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Methoxychlor Increases Susceptibility to Predation in the Salamander *Ambystoma macrodactylum*

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Most studies of the toxicological effects of environmental contaminants consider few end-points in single species, often with little reference to ecological principles and possible effects on populations and communities (Calow 1998). While certainly valuable, such studies clearly fall short of the most aspiring goal of the science of ecotoxicology. This is to provide an integrated view of effects on individuals, populations, and communities through both laboratory- and field-based investigation (Banks and Stark 1998).

End-points considered for contaminated animals should have relevance for biological fitness. Studies of behavioral end-points may be especially important in this respect. Behavior patterns often are the means by which animals interact with their environments in order to survive and reproduce. In addition, as noted by Smith and Logan (1997), behavior patterns may be especially sensitive end-points, responding rapidly even to concentrations of contaminants that are non-lethal.

One behavioral end-point of clear significance to individuals, and also important at population and community levels, is the ability to survive an encounter with a predator. Effects of contaminants and other environmental stressors on susceptibility to predation have long been of interest to ecotoxicologists. As reviewed by Walker et al. (1996) most studies have placed exposed prey with unexposed predators, with the common result of increased susceptibility in exposed prey relative to controls.

With their permeable skins, typically biphasic life cycles, and intermediate positions in food webs, amphibians may be especially vulnerable to environmental contamination (Pough et al. 1998). Chemicals used to control both animal and plant pests may be particularly problematic, reaching amphibians by direct application, run-off, and/or drift. Berrill et al. (1997) reviewed the effects of exposure of larval amphibians to a variety of insecticidal and herbicidal compounds. Even with exposure to sublethal doses, intoxicated larvae exhibited symptoms including paralysis and attenuated startle responses to unpredictable stimuli. Few studies of amphibian larvae have gone as far as staging predator-prey encounters. An exception is the work of Cooke

(1970, 1971), who observed increased susceptibility of frog larvae to newt predators after exposure of the former to DDT.

Together with premature hatching of eggs, paralysis and attenuated startle response (perhaps due to compromised cardiovascular performance) are shown by larval long-toed salamanders, *Ambystoma macrodactylum,* exposed *in ovo* to methoxychlor (MXC), a chlorinated hydrocarbon insecticide (Ingermann et al. 1997). MXC (l,l'-[2,2,2-trichloroethylidene] bis[4-methoxyl]-benzene) was developed as a replacement for DDT, and is in wide use due to its relatively low toxicity to mammals (Kapoor et al. 1970). It is employed to control hemophagous and chewing insects in wetlands, forests, agricultural areas, and livestock facilities. MXC also is weakly estrogenic, and its capacity to disrupt the endocrinological control of reproduction has been studied extensively in mammals (e.g., Cummings 1997). However, the effects of MXC on salamander larvae appear to be independent of endocrine disruption, for they are not mimicked by exposure to exogenous estradiol (Ingermann et al. 1999).

This paper attempts to extend our understanding of the effects of MXC on larval salamanders in the more holistic-integrative direction advocated by Banks and Stark (1998). I sought to determine lowest observed effects concentrations and dose-response relationships for MXC using embryonic survival and larval cardiovascular and antipredator performance as end-points of likely ecological relevance.

MATERIALS AND METHODS

The long-toed salamander is a common amphibian of the Pacific and Inland Northwest of North America, where it breeds in stationary and slow-moving bodies of water. In southeast Washington, eggs are laid as early as February, and larvae may not complete metamorphosis until late summer (Nussbaum et al. 1983; Verrell and Pelton 1996). Thus, there is a broad window of time available for exposure of larval salamander populations to MXC, which is used extensively in Washington, especially by apple growers east of the Cascade Mountains (USGS 1998).

Eggs used in this study were laid in the laboratory by females collected from a local breeding pond in spring 1999. Only eggs that showed macroscopic signs of early development were used in the experiment. Approximately seven days after eggs were laid, developing embryos were separated into groups of approximately 50. Each group was placed into incubation containers containing 1 L of aged tap water (water-control), 0.4 mL of dimethyl sulfoxide/L (0.04% DMSO vehicle-control), or 0.01, 0.1, 1.0 or 10.0 mg MXC/L dissolved in 0.04% DMSO. Only MXC that had been base-washed and recrystallized was used (Bulger et al. 1978). Eggs were maintained in darkness at a temperature of approximately 8 °C, and were checked regularly for mortality and hatching. All incubation media were changed once per week.

After hatching, individual larvae were transferred to beakers containing approximately 100 mL of their respective solutions, and were maintained in darkness until they were no older than three days post-hatch. At this time, 10 individuals were selected randomly from each of the six treatments. Each larva was placed in a watch-glass containing aged tap water and allowed 10 min to acclimate at 8 °C. The number of pulses of blood flow passing through a single gill capillary for two consecutive 30-s periods per larva was determined, using a binocular dissecting scope at 40X magnification. Rate of blood flow through the gill is considered to be an indirect measure of cardiovascular performance (Boutilier et al. 1992). Larvae were euthanized in chloretone after use.

At no more than five days post-hatch, 20 individual larvae were selected randomly from each of the treatments except for 10 mg MXC/L (in which mortality was high; see below). Each larva was placed in a beaker containing approximately 100 mL of aged tap water and a single dragonfly naiad of the genus *Aeshna*. Naiads are the aquatic immature stages of these insects, and are voracious predators in pond communities (Caldwell et al. 1980). All naiads were collected from the same pond as were the salamanders used to provide eggs. Each naiad was deprived of food for at least five days before use. All interactions between potential prey and predators were filmed under dim red illumination over a period of 12 hours using a low-light video system (Panasonic). Surviving salamander larvae were euthanized in chloretone after use.

Data from all parts of this study were analyzed using nonparametric statistical tests given in Siegel and Castellan (1988), with alpha = 0.05.

RESULTS AND DISCUSSION

Because all eggs used in this study were alive when treatments were initiated, the proportion of eggs that hatched is an index of treatment effects on embryonic survival (Table 1). The percentage of eggs hatching was high and consistent among all treatments, except incubation in 10 mg MXC/L. Of 132 fertilized eggs incubated at the highest concentration of MXC, less than 10% hatched.

In agreement with the studies of Ingermann et al. (1997, 1999) my data suggest that exposure to MXC may be directly toxic to embryonic salamanders only at high concentrations. However, 10 mg MXC/L may be unrealistically high in terms of exposure in nature. Few data on concentrations of MXC in the field are available. The target concentration of MXC sought to control insect pests in riverine habitats is only 0.3 mg/L (ACSCEQ 1983). This value clearly is within the range of non-lethal concentrations included in my treatments.

Comparisons of rates of pulsatile blood flow through gill capillaries provide an

Table 1. Effects of two control and four experimental treatments on the percent survival of developing embryos to hatching.

Treatment	No. incubated	% hatching
Water-control	103	80.6
DMSO-vehicle	98	73.5
0.01 mg MXC/L	96	69.8
0.1 mg MXC/L	97	67.0
1.0 mg MXC/L	90	66.7
10.0 mg MXC/L	132	9.8

index of treatment effects on the cardiovascular performance of newly-hatched larvae (Table 2). Comparing the two control treatments, median rate of blood flow was significantly lower in DMSO-vehicle larvae versus water-control larvae (P = 0.008; two-tailed Mann-Whitney test for large samples). Because all MXC-treated larvae also had been exposed to DMSO, effects of MXC on rate of blood flow were determined with reference to the DMSO-vehicle treatment. Significant heterogeneity in median rates of blood flow was found among DMSO-control, 0.01 mg MXC/L, 0.1 mg MXC/L and 1.0 mg MXC/L treatments (KW = 11.32, 3 df, $P < 0.04$; two-tailed Kruskal-Wallis one-way ANOVA). Six pairwise comparisons were then made among these four treatments using the method of Siegel and Castellan (1988). The only significant pairwise comparison was between the 0.1 mg MXC/L and 1.0 mg MXC/L treatments (observed difference in mean ranks = 16.8; critical value of difference = 13.79; $P < 0.05$).

Of the 13 larvae that hatched from eggs incubated in 10 mg MXC/L, a measure of rate of blood flow was obtained for a single individual only (14 pulses/min). The gills of the remaining larvae were very small, unbranched structures, within which I could find no capillaries carrying blood. Furthermore, none of these larvae showed any locomotor activity, despite repeated prodding, and all exhibited clear signs of decay within five days of hatching.

To the extent that rate of gill blood flow is truly a measure of cardiovascular performance that has ecological relevance (Boutilier et al. 1992), my data suggest that the effects of MXC are quite modest. Indeed, any depressive effect of MXC is difficult to separate from the depressive effect detected for DMSO (the vehicle used to facilitate MXC uptake). Future work should address the extent to which MXC may affect more direct measures of organismal respiration and metabolism, such as O_2, uptake from and CO_2 removal to the external aquatic medium.

Table 2. Effects of two control and three experimental treatments on rates of blood flow (pulses/min) through gill capillaries of young larvae (N = 10 larvae/treatment).

Treatment	Median	Range
Water-control	24.5	21 - 29
DMSO-vehicle	19.0	15 - 28
0.01 mg MXC/L	19.0	17 - 25
0.1 mg MXC/L	21.5	20 - 27
1.0 mg MXC/L	18.0	13 - 23

Larval long-toed salamanders appear to be both accessible and palatable prey for various aquatic predators occurring in syntopy, including cannibalistic conspecifics, other ambystomatid taxa, garter snakes and various aquatic insects. Pre-adult mortality, to which larval predation makes a large contribution, is a major determinant of demographic fluctuations in populations of salamanders (e.g., Anderson et al. 1971).

Dragonfly naiads are voracious predators of amphibian larvae, and are present throughout the year in the pond from which the salamanders that produced the eggs used in this study were collected. Across the five treatments in which larvae were placed with dragonfly naiads, almost all (93%) failed to survive to the end of their 12 hour observation sessions. Fifty five of the 93 larvae were eaten completely, the remainder eaten partially but lethally. Comparisons of antipredator performance among treatments were made using data on latency to complete/partial ingestion (Table 3).

No significant difference was found between water-control and DMSO-vehicle treatments in median latency to ingestion ($P = 0.94$; two-tailed Mann-Whitney test for large samples). Using DMSO-vehicle larvae as the appropriate control for the three MXC treatments, significant heterogeneity in median latencies to ingestion was found (KW = 20.4, 3 df, $P < 0.002$; two-tailed Kruskal-Wallis one-way ANOVA).

Six pairwise comparisons among these four treatments revealed significant differences between the DMSO-vehicle treatment and all MXC treatments (observed differences in mean ranks = 20.8-30.8; critical values of difference = 17.5-17.7; $P < 0.05$ in all three tests). However, no significant differences were apparent in comparisons among MXC treatments (observed differences in mean ranks = 2.5-9.9; critical values of difference = 16.5-17.5; $P > 0.05$ in all three tests).

Table 3. Effects of two control and three experimental treatments on latencies (min) to complete or partial ingestion of young larvae by predatory dragonfly naiads (N = number of larvae ingested).

Treatment	Median	Range
Water-control (N = 18)	324	18 - 690
DMSO-vehicle (N = 16)	261	12 - 660
0.01 mg MXC/L (N = 20)	69	12 - 372
0.1 mg MXC/L (N = 19)	42	6 - 318
1.0 mg MXC/L (N = 20)	42	6 - 120

These results suggest that larval long-toed salamanders hatched from eggs exposed to ecologically-realistic concentrations of MXC may be compromised in terms of their ability to avoid/escape predation by dragonfly naiads (of course, my experimental design offered very limited opportunities for evasion). I found no effect of MXC concentration on median latency to ingestion; exposure to 0.01 mg/L was as detrimental as exposure to 1.0 mg/L. Thus, I assume that the lowest observed effects concentration for this end-point lies below 0.01 mg MXC/L. In addition, I found no dose-response relationship between MXC concentration and median latency to ingestion. Ingermann et al. (1997) reported that the minimum concentration of MXC needed to attenuate larval startle response lies between 0.03 and 0.1 mg/L, somewhat higher than the low dose of 0.01 mg/L needed to increase susceptibility to predation.

Although I did not collect quantitative data on behavior patterns exhibited by larvae exposed to predators, qualitative differences among some treatments were readily apparent. Both water-control and DMSO-vehicle larvae moved around their observation arenas quite extensively, especially when approached by a predator. These latter movements appeared to be both rapid and unpredictable in terms of direction, which may facilitate successful evasion in nature. In contrast to such “darting,” larvae in all three MXC treatments did little more than twitch their bodies sporadically while lying on the substrate, if they moved at all. I obtained the impression that such twitchy movements rendered MXC-treated larvae more conspicuous to the predators with which they were placed. Many chlorinated insecticides directly affect the nervous systems of both target and non-target species (Joy 1994). It seems likely that the negative effects of MXC on the capacity for movement of larval salamanders are due to neurotoxic action.

In conclusion, my data and those of Ingermann et al. (1997, 1999) suggest that exposure of young long-toed salamanders to non-lethal and ecologically-realistic concentrations of MXC may negatively impact their survival. Contaminated eggs hatch prematurely to produce larvae that move little and are susceptible to predation. I obtained no evidence that compromised cardiovascular performance may be responsible for these effects. While the conservation status of *A. macrodactylum* presently is considered to be secure in the Northwest, growing evidence implicates exposure to pesticides as one of many factors that may contribute to declines of amphibian species (Blaustein and Wake 1995). Additional experimental studies that bridge the gap between observed effects of pesticides on individuals, and possible consequences for populations and communities, are sorely needed.

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