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Methoxychlor Alters Hatching and Larval Startle Response in the Salamander *Ambystoma macrodactylum*

R. L. Ingermann,[1] D. C. Bencic,[1] V. P. Eroschenko[1,2]

[1]Department of Biological Sciences, University of Idaho, Moscow, Idaho 83844-3051, USA
[2]WAMI Medical Education Program, University of Idaho, Moscow, Idaho 83844-3051, USA

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Due to its lower toxicity to mammals and its shorter half-life, the estrogenic pesticide methoxychlor (MXC; 1,1'-[2,2,2-trichloroethylidene]bis[4-methoxy]-benzene) has been widely used as a replacement for DDT (its structural analog) in the control of a variety of insect pests. It has been applied to forests and crops by aerial spraying, a process which has contaminated nearby waters. MXC has also been applied to wetlands to control fly and mosquito larvae, particularly in the spring and summer (Stoltz and Pollock 1982; ATSDR 1994). Depending upon biotic and abiotic conditions, its half-life in water can range from a few hours to a year (Merna et al. 1972; Wolfe et al. 1977; ATSDR 1994). Because of its biological actions on target species, sites of application, and potential persistence in the environment, MXC may have significant actions on non-target aquatic organisms. It has major and variable effects on non-target fish including influences on mortality and fecundity (Merna et al. 1972; Lee et al. 1975; Lockhart et al. 1977; Holdway and Dixon 1986; Heming et al. 1988; 1989); however, its effects on other non-target organisms have received little attention. To our knowledge, the effect of MXC on amphibians has not been studied. Consequently, we examined the influence of MXC on pre-hatch mortality and time-of-hatch of the embryos and post-hatch startle response of the larvae of the salamander *Ambystoma macrodactylum.*

MATERIALS AND METHODS

Eggs of *Ambystoma macrodactylum* Biard were collected from local ponds in late March/early April and identified as previously described (Berner and Ingermann 1990). Eggs were stored for about 10 days at 0 - 1 °C in darkness. Just prior to initiating the experiment, clusters of eggs were separated into smaller groups containing 1 to 6 eggs. These were mixed and subsequently 30 to 34 eggs were placed into each of 8 bowls containing 800 mL aged tap water containing no additives, 0.4 mL dimethyl sulfoxide (DMSO)/L (vehicle), or 0.03, 0.1, 0.3, 1.0, 3.2, or 10.0 mg MXC/L dissolved in 0.4 mL DMSO/L. MXC was 98% pure from Sigma Chem. Co., St. Louis, MO; DMSO and MXC had no effect on water pH; day 1 of the experiment was 10 April 1996. Bowls and eggs were maintained in darkness at 10 - 11 °C and checked daily for mortality and hatching. After hatching, up to 4 larvae were transferred to a beaker containing approximately 50 mL of their respective solutions and were maintained in darkness at 10 - 11 °C. Larvae were subsequently monitored for mortality for 10 days after hatch. Also, 2 days after hatch, 3 larvae from the water, DMSO, 0.3 mg MXC/L, and 10 mg MXC/L groups were preserved in 10% neutrally buffered formalin for subsequent physical measurement and staging, according to embryonic stages for *Ambystoma*

Correspondence to: R. L. Ingermann

maculatum (identified as *Amblystoma punctatum* by Harrison [1969]). Stage and measurement data were collected without knowledge of treatment group identity.

At 10 days after hatch, 1 to 6, but usually 3 larvae were randomly selected from each treatment group and transferred to a container holding 38 cm x 38 cm x 3 cm water at 10 °C. Larvae were left undisturbed for about 0.5 hr, after which a 450 g lead ball was dropped against the side of the container and the movements of the larvae recorded with a video recorder. Larvae were left undisturbed for about 10 min and then startled again; this procedure was repeated once more for a total of three times. Subsequently, the number of larvae which responded and the distance traveled by the responding larvae were determined.

Additional eggs were maintained in darkness in water, DMSO, or 0.3 mg MXC/L until 2 days after peak hatch, as determined in the experiment above (and shown in Fig. 1). Larvae were then transferred to outdoor containers (26 L) with water only. Twenty-six larvae from the water group were transferred on day 28, 26 DMSO-exposed larvae on day 23, and 24 MXC-exposed larvae on day 20/21. Water contained zooplankton and other normal prey items, and in addition, frozen brine shrimp and live aphids were added to the containers several times per week. The containers were kept doubly covered in shade. Weight of larvae was determined on experimental day 78 (26 June) and larvae analyzed for startle response on days 79 and 80.

Day-of-hatch, distance of movement after startle response, and day 78 wet weights were analyzed by a one-way ANOVA followed by a Student-Newman-Keuls test for significance. Significance was taken as $P<0.05$.

RESULTS AND DISCUSSION

During the period of embryonic development and through 10 days after hatching, MXC at concentrations up to 10 mg/L did not result in appreciable mortality in *A. macrodactylum* embryos or larvae and mortality was not dose-dependent (Table 1). These urodele embryos were therefore less sensitive to acute MXC toxicity than are the adults or juveniles of numerous teleosts which have $LC50$ values of 0.002 to 0.290 mg MCX/L for 96 hr exposures (Henderson et al. 1959; Merna et al. 1972; Heming et al. 1989). Concentrations of MXC at and above 0.1 mg/L, however, promoted early hatching (Fig. 1). This appeared to be due to precocial hatching rather than due to accelerated development (Table 2). Hatching in embryonic salamanders is prompted by environmental stress, usually oxygen limitation, which triggers the release of hatching enzyme (Petranka et al. 1982). As such, this mechanism is analogous to that which normally prompts the hatching of fish embryos (DiMichele and Taylor 1980: Iuchi et al. 1985; Oppen-Berntsen et al. 1990). However, Cloud (1981) has shown that deoxycorticosterone (as well as some other steroids including pregnenolone and progesterone but not testosterone or estradiol) induces precocial hatching in the teleost *Oryzias latipes*, suggesting that different types of stressors prompt hatching in fish. Because of its toxic, pesticidal properties, the induction of precocial hatching by MXC in *A. macrodactylum* embryos may be a similar, oxygen-independent stress response.

Ten days after hatch, embryos/larvae maintained in 0.3 mg MXC/L or greater showed alterations in their startle response (Fig. 2A and B). It is not clear at this time whether the decreased responsiveness and shorter distances traveled were due to precocial

Table 1. Mortality of embryos and larvae prior to the day 10 startle response measurements.

Group	Pre-Hatch Deaths		Number of Hatches	Deaths during 10 Days after Hatch
	Day 1	Days 2-35		
H_2O	1	1	32	0
DMSO	4	2	28	0
0.03 mg MXC/L	4	1	28	0
0.1	14	5	14	0
0.3	2	1	30	1
1.0	4	4	24	1
3.2	5	2	26	0
10.0	1	2	27	2

Table 2. Characteristics of larvae preserved 2 days after hatch.

Group	Stage	Length of Body	Width of Head	Length of Balancer	Wet Weight
H_2O	41-42	12.5 ± 0.1	2.6 ± 0.1	1.3 ± 0.1	15.4 ± 0.3
DMSO	40-41	12.6 ± 0.1	2.3 ± 0.1	1.3 ± 0.1	14.8 ± 0.3
0.3 mg MXC/L	40	11.6 ± 0.3	2.0 ± 0.1	1.0 ± 0.1	10.5 ± 0.9
10.0	39	11.0 ± 0.1	2.0 ± 0.1	0.8 ± 0.1	10.5 ± 0.4

Stage is range of developmental stages described by Harrison (1969) for *A. macularum* (identified as *A. punctatum)*. Length in mm. wet weight in mg. Data are mean ± SEM, $N = 3$.

hatching or due to toxic effects of the MXC, or to a combination of both. However, embryos/larvae maintained at 0.3 mg MXC/L for the initial 20/21 day period and then transferred to outdoor enclosures with just water until days 79 and 80 showed a comparable startle response to water controls (Fig. 3). (There was no consistent and compelling evidence of habituation or sensitization among any of the startle response trials.) Thus, the behavioral effects of early 0.3 mg MXC/L-exposure on distance traveled following a startle stimulus appeared reversible.

When embryos/larvae were maintained until 2 days after hatch in water, DMSO, or 0.3 mg MXC/L and then transferred to shaded and covered outdoor enclosures with just water, final body weights of the MXC-treated larvae on day 78 were less than those of water or DMSO controls. Mean wet weights of the MXC-treated, DMSO, and water larvae were 340 ± 40, 530 ±80, and 480 ± 70 mg (mean ± SEM, $N = 20, 12, 12$). respectively. The weight of the MXC-treated larvae was significantly less than that of

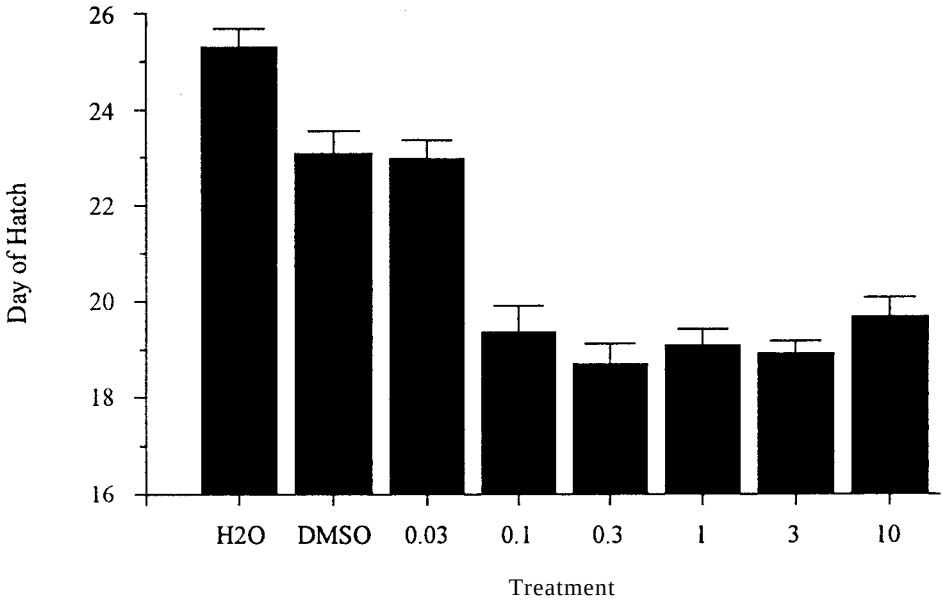

Figure 1. Mean day of hatch. Shown are means ± SEM; *N* values are given in Table 1 as Number of Hatches. Day of hatch for water controls was significantly greater than for any other group; day of hatch for DMSO and 0.03 mg MXC/L-treated embryos did not differ but were significantly greater than for embryos maintained with 20.1 mg MXC/L.

water and DMSO larvae; the water and DMSO larvae did not differ in weight. This suggests that the initial MXC-treatment did have a long-term impact on the animal.

DMSO (at 0.4 mL/L) alone significantly accelerated the time of hatch (Fig. 1) and the larvae of this group tended to swim longer distances than the water controls in response to the startle stimulus (Fig. 2B, 3). That DMSO influenced salamander embryo and larval development is not unexpected as DMSO at or below the concentration used in this study has been previously shown to have biological/biochemical effects, such as on cell chemotaxis and depolymerization of hyaluronic acid (Antony et al. 1983; Fox and Fox 1983). It remains to be seen, however, whether the actions of DMSO on *A. macroalactylum* embryos and larvae have longer-term consequences.

MXC, at up to 10 mg/L, did not increase embryo or larval mortality for at least 10 days after hatch and the dose of 0.3 mg/L appeared to have few substantial long-term deleterious impacts on the organism. Nonetheless, as shown by Holdway and Dixon (1986) for a teleost, it is possible that even low concentrations of MXC may have profound impacts on reproductive success and population stability of this salamander. Further, as shown by the work of Arnold et al. (1996) with estrogenic xenobiotics, exposures to low levels of MXC may have significant detrimental consequences in the presence of low concentrations of other pesticides due to synergistic interactions. Therefore, although *A. macrodactylum* embryos and larvae appear substantially less

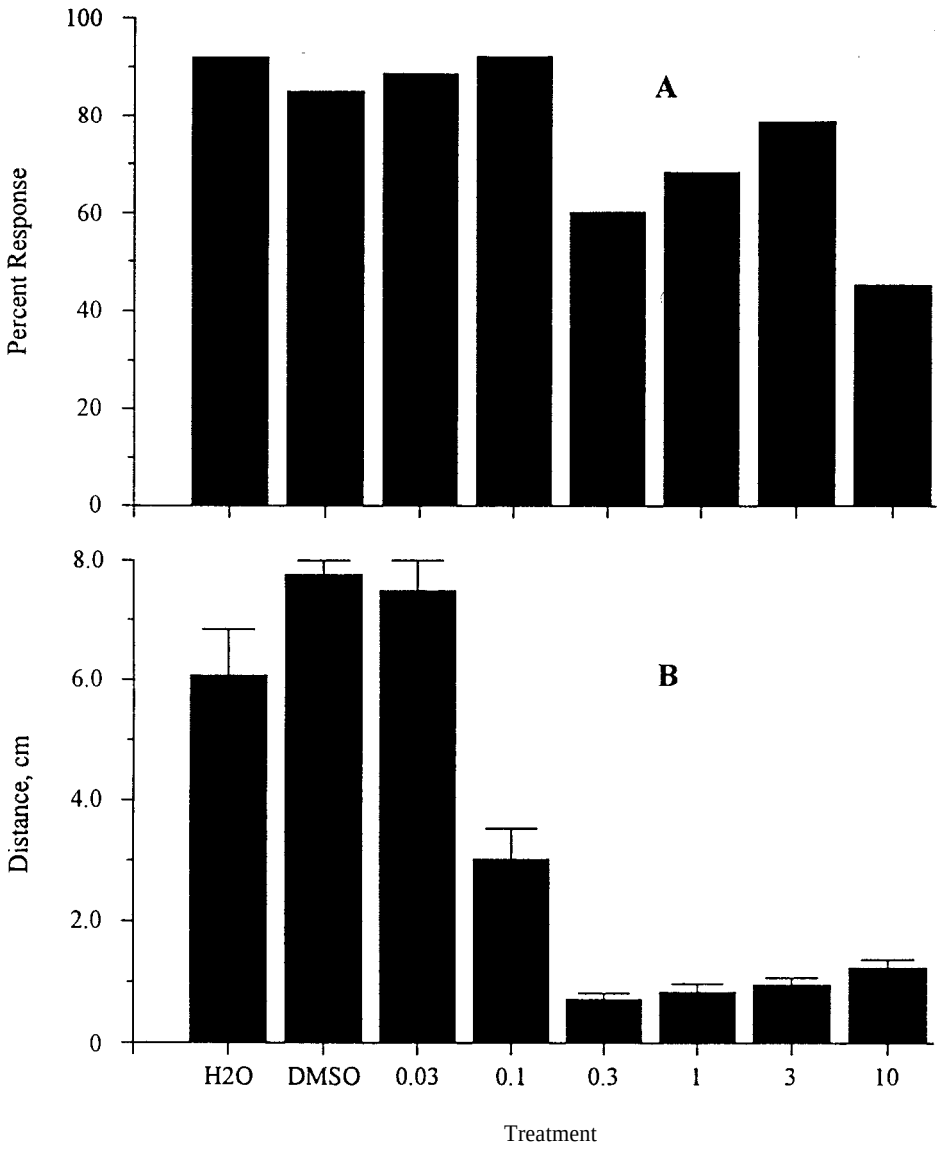

Figure 2. A) Percent of trials in which larvae responded (with at least a visual twitch) to the startle response stimulus. For the water group, 26 larvae were tested a total of 76 times and responded 70 times (92%). For the other groups, the number of individual larvae, number of total tests, and number of total responses were: DMSO = 21, 60, 52; 0.03 mg MXC/L = 27, 80, 71; 0.1 mg/L = 14, 40, 37; 0.3 mg/L = 24, 70, 43; 1.0 mg/L = 23, 69, 47; 3.2 mg/L = 22, 66, 48; 10.0 mg/L = 18, 53, 24, respectively. **B)** Mean distance + SEM traveled by larvae which responded to the stimulus. Distances traveled by water, DMSO, and 0.03 mg MXC/L-treated larvae did not differ but were significantly greater than the distance traveled by the 0.1 mg/L-treated larvae; larvae in these four groups traveled significantly further than did larvae exposed to ≥ 0.3 mg/L.

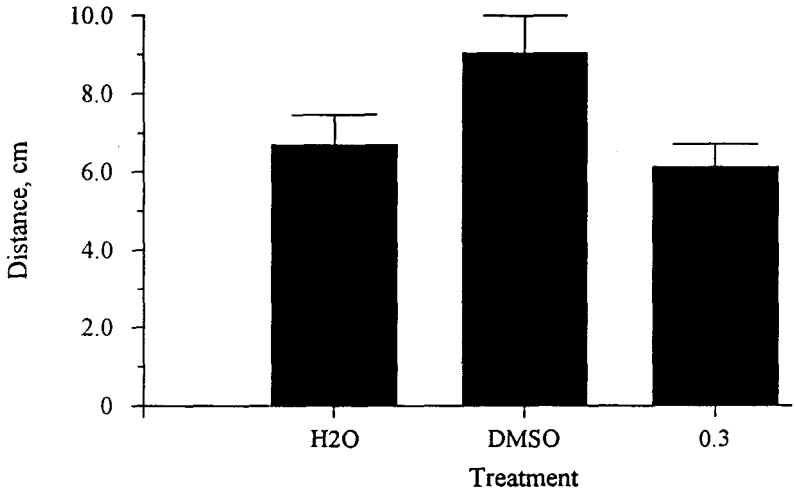

Figure 3. Mean distance + SEM traveled by larvae responding to the startle stimulus on day 79-80. The number of individual larvae, number of total tests. and number of total responses were: water = 12, 36, 30; DMSO = 12, 36, 34; 0.3 mg MXC/L-treated = 20, 59, 52, respectively. Distances traveled by the MXC-treated and water control larvae were not different but both were significantly less than that of the DMSO larvae.

sensitive to MXC than teleosts in terms of mortality, the results of the present study cannot be interpreted as indicating that low concentrations of MXC will not have major and/or long-term influences on this amphibian in its native habitat.

Overall, the results of the present study indicate that exposure of embryos and early larvae to MXC at or above 0.1 mg/L results in the precocial hatch of the embryos, probably a reduced likelihood of the larvae responding to a startle stimulus, and a significantly shorter distance traveled in response to the stimulus at 10 days after hatch. This latter effect was reversible as transfer of larvae from 0.3 mg MXC/L to only water shortly after hatch for about 2 months resulted in a startle response indistinguishable from that of the water control. However, maintenance of these MXC-exposed larvae in only water for this period produced a lower larval growth rate. In conclusion, our data indicate that low, nonlethal MXC doses produce pronounced but variable effects on this developing salamander and at least some of these effects are reversible early in life. However, whether early exposures to MXC will have any long-term effects on amphibian physiology and reproductive success remains to be established.

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