroid activity, a separate set of NF stage 54 specimens, 5 each from the control and 0.1 mg/l methoxychlor treatment groups, were fixed in Bouin’s solution for 4 days, rinsed in 70% ethanol, and preserved in 10% neutral buffered formalin for histological examination. After trimming, larval tissue samples were placed in cassettes, and step sections (24 μm) were taken caudal to rostral. Once confirmation of the presence of adequate thyroid tissue was made, slides were stained with hematoxylin and eosin (H-E).

Exposure Study 3—Metamorphic Climax
Assay methodology. Tail resorption exposure studies were conducted in general accordance with previous methods (ASTM, 1998; Fort et al., 2000; Fort and McCaughan, 2003; Fort and Stower, 1997). Experiments performed to evaluate effects of methoxychlor exposure on tail resorption were performed for approximately 18 days, from NF stage 58 through NF stage 66. Five NF stage 58 larvae were placed in each of four 7-liter vessels containing varying constant concentrations of the toxicants. The methoxychlor stock solution was diluted in appropriate volumes of dechlorinated tap water to prepare the appropriate test dilutions. Test concentrations included 0.0001, 0.001, 0.01, and 0.1 mg/l. Four separate vessels containing five larvae each were exposed to dechlorinated tap water alone. Treatment and control dishes contained a total of 5 l of solution. The pH of the test solutions was maintained between 7.8 and 8.0.

All solutions were administered through a flow-through delivery system to provide a complete volume replacement every 24 h during the test period. Staging was performed daily, and dead organisms were removed and the numbers recorded. Tests were terminated once 80% of the control larvae reached stage 66 for the evaluation of tail resorption. Two separate definitive tests were performed. At the completion of the exposure, larvae were examined for gross effects on tail resorption, as well as for evaluation of the thyroid glands, and they were prepared for T3 analysis.

Triiodothyronine (T3) analysis. T3 levels were determined in homogenized tissue samples from the control and 0.1 mg/l control treatments at stages 56 (control only), 58, 60, 62, 64, and 66 using conventional radioimmunoassay (RIA) in general accordance with standard methods (Huang et al., 2001; Tagawa and Hirano, 1987). An additional set of 30 organisms in each of six replicates (five organisms per replicate) were cultured as described for the metamorphic climax assay in either dechlorinated tap water or 0.1 mg/l methoxychlor. Anesthetized specimens (0.01 % [w/v] 3-aminobenzoic acid ethyl ester [MS-222]) from each of the respective stages cited were macerated at 4°C. Tissues were homogenized at 4°C with a Polytron tissue homogenizer (Brinkman Instruments Company, Westbury, NY) and prepared for RIA (Huang et al., 2001). Reconstituted precipitates were transferred to AB-coated tubes (ICN, Irvine, CA), incubated for 2 h at 37°C, and radioactivity measured. A standard curve was generated by spiking physiologically based concentrations of T3 (Sigma Chemical Company) to an aliquot of the control homogenates and measuring radioactivity as described for the previous treatments. Two separate assays were performed. For each assay, the 0.1 mg/l methoxychlor treatment and control were evaluated in triplicate. Duplicate standards were used. Values in all assays ranged from 7.3% to 15.8% for the controls and 17.8% to 34.4% for the 0.1 mg/l methoxychlor treatments, respectively.

Adult Exposure Studies
Experimental animals. For the reproductive toxicity studies, sets of 96 adult X. laevis (48 female and 48 male) were obtained (Xenopus 1, Dexter, MI) for trial 1. Based on the results of trial 1, concentrations were refined such that 64 adult X. laevis (32 female and 32 male) were used for trial 2. Frogs were maintained in groups of eight (by sex) in polyethylene tubs in dechlorinated tap water and were fed Salmon chow ad libitum, as described previously.
After 14 days of acclimation, eight female and eight male frogs were administered either dechlorinated tap water with 0.5% (v/v) DMSO or methoxychlor at varying concentrations via the culture water as described in the following sections.

**Exposure Study 4—Adult Reproduction**

**Female exposure, sample collection, and breeding.** Eight healthy female frogs per treatment group were superovulated by injection of 750 IU of human chorionic gonadotropin (hCG) into the dorsal lymph sac. Within 3 to 4 h post injection, ovulation commenced. For breeding, the superovulated females were paired with males injected with ~500 IU hCGs.c. Each mating pair was placed in a breeding chamber and allowed to breed overnight. 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 theoviduct so as to strip the ovary of oocytes not yet released. Each female was then placed into her respective treatment for 30 days, at which time a thorough evaluation of reproductive status was performed, which included an assessment of general physical health, including body weight, ovary health and weight, total oocyte content, oocyte stage distribution, and oocyte maturation capacity. Exposure via the culture water was selected for the reproductive toxicity evaluation because it represented an environmentally realistic route of exposure. All solutions were administered through a flow-through delivery system set to provide a complete volume replacement every 24 h during the test period.

Test concentrations were based on a range of sublethal concentrations determined from a preliminary experiment evaluating acute lethality. Five separate concentrations (0.0 [control], 0.001, 0.005, 0.01, 0.05, and 0.1 mg/l) were tested in the first trial, and three test concentrations (0.0 [control], 0.001, and 0.1 mg/l) were evaluated in the second trial. Four of the eight females from each treatment were paired with unexposed males to evaluate breeding response as previously described. The remaining four females were weighed and anesthetized with 1% (w/v) MS-222. The ovaries 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 examined. Ovaries from two frogs were further processed for germinal vesicle breakdown (GVBD) assay.

**Female gametogenesis.** Oocyte staging was performed in accordance with the method of Dumont (1972), in which stages are based on maturity and numbered from I to VI, with I representing the immature oocytes and VI representing fully mature, banded oocytes. A thorough count of all oocytes was then performed. Once this information was collected, the process was repeated twice to verify the data collected. The number of necrotic oocytes was also determined.

**In Vitro oocyte maturation—germinal vesicle breakdown (GVBD) assay.** Ovaries from two female frogs from the 0.0 (control) and 0.001 mg/l treatment were excised and placed in sterile, chilled OR2 buffer, pH 7.6, in a system set to provide a complete volume replacement every 24 h during the exposure. All solutions were administered through a flow-through delivery system set to provide a complete volume replacement every 24 h during the test period.

**Sperm cell count.** Prior to homogenization, the testes were grossly examined for external abnormalities. For the total sperm count, saline-methiolate-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 (Fort et al., 2001a). Testes were placed in a clear scintillation vial with SMT, minced with scissors, and homogenized (Powergen 125, Fisher Scientific) for 2 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.

**Data Collection and Analysis**

**Exposure Studies 1 and 2—FETAX and Hind Limb Development**

For each embryo-larval or limb development test, frequencies of mortality and malformation were determined. Head–tail length of surviving embryos was measured as an index of growth using Sigma Scan (SPSS, Corte Madera, California) digitizing software for the embryo-larval development tests. The length data were then used to calculate the minimum concentration to inhibit growth (MCIG) value for each experiment using ANOVA (Bonferroni t-test, p < 0.05).

**Exposure Study 3—Metamorphic Climax**

Video images were captured using a Sony CCD-iris high-resolution color digital video camera (Fort et al., 2000). Image processing software and a FlashPoint 128 (Integral Technologies, Inc., Indianapolis, IN) video frame grapper were used to digitize the tail length at developmental stages 58, 60, 62, 64, and 66. A ruler videotaped with the larvae was used to monitor image distortion and calibrate the length-measuring program to ensure accurate measurements of the larvae. Tail lengths were measured using Sigma Scan (SPSS, Corte Madera, CA). The mean tail lengths for each concentration of methoxychlor at each specific NF stage were corrected for the starting tail length at day 0 (stage 58). The median time required for half of the tail to resorb (T50) was determined for each test concentration using isotonic regression of monotonic data using SigmaStat (SPSS, Corte Madera, CA).

**Exposure Study 4—Reproduction**

Reproductive status, including ovary and testis weights and morphology, total egg count, oocyte necrosis, oocyte stage distribution, maturation capacity, and sperm counts were determined for each adult. Breeding success, fertilization rates, and embryonic viability were also determined. Comparisons of reproductive fitness evaluations and determination of No Observed Adverse Effect Concentration (NOAEC) and Lowest Observed Adverse Effect Concentration (LOAEC) values were performed using ANOVA (Dunnett’s Test, p < 0.05). Chronic values (ChV) were determined by calculating the geometric mean of the NOAEC and LOAEC values. Because NOAEC and LOAEC are concentration-based endpoints, and are thus influenced by the test concentrations, ChV provided a means of normalizing the concentration-based endpoints.

**Chemical Analysis**

Methoxychlor concentrations were determined by gas chromatography using electron-capture detection (GC-ECD). Practical quantitation limits (method...
detection limit * dilution factor) for methoxychlor were 0.1 µg/l and 50.0 ng/g, for water samples and tissue samples, respectively. Nominal test concentrations were used for the short-term embryo-larval development and metamorphic climax tests, thus no analytical measurement of methoxychlor was performed. Stock test solutions for the limb development assay and reproductive toxicity tests were measured weekly throughout the exposure period. Tissue samples from the reproductive studies including ovary, testis, and carcass (whole body without ovaries or testes) were collected from each trial and frozen until analysis. Tissue samples from four specimens (eight for carcass samples) were composited into one sample for each concentration per trial.

RESULTS

Developmental Assessment

Exposure Study 1 – FETAX

Control results. In each test, the FETAX solution and FETAX solution with 0.5% (v/v) DMSO control mortality and malformation were ≤2.5% and ≤5.0%, respectively. The incidences of mortality and malformation for the 5.5 mg/l 6-aminonicotinamide positive control treatment ranged from 0% to 10% and 42.5% to 62.5%, respectively. The incidences of mortality and malformation for the 2,500 mg/l 6-AN positive control treatment ranged from 52.5% to 82.5%, and 100%, respectively. The performance results met the requirements established in ASTM E1439–98 (ASTM, 1998).

Methoxychlor. The 4-day (stage 46) LC50 and EC50 (malformation) for methoxychlor were both ≤1.0 mg/l (maximum soluble concentration in 0.5% v/v DMSO). Methoxychlor at a concentration of 1.0 mg/l induced no mortality and 7.5% malformation. Of the malformations induced, only visceral edema, craniofacial dysmorphogenesis, and subtle notochord lesions appeared to be characteristic of methoxychlor exposure. Growth was not affected by methoxychlor exposure (ANOVA, Bonferroni t-test, p < 0.05).

Exposure Study 2—Hind Limb Development

Control results. In each test, the FETAX solution and dechlorinated tap water with 0.5% (v/v) DMSO control mortality and hind limb malformation was ≤12.5% and ≤5.0%, respectively. The incidences of mortality and hind limb malformation for the 5.5 mg/l 6-AN positive control treatment ranged from 0% to 20.0% and 25.0% to 42.5%, respectively. The performance results met the requirements established in ASTM E1439–98 (ASTM, 1998).

Methoxychlor. The 30-day (stage 54) EC50 (hind limb malformation) for methoxychlor was also > 0.1 mg/l. Methoxychlor at a concentration of 0.1 mg/l induced nominal mortality and ≤5.0% hind limb malformation. Of the malformations induced, a shortening of the femur appeared to be characteristic of methoxychlor exposure. Although limb development was apparently not affected morphologically, methoxychlor exposure did significantly slow the rate of development (Fig. 1) (ANOVA, Bonferroni t-test, p < 0.05), most specifically marked by the differentiation of the digits. A concentration-dependent retardation in digit differentiation was observed, resulting in a NOAEC value of 0.005 mg/l and a LOAEC value of 0.01 mg/l (ChV = 0.008 mg/l). No digit differentiation was noted at concentrations as low as 0.05 mg/l. Results from histological examination (Fig. 2) of the paired thyroid glands confirmed the general
enlargement of the thyroid glands and follicular hyperplasia in specimens exposed to 0.1 mg/l methoxychlor. No apparent effect on the homogeneity of colloid was noted in the methoxychlor treated specimens, however.

Exposure Study 3—Metamorphic Climax

Results of metamorphic climax (stages 58–66) culture studies during which time the tail resorbed and the larvae transgressed into a young juvenile frog are presented in Figure 3. In this case, the time required for 50% of the tail to resorb ($T_{0.5}$) was determined for each concentration tested. A concentration-dependent increase in the $T_{0.5}$ was detected, which suggested that methoxychlor inhibited the rate of tail resorption. The mean $ChV$ was determined to be 0.06 mg/l (ANOVA, Bonferroni $t$-test, $p < 0.05$). Although no histological examination was performed, NF stage 61–62 specimens exposed to 0.1 mg/l were qualitatively found to have enlarged thyroid glands compared to controls, which were visualized without staining directly through the transparent larvae under a dissecting microscope.

Results of $T_3$ profile analysis immediately prior to and throughout metamorphic climax in the control specimens and the 0.1 mg/l methoxychlor treatment are presented in Figure 4. Interestingly, the $T_3$ surge in the 0.1 mg/l methoxychlor treatment was somewhat lower in amplitude and somewhat shifted to the right of the $T_3$ surge curve for the control organisms.

Exposure Study 4—Adult Reproduction

Female evaluation

Tissue accumulation. Accumulation of methoxychlor was noted in the carcass, but not to the extent found in...
the ovary samples (Table 2). This trend was consistent in both trials.

Reproductive endpoints. The effects of 30-day methoxychlor via culture water on female reproductive endpoints are provided in Tables 3 and 4. The ChV for reduction in ovary weight were 0.008 mg/l and 0.006 mg/l methoxychlor, respectively, for trials 1 and 2 (ANOVA, Dunnett’s Test, $p < 0.05$). Necrosis of the ovary was observed at 0.05 mg/l and 0.01 mg/l methoxychlor, respectively, for trials 1 and 2. Chronic values for reduction in total oocyte counts were 0.008 mg/l and 0.006 mg/l methoxychlor, respectively, for trials 1 and 2. Chronic values for reduction in total oocyte counts were 0.008 mg/l and 0.006 mg/l methoxychlor, respectively, for trials 1 and 2 (ANOVA, Dunnett’s Test, $p < 0.05$). The ChV for an increase in the proportion of immature oocytes (<stage III) were 0.008 mg/l and 0.006 mg/l methoxychlor for trials 1 and 2, respectively (ANOVA, Dunnett’s Test, $p < 0.05$). Chronic values for increased oocyte necrosis from trials 1 and 2 were 0.003 mg/l and 0.006 mg/l methoxychlor, respectively. Oocytes collected from females exposed to 0.1 mg/l methoxychlor were completely necrotic in both trials.

Successful breeding responses as determined by amplexus occurred with each of the females from the control and the 0.001 mg/l methoxychlor treatment. Three of the four (75%), two of the four (50%), and one of the four (25%) females exposed to 0.005, 0.01, or 0.05 mg/l methoxychlor for 30 days via the culture media successfully amplexed in trial 1. Similar results were observed in trial 2 with the exception of the 0.01 mg/l methoxychlor treatment, in which only one of the four females successfully amplexed. None of the four females exposed to 0.1 mg/l methoxychlor in either trial initiated breeding activity.

No effect on fertilization rates was observed with females exposed to methoxychlor concentrations ranging from 0.0 to 0.01 mg/l for 30 days in the culture media; however, a significant

![FIG. 3](image3.png)

**FIG. 3.** Median time required for *X. laevis* larvae to reach 50% tail resorption ($T_{0.5}$) for each methoxychlor treatment. Error bars represent standard error of the mean (SEM).

![FIG. 4](image4.png)

**FIG. 4.** Results of T₃ profile analysis of *X. laevis* control and 0.1 mg/l methoxychlor treatment specimens during the conclusion of prometamorphosis (stages 56–58) and throughout metamorphic climax.

**TABLE 2**

<table>
<thead>
<tr>
<th>Treatment (mg/l)</th>
<th>Carcass</th>
<th>Ovary</th>
<th>Testis</th>
</tr>
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<tbody>
<tr>
<td><strong>Trial 1</strong></td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td></td>
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<tr>
<td>0.001</td>
<td>0.001</td>
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<td>0.30</td>
</tr>
<tr>
<td>0.005</td>
<td>—</td>
<td>0.22</td>
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<tr>
<td>0.01</td>
<td>0.01</td>
<td>0.38</td>
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<tr>
<td>0.05</td>
<td>—</td>
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</tr>
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<td>0.1</td>
<td>0.1</td>
<td>1.32</td>
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</tr>
<tr>
<td><strong>Trial 2</strong></td>
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<tr>
<td>0.1</td>
<td>0.1</td>
<td>1.32</td>
<td>1.07</td>
</tr>
</tbody>
</table>

<sup>a</sup>Expressed as µg/g. Represents composite samples for each treatment concentration. N = 8 for carcass samples. N = 4 for reproductive tissue samples. MDL = 10 ng/g.

<sup>b</sup>Whole body without ovaries or testes.
reduction (ANOVA, Dunnett’s Test, \( p < 0.05 \)) in fertilization rates was found in the 0.05 mg/l treatment in the first trial and 0.01 mg/l methoxychlor in the second trial. A significant reduction (ANOVA, Dunnett’s Test, \( p < 0.05 \)) in 96-h embryo viability (matured or dead) was detected at 0.005 mg/l and 0.001 mg/l methoxychlor in trials 1 and 2, respectively. Embryos from females treated with 0.01 mg/l were completely nonviable in both trials.

The effect of maternal methoxychlor exposure on early embryo-larval development is provided in Tables 5 and 6. A statistically significant (ANOVA, Dunnett’s Test, \( p < 0.05 \)) increase in embryolethality was detected in the 0.005 mg/l (ChV = 0.003 mg/l) and the 0.01 mg/l (ChV = 0.006 mg/l) methoxychlor treatments in trials 1 and 2, respectively. In addition, a significant increase (ANOVA, Dunnett’s Test, \( p < 0.05 \)) in abnormal development was observed in fertilized embryos collected from females exposed to 0.005 mg/l methoxychlor for 30 days and cultured in FETAX Solution for 4 days in trial 1. In trial 2, baseline levels of malformation were detected in the 0.001 mg/l methoxychlor treatment. The 0.01 mg/l methoxychlor treatment in trial 2 induced complete lethality. Finally, successful breeding did not take place in the 0.1 mg/l methoxychlor treatment from trial 2. Thus, limited information regarding malformations induced was generated from trial 2 data. Larvae from females exposed to \( \geq 0.005 \) mg/l methoxychlor for 30 days possessed abnormal guts, lesions within the notochord, and craniofacial dysmorphism. Larvae from females exposed to \( \geq 0.01 \) mg/l methoxychlor for 30 days also demonstrated hydrocephalus, microcephaly, axial flexure resulting from the notochord lesions, microphthalmia, cleft palate, and hypognathia.

In Vitro Oocyte Maturation (GVBD Assay). As an extension of studies performed by Pickford and Morris (1999), in which the propensity of methoxychlor to inhibit progesterone-induced GVBD was demonstrated, the effect of maternal methoxychlor exposure on the capacity to produce mature oocytes required for fertilization was evaluated. Normal-appearing banded stage V and VI oocytes were removed from two females from the 30 days 0.001 mg/l methoxychlor treatment from trial 2 and co-cultured with 100 nM progesterone. The majority (83.2% ± 4.8%) of stage VI oocytes from the control females successfully completed GVBD when cultured with 100 nM progesterone. However, a significant proportion (ANOVA, Bonferroni \( t \)-Test, \( p < 0.05 \)) of the stage VI oocytes from the methoxychlor-treated female group did not undergo GVBD (34.1% ± 5.2%), indicating that they were not as progesterone responsive as the control treatment.

### Table 3

**Trial 1—Summary of the Effects of Methoxychlor on Reproductive Endpoints in X. laevis**

<table>
<thead>
<tr>
<th>Concentration (mg/l)</th>
<th>Ovary weight (%)</th>
<th>Egg mass</th>
<th>Male parameters</th>
<th>Breeding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>% &lt;Stage 3 (%)</td>
<td>% &gt;Stage 3 (%)</td>
</tr>
<tr>
<td>0.0</td>
<td>0.12 (0.03)</td>
<td>2987</td>
<td>23.9</td>
<td>76.1</td>
</tr>
<tr>
<td>0.001</td>
<td>0.13 (0.03)</td>
<td>2845</td>
<td>25.9</td>
<td>74.1</td>
</tr>
<tr>
<td>0.005</td>
<td>0.08 (0.02)</td>
<td>1456</td>
<td>31.4</td>
<td>68.6</td>
</tr>
<tr>
<td>0.01</td>
<td>0.05 (0.01)</td>
<td>437</td>
<td>44.9</td>
<td>55.1</td>
</tr>
<tr>
<td>0.05</td>
<td>0.04 (0.01)</td>
<td>253</td>
<td>63.3</td>
<td>36.7</td>
</tr>
<tr>
<td>0.1</td>
<td>0.01 (0.002)</td>
<td>14</td>
<td>100.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*Concentration in dechlorinated tap water. Adults were exposed to methoxychlor for 30 d prior to evaluation. N = 4 for reproductive endpoint evaluation and 4 for breeding response. 0.0 represents 0.5% (v/v) DMSO in dechlorinated tap water. Values are expressed as means, with standard error of the means in parentheses.

Expressed as % of body weight.

Breeding response.

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

FETAX assessment culturing embryos in FETAX Solution alone.

*Based on Dumont stages (Dumont, 1972).

*Value significantly different from control (ANOVA, \( p < 0.05 \)).
Trial 2—Summary of the Effects of Methoxychlor on Reproductive Endpoints in *X. laevis*.

**TABLE 4**
Trial 2—Summary of the Effects of Methoxychlor on Reproductive Endpoints in *X. laevis*.

<table>
<thead>
<tr>
<th>Concentration&lt;sup&gt;a&lt;/sup&gt;(mg/l)</th>
<th>Ovary weight&lt;sup&gt;b&lt;/sup&gt; (%)</th>
<th>Egg mass</th>
<th>Male parameters</th>
<th>Breeding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total&lt;sup&gt;c&lt;/sup&gt; (mg/l)</td>
<td>&lt;Stage 3&lt;sup&gt;d&lt;/sup&gt; (%)</td>
<td>≥Stage 3&lt;sup&gt;d&lt;/sup&gt; (%)</td>
<td>Necrotic (%)</td>
</tr>
<tr>
<td>0.0</td>
<td>(0.02) 2787 (5.3)</td>
<td>31.1</td>
<td>69.0</td>
<td>6.9</td>
</tr>
<tr>
<td>0.001</td>
<td>(0.01) 245 (3.5)</td>
<td>31.1</td>
<td>68.9</td>
<td>11.4</td>
</tr>
<tr>
<td>0.01</td>
<td>(0.02) 153 (4.0)</td>
<td>43.2</td>
<td>56.8</td>
<td>65.1&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.1</td>
<td>(0.007) 20 (0.0)</td>
<td>100.0&lt;sup&gt;e&lt;/sup&gt; (0.0)</td>
<td>0.0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>100.0&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Concentration in dechlorinated tap water. Adults were exposed to methoxychlor for 30 d prior to evaluation. N = 4 for reproductive endpoint evaluation and 4 for breeding response. 0.0 represents 0.5% (v/v) DMSO in dechlorinated tap water. Values are expressed as means, with standard error of the means in parentheses.

<sup>b</sup>Expressed as % of body weight.

<sup>c</sup>Breeding response.

<sup>d</sup>Presented as treated females bred with untreated males/treated males bred with untreated females.

<sup>e</sup>FETAX assessment culturing embryos in FETAX Solution alone.

<sup>f</sup>Based on Dumont stages (Dumont, 1978).

<sup>g</sup>Value significantly different from control (ANOVA, p < 0.05).

**TABLE 5**
Trial 1—Summary of Effect of Maternal Methoxychlor Exposure on Early Embryo-Larval Development in *X. laevis* Progeny.

<table>
<thead>
<tr>
<th>Concentration&lt;sup&gt;a&lt;/sup&gt;(mg/l)</th>
<th>Mortality&lt;sup&gt;b&lt;/sup&gt; (%)</th>
<th>Malformation&lt;sup&gt;c&lt;/sup&gt; (%)</th>
<th>Type of terata</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>2.5</td>
<td>0.9</td>
<td>Gut maldevelopment</td>
</tr>
<tr>
<td></td>
<td>(1.4)</td>
<td>(0.2)</td>
<td></td>
</tr>
<tr>
<td>0.001</td>
<td>5.0</td>
<td>2.1</td>
<td>Gut maldevelopment</td>
</tr>
<tr>
<td></td>
<td>(2.0)</td>
<td>(0.5)</td>
<td></td>
</tr>
<tr>
<td>0.005</td>
<td>41.7&lt;sup&gt;e&lt;/sup&gt;</td>
<td>23.4&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Gut maldevelopment,</td>
</tr>
<tr>
<td></td>
<td>(6.7)</td>
<td>(1.6)</td>
<td>notochord lesions,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>craniofacial defects</td>
</tr>
<tr>
<td>0.01</td>
<td>60.0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>100.0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Gut maldevelopment,</td>
</tr>
<tr>
<td></td>
<td>(10.0)</td>
<td>(0.0)</td>
<td>notochord kinking,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>microcephaly,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>hydrocephalus,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>microophthalmia,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>cleft palate,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>hypognathia</td>
</tr>
<tr>
<td>0.05</td>
<td>100.0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>(0.0)</td>
<td>—</td>
</tr>
<tr>
<td>0.1</td>
<td>(0.0)</td>
<td>(0.0)</td>
<td>(0.0)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Concentration in dechlorinated tap water with 0.0 representing 0.5% (v/v) DMSO in FETAX solution.

<sup>b</sup>Values expressed as means, with standard error of the means in parentheses.

<sup>c</sup>Value significantly different from control (ANOVA, p < 0.05).

**Male assessment**

Tissue accumulation. Accumulation of methoxychlor in the testes was noted in the male specimens used in the study, but accumulation was somewhat less than found in the ovaries of the exposed female specimens.

Reproductive endpoints. The effects of methoxychlor on male reproductive endpoints and reproductive performance are presented in Tables 3 and 4. The ChV for reduction in testis weight were 0.008 mg/l and 0.006 mg/l methoxychlor, respectively for trials 1 and 2 (ANOVA, Dunnett’s Test, p < 0.05). Methoxychlor exposure for 30 days significantly reduced (ANOVA, Dunnett’s Test, p < 0.05) testis weight, although, externally, lesions were only noted in males exposed to 0.1 mg/l...
methoxychlor. The ChV for reduction in sperm count were also 0.008 mg/l and 0.006 mg/l methoxychlor, respectively, for trials 1 and 2 (ANOVA, Dunnett’s Test, $p < 0.05$).

No effect on breeding response was noted in the 0.001 and 0.005 mg/l methoxychlor treatments from trial 1 or the 0.001 mg/l methoxychlor treatment from trial 2. At 0.1 mg/l methoxychlor, amplexus was not induced in any of the males bred in either trial. In addition, a significant reduction (ANOVA, Dunnett’s Test, $p < 0.05$) in fertilization and embryonic viability at 96 h was observed in the 0.05 mg/l methoxychlor treatment group in trial 1. No reduction in fertilization or embryo viability was found in trial 2 studies, although 0.01 mg/l methoxychlor was the greatest concentration tested in which embryos were produced.

**DISCUSSION**

Results from the present study indicated that *X. laevis* demonstrated stage-specific sensitivity to methoxychlor. Moreover, the sensitivity was manifested most prominently during the reproductive phase of the life cycle and during metamorphosis. However, little effect was noted during early embryo-larval development. The effects observed during early metamorphosis at which time the transition from premetamorphosis to metamorphosis was occurring were based primarily on a slowing of hind limb development. Anuran metamorphosis is separated into three distinct periods, premetamorphosis, metamorphosis, and metamorphic climax (Dodd and Dodd, 1976; Etkin, 1964; 1968). Premetamorphosis refers to a period of embryonic and early larval development that takes place without thyroid hormone (TH). Some advanced morphological development occurs during this stage, including hind limb bud development. More specific morphogenesis, such as differentiation of the toes and rapid growth (elongation) of the hind limbs, occurs during premetamorphosis. Biochemically, premetamorphosis is characterized by rising concentrations of endogenous TH. The final period is metamorphic climax, in which a surge of TH triggers the final processes associated with metamorphosis, including forelimb development and resorption of the tail. Drastic internal transformations at the organ system, tissue, and biochemical levels are also taking place during metamorphosis and metamorphic climax.

Although development of the primordial limb bud is not under the control of the thyroid axis, differentiation of the hind limb, including digits, is controlled by thyroid activity. In the present study, abnormal development of the hind limbs was minimal. However, the rate of development, specifically the differentiation of the hind limb digits was slowed in a concentration-dependent manner. Alone, these results did not specifically indicate that the developmental delay observed was the direct result of disruption of thyroid axis activity. However, the identification of enlarged thyroid glands with marked follicular hyperplasia in the 0.1 mg/l methoxychlor treated

organisms (Fig. 2) suggested that the thyroid may be a site of action. The effects of methoxychlor on delayed resorption of the tail during metamorphic climax and shifts in dynamics of the $T_3$ surge that marks the onset of metamorphic climax further suggested that the thyroid axis may have been involved in the development delay observed in the methoxychlor treated organisms. More work, including evaluation of the expression profiles of thyroid receptors, will be required to elucidate the specific mode of action of methoxychlor.

Methoxychlor exerted a relatively strong effect on delaying tail resorption during metamorphic climax. This response was much stronger than that observed with several other organochlorine pesticides, including o,p-DDE, p,p-DDE, dieldrin, and aldrin (Fort, unpublished data). In addition to inhibition of tail resorption, alteration in the form of a reduction and rightward shift in the $T_3$ surge implied that the delay in metamorphic completion might be the result of thyroid axis disturbance. Again, it is difficult to provide a direct connection between the apical morphological effects and changes in TH profiles without further study. However, it appeared that methoxychlor altered the rate of development during metamorphosis by disrupting the thyroid axis.

The effects of methoxychlor on reproductive fitness in *X. laevis* were the most striking. The effect of methoxychlor on male reproductive fitness parameters only influenced reproductive success, as measured by fertilization and embryo-larval viability, at a concentration of 0.05 mg/l. However, decreased reproductive success in exposed females was observed at 0.005 mg/l methoxychlor. Thus, although methoxychlor appeared to target both the female and the male reproductive systems, the effects on the female reproductive tract may be the most biologically significant. Further, each of the female reproductive endpoints was adversely affected by exposure to methoxychlor. The increase in the number of necrotic oocytes appeared to be slightly more sensitive than the other reproductive endpoints. Overall, ovarian health was adversely affected to a great extent. Male reproductive health was also affected by methoxychlor exposure. This effect included a concentration-dependent influence on testis weight and sperm count. Further study will be needed to determine if methoxychlor alters spermatogenesis, producing abnormal sperm cells.

Breeding response was also affected, although some of the effects noted here may have been the result of general toxicological insult and unrelated to the reproductive fitness parameters measured in the present study. Although the trend was more obvious in trial 2 than in trial 1, female exposure appeared to have a greater impact on breeding success (induction of amplexus and oocyte fertilization). Fertilization was one of the least sensitive reproductive endpoints measured. Fertilization was more dramatically reduced in exposed female cross-bred with unexposed males than in exposed males cross-bred with unexposed females. Embryos produced from exposed females cross-bred with unexposed males were markedly less viable than the reciprocal cross. Thus, the biological impact of
female methoxychlor exposure was greater than male exposure. Although males exposed to methoxychlor exhibited reproductive stress from a statistical standpoint in the present study, the effects induced were not necessarily biologically significant.

Methoxychlor demonstrated a propensity to accumulate in the adult *X. laevis* exposed in the reproductive studies. Hall and Swineford (1979) found relatively significant uptake of methoxychlor from food and water in the American toad, *Bufo americanus*. On a per mass basis in the present study, the ovary accumulated greater quantities of methoxychlor than the remainder of the body. The testis also accumulated methoxychlor, but not to the same extent as found in the ovary. Based on these accumulation profiles, it appeared that the ovary, and to a lesser extent the testis, were target sites for methoxychlor. In terms of expressing the reproductive effects observed in the adult specimens as a carcass concentration, the majority of effects were observed between 0.2 and 0.5 μg/g. However, these effects were observed in exposed females at ovary concentrations ranging from 0.4–1.9 μg/g, and in exposed males at testis concentrations ranging from 0.5–1.0 μg/g. This accumulation may account for the marked effects on reproductive fitness. Very few studies have evaluated the toxicological effects of methoxychlor on early development in amphibians (Bevan et al., 2003). Although these investigators found that other environmental estrogens, such as, nonylphenol and octylphenol, as well as the natural estrogen, 17β-estradiol, were capable of inducing gross abnormalities at NF stage 37 (48 h of development) at the natural estrogen, 17β-estradiol, were capable of inducing gross abnormalities at NF stage 37 (48 h of development) at

Methoxychlor has been shown to alter episodic prolactin release in adult male Sprague-Dawley rats by sensitizing the pituitary to thyrotropin releasing hormone (TRH) (Lafuente et al., 2003). Amstislavsky et al. (2003) found that administration of 16.5 mg/kg methoxychlor to pregnant mice on days 2–4 did not interfere with tubal embryo transfer or induce malformations. However, these investigations did suppress embryo development and increased the frequency of nuclear fragmentation and the formation of micronuclei. Borgeest et al. (2002) determined that administration of methoxychlor i.p. for 10–20 days at a dose of 32 mg/kg per day caused an increase in the ovarian surface epithelial height and increased antral follicular atresia. Adverse effects of methoxychlor exposure on the testis have also been demonstrated by Johnson et al. (2002) and Latchoumycandane and Mathur (2002). Johnson et al. (2002) found that perinatal rat dams gavaged with 5–150 mg/kg per day for one week prior to and one week after birth, followed by a subsequent exposure of male rats to methoxychlor from postnatal days 7 to 42 experienced reduced testicular size and epididymal spermatozoa, as well as a reduction in the number of Sertoli cells. Latchoumycandane and Mathur (2002) found similar morphological effects in male rats, as well as signs of increased oxidative stress in the testis.

Gray et al. (1989), Wade et al. (2002), and Zhou et al. (1995), and each found that methoxychlor had a thyroid toxic effect. Gray et al. (1989) found that in addition to endocrine disturbance at the gonadal level, pituitary levels of prolactin, follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH) were altered. Serum TSH was reduced by at least 50% compared to controls at 100 mg/kg per day, while pituitary levels increased. In conclusion, exposure of *X. laevis* to sublethal levels of methoxychlor repressed metamorphic events, including hind limb differentiation and tail resorption, as well as, interfered with normal reproductive processes in both adult female and male specimens. However, no gross morphological effects were observed on early embryo-larval development. These results were reasonably consistent with results obtained in mammals. Furthermore, they shed light on the practical aspects of evaluating environmental chemicals with EDC activity, like methoxychlor. Although it is generally thought that early life stage tests utilizing an embryo-larval exposure period provide evaluation at the most sensitive period in an organism’s life cycle, this may not necessarily be the case for EDCs. Although Bevan et al. (2003) demonstrated that early exposure to some environmental estrogens may have early consequences in developing embryos, it would appear that exposure during the latter phases of
development, including primary sexual development, metamorphosis, secondary sexual development, and reproduction would be a larger target of the life cycle. Although somewhat controversial, this is apparently the case with the estrogenic herbicide atrazine, which induces abnormal embryo-larval development in *X. laevis* at concentrations ≥ 5.0 mg/l (Fort et al., in press a, in press b), but induces severe effects on secondary sexual development at concentrations orders of magnitude less then 5.0 mg/l (Carr et al., 2003; Hayes et al., 2002). Overall, these data, along with the data from the present study, support the use of either extended partial life cycle–based or full life cycle–based bioassays for the evaluation of toxicants that act by disrupting endocrine function.

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REFERENCES


