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Methoxychlor Effects on Hatching and Larval Startle Response in the Salamander *Ambystoma macrodactylum* Are Independent of Its Estrogenic Actions

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The pesticide methoxychlor (MXC; l, l '-[2,2,2-trichloroethylidene]bis[4-methoxyl-benzene) has been widely used as a replacement for DDT (its structural analog) due to its lower toxicity to mammals and its shorter half-life. It has been applied to terrestrial vegetation 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). In Canadian rivers, MXC has been introduced to yield a concentration of 0.9 µ*M* (300 µg/L) in the river cross section (ACSCEQ 1983). Such general applications expose non-target organisms to this pesticide, however, the consequences of such exposures on these organisms have received relatively little attention. We have previously shown that exposure of the egg/embryo to sublethal concentrations of MXC promotes precocial hatching and attenuated larval startle response in the salamander *Ambystoma macrodactylum* (Ingermann et al.1997). Since MXC exerts estrogenic effects on mammals (e.g., Cummings and Metcalf 1995; Eroschenko et al. 1995; vom Saal et al. 1995), the present study was designed to examine whether the actions of MXC on *A. macrodactylum* eggs, embryos, and free swimming larvae were due to its estrogenic actions. Consequently, the actions of MXC were compared to those of 17β-estradiol (E_2). Estrogenic contaminants exist even in commercial, 99% pure MXC preparations (Bulger et al. 1978). Therefore, 98% pure MXC was base-washed and recrystallized from hexane, and its actions examined. Also, since the estrogenic actions of MXC in mammals are largely attributed to its metabolite, HPTE (2,2-bis[p-hydroxyphenyl]-1,1,1-trichloroethane) (Cummings and Metcalf 1994, 1995; Shelby et al. 1996), the actions of HPTE on hatching and the startle response were examined as well. Finally, since Cloud (1981) has demonstrated that deoxycorticosterone (DOC) induces precocial hatching in fish embryos, and since DDT and another stress hormone, corticosterone, share some biological actions on anuran tadpoles (Hayes et al. 1997), the actions of DOC on *A. macrodactylum* eggs/embryos were also investigated.

MATERIALS AND METHODS

Eggs of *Ambystoma macrodactylum* Biard were collected as previously described (Ingermann et al. 1997). Just prior to initiating the experiment, clusters of eggs were separated into smaller groups containing 1 to 5 eggs. These were mixed and subsequently about 25 eggs were placed into containers containing 1400 or 1600 mL aged tap water without additives, 0.4 mL dimethyl sulfoxide (DMSO)/L (vehicle), or 0.2, 1.0, or 5.0 µ*M* final concentration of 98% MXC, E_2, or DOC. Experiments were also conducted with

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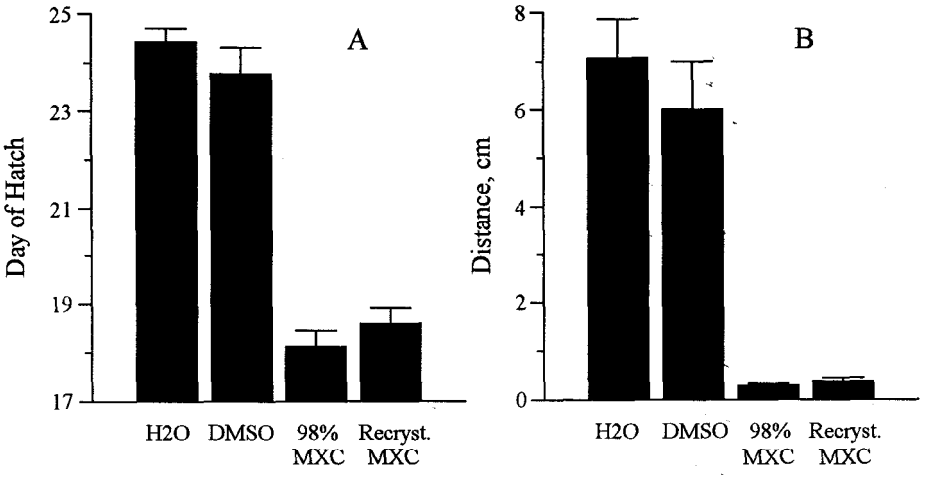

Figure 1. Effect of commercial, 98 % MXC and base-washed and recrystallized MXC on day of hatch and distance swum in response to a startle stimulus. The MXC (1.0 μ*M*) treatments were not different from each other but both differed from controls. (Mean ± SEM, **A:** N = 25-26; **B:** N = 29-42.)

MXC, which had been base-washed and recrystallized from hexane (Bulger et al. 1978), and with 1μM HPTE (using DMSO as the vehicle). All solutions were replaced weekly. With the exception of HPTE, obtained from Cedra Corp. (Austin, TX), all reagents were from Sigma Chem. Co. (St. Louis, MO). Day 1 of the experiments was 9 April 1997 and 27 March 1998. Containers and eggs were maintained in darkness at 10 - 11 °C and checked daily for mortality and hatching. (Mortality was minimal and showed no pattern among treatment groups; data not shown.) After hatching, up to 3 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. At 10 days after hatch, groups of 1 to 3 larvae were analyzed for startle response in triplicate, primarily as described (Ingermann et al. 1997). The difference from the previous startle response experiment was that each N in the present study was the mean of the 1 to 3 larval responses per startle (i.e., individual responses per startle were not kept separate). As noted previously, there was no evidence of habituation or sensitization among any of the three startle response trials.

Day-of-hatch and distance of movement after startle response 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

MXC (98 %) has been shown to induce precocial hatching and a blunted startle response in *A. macrodactylum* (Ingermann et al. 1997). Recrystallized MXC was as effective in inducing precocial hatch and altering the startle response as was the 98% MXC (Fig. 1). These results suggest that the active ingredient is MXC itself and not the presence of contaminants of the relatively pure commercial product.

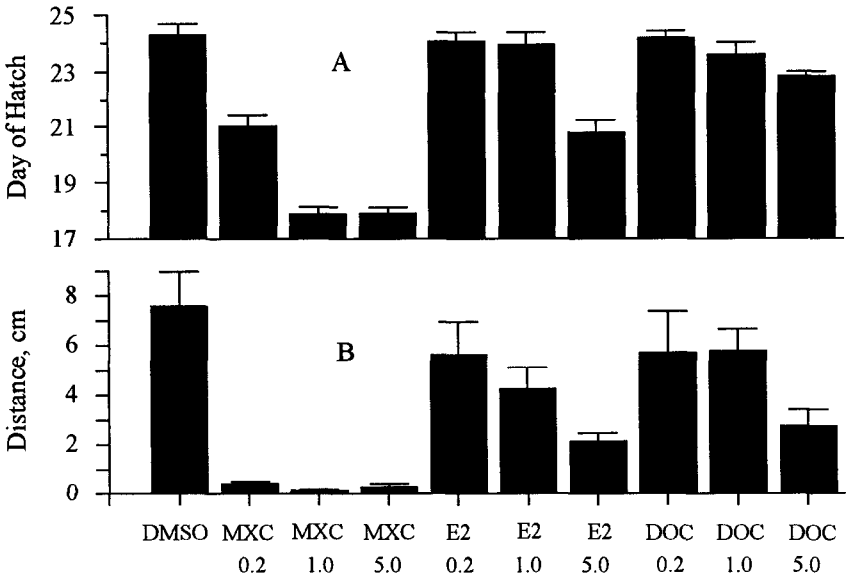

Figure 2. Influence of 0.2, 1.0 and 5.0 µ*M* 98 % MXC, E_2, and DOC on day of hatch and distance swum in response to a startle stimulus. **A:** All MXC treatment groups and the 5.0 µ*M* E_2 group differed from the DMSO control group; with the exception of the 5.0 µ*M* E_2 group, all groups differed from the 0.2 µ*M* 98% MXC group. (Mean ± SEM, *N* = 21-25 with exception of the 0.2 µ*M* DOC group where *N* = 49) **B:** Groupings for distance swum by all MXC doses, 5.0 µ*M* E_2, and 5.0 µ*M* DOC were significantly different from the DMSO group. Distance swum by the 0.2 µ*M* 98% MXC was different from the 0.2 µ*M* E_2 and 0.2 and 1.0 µ*M* DOC groups. (*N* = 30-36 except for 5.0 µ*M* E2: 27, 5.0 µ*M* DOC: 21, 5.0 µ*M* MXC: 14).

MXC binds to the E_2 receptor and exerts a variety of estrogenic actions in rodents (e.g., Cummings and Metcalf 1995; Eroschenko et al. 1995; vom Saal et al. 1995). To assess estrogenic influences of MXC on the salamander egg/embryo, the effects of E_2 were compared to those of 98% MXC. Neither 0.2 nor 1.0 µ*M* E_2 had any effect on hatch time or startle response at 10 d after hatch. In contrast, maintaining eggs/embryos in 5.0 µ*M* E_2 until hatch, resulted in an earlier hatch and a reduced startle response, relative to DMSO controls (Fig. 2). The actions of 5.0 µ*M* E_2 were approximately comparable to those of 0.2 µ*M* 98% MXC. The actions of E_2 on startle response, however, appeared qualitatively different from those of MXC. Larvae of embryos exposed to 5.0 µ*M* E_2 often responded by swimming in tight circles, a phenomenon not seen in larvae of embryos exposed to 0.2 µ*M* 98% MXC. (Such a spinning response was noted in 18 of 35 responses to the startle stimulus when 23 E_2-exposed larvae were startled in triplicate. In contrast, 0 of 35 responses from 24 MXC-exposed larvae, startled in triplicate, showed such tight, circular swimming.) Since elevated concentrations of E_2 are toxic to amphibians (Nishimura et al. 1997), it is possible that 5.0 µ*M* E_2 and 0.2 µ*M* 98% MXC share toxic actions rather than estrogenic actions.

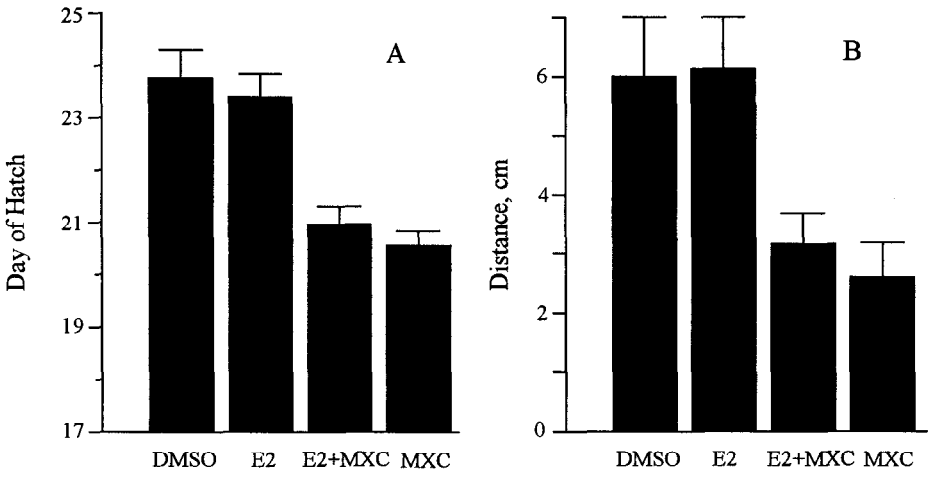

Figure 3. Effect of 1.0 µ*M* E_2 on the actions of 0.2 µ*M* 98% MXC on day of hatch and distance swum in response to a startle stimulus. In both **A** and **B:** The MXC treatment groups did not differ from each other but both differed from DMSO and E_2 groups. (Mean ± SEM, **A:** *N* = 25-26; **B:** *N* = 30-42)

To examine the relationship between E_2 and MXC further, eggs/embryos were maintained in 1.0 µ*M* HPTE until hatch. HPTE, the primary estrogenic metabolite of MXC (Cummings and Metcalf 1994, 1995; Shelby et al. 1996), had no effect on date of hatch or on startle response at 10 d after hatch. Finally, MXC may be antiestrogenic, as recently proposed for its structural analog, DDT (Clark et al. 1998). Eggs/embryos were maintained until hatch in 0.2 µ*M* 98% MXC, 1.0 µ*M* E_2, or 0.2 µ*M* 98% MXC plus 1.0 µ *M* E_2. Addition of E_2 had no effect on the actions of 0.2 µ *M* 98% MXC (Fig. 3), suggesting that MXC and E_2 do not have antagonist effects relative to those biological actions monitored in this study. Further, these data do not demonstrate any additive (or synergistic) effects of MXC and E_2. Thus, although MXC has estrogenic actions in a variety of organisms, its induction of precocial hatching and its attenuation of the startle response of the *A. macrodactylum* hatchling appear independent of estrogenic actions.

Although low oxygen tensions stimulate hatching in fish and salamanders (DiMichele and Taylor 1980; Petranka et al. 1982; Iuchi et al. 1985; Oppen-Berntsen et al. 1990), Cloud (1981) has shown that DOC (as well as steroids such as pregnenolone and progesterone, but not testosterone or E_2) induces precocial hatching in the teleost *Oryzias latipes*. 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. To test this possibility, eggs/embryos were maintained until hatch in 0.2, 1.0, and 5.0 µM DOC. These concentrations had no effect on time until hatch, although the highest dose reduced the distance swum in response to a startle stimulus (Fig. 2).

These results suggest that early exposure to MXC promotes precocial hatching and a reduced startle response in *A. macrodactylum* hatchlings which cannot be attributed to the actions of contaminants of commercial, 98% MXC. Further, although MXC exerts estrogenic actions on a variety of vertebrates, its actions on the parameters studied in this investigation cannot be attributed to its acting as an estrogen mimic. Finally, although

DOC induces precocial hatch in a teleost, it has no similar action on hatching in this salamander (at a dose of 1.0 μ*M*).

At the concentrations used in this study, neither 98% nor recrystallized MXC induced any discernible mortality for at least 30 days after initiation of the experiment (data not shown). Nonetheless, it is possible that survivorship of exposed larvae is compromised chronically, for example, by reducing the ability of such larvae to avoid predation. By hatching earlier and having a blunted startle response, MXC-exposed larvae may demonstrate increased susceptibility to predation. Indeed, Cooke (1971) demonstrated selective predation by salamanders (*Triturus cristatus*) on frog (*Rana temporaria*) tadpoles treated with sublethal concentrations of DDT. An increase in pesticide-induced predator susceptibility may depress recruitment and so exert a negative influence on population viability (even if the survivors court and mate, and lay viable eggs).

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