Developmental disorders induced by pesticide degradation products
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Teratogenic Effects of Amitraz, 2,4-Dimethylaniline and Paraquat on Developing Frog (Xenopus) Embryos

Abstract

Developmental effects of amitraz (acaricide), its metabolite (2,4-dimethylaniline), and paraquat (herbicide) on embryos of a non-target organism, Xenopus laevis, were investigated. Following the standard protocol of the American Society for Testing and Materials (ASTM), the experiments were carried out using native Xenopus frogs. There was a drastic increase in mortality from 24 h to 96 h for paraquat, while 2,4-dimethylaniline showed no mortality at the highest concentration tested (100 mg/L). The 96-h LC50 values were 0.67, 3.27 and >>100 mg/L for paraquat, amitraz and 2,4-dimethylaniline, respectively. At concentrations higher than 0.2 mg/L of paraquat all the embryos were malformed, while growth reduction was apparent at all test concentrations (0.1 - 5 mg/L). The most common teratogenic effects were flexures of the notochord and stunting of growth. Edema was the most common effect of amitraz on the embryos and 100% of the surviving embryos in 5 mg/L were edematous. The 96-h EC50 (malformation) values were 1.21 (95% CI 0.48 - 3.03) and 0.18 (95% CI 0.16 - 0.20) mg/L for amitraz and paraquat, respectively. The ratio of 96-h LC50 to 96-h EC50 (malformation), i.e the teratogenicity index (TI), were 2.7 and 3.72 for amitraz and paraquat, respectively, while for 2,4-dimethylaniline (TI>5) all the embryos in 25 mg/L showed observable pigment loss and encephalomegaly. This shows that paraquat and the degradation product of amitraz, 2,4-dimethylaniline, should be classified as teratogens. Teratogenic risks of massive application of these pesticides on Kenyan farms should therefore be considered.
Introduction

The formamidine amitraz (N-2,4(dimethylphenyl)-N-([(2,4-dimethylphenyl)-imino)methyl]-N-methanimidamine) is currently the acaricide of choice in Kenya. This compound and its biologically active metabolite BTS27271 are α₂-adrenoceptor agonists causing contractions of mammalian cardiac and uterine muscles (Hsu and Kakuk 1984; Shin and Hsu 1994). It is metabolised into 2,4-dimethylaniline, and other degradation products, in arthropods and mammals (Schuntner and Thompson 1978; Knowles and Benezet 1981; Knowles and Gayen 1983; Kimmel et al. 1986; Knowles and Hamed 1989). Paraquat (1,1’-dimethyl-4-4’-bipyridium dichloride, commercially available as Gramoxone®) is one of the most heavily used herbicides in commercial maize, sugar, and coffee farming in the Lake Victoria basin. Like the formamidines and the chloroacetanilides, paraquat is a chlorinated hydrocarbon. It is mainly photochemically degraded on plants by the ultraviolet light from the sun into 4-carboxy-1-methylpyridinium chloride and methylamine hydrochloride (Slade 1965, 1966).

Early developmental stages of amphibians have been used to monitor environmental contamination due to their sensitivity to a wide variety of toxic agents (Fort et al. 1999b; Fort et al. 1999a; Prati et al. 2000; Tietge et al. 2000). Early stages are the most sensitive of all life stages to xenobiotics and the frog embryo teratogenesis assay-Xenopus (FETAX) provides information on mortality, malformation, and growth inhibition (ASTM 1994). Susceptibility of various developmental stages of amphibians to pesticides has been reported (Dial and Bauer 1984). Paraquat targets the muscle cell cytoskeleton of amphibians leading to marked loss of actin bundles, thus affecting the spatial organization of the cytoskeleton actin structures (Vismara et al. 2000). This explains the teratogenic effects like growth stunting and flexures observed after exposures of frog embryos to paraquat in previous studies (Dial and Bauer 1984; Dial and Dial 1987; Vismara et al. 2000). The effects of amitraz and its environmentally stable aniline metabolite 2,4-dimethylaniline on amphibians are unknown.

The present study compares survival, growth, developmental and gross teratogenic effects of amitraz, its metabolite (2,4-dimethylaniline) and paraquat on Xenopus embryos. Xenopus laevis, a claw-footed and tongue-less frog, is native to the Lake Victoria basin. Ponds that drain the
pesticides-laden farmlands in the Lake Victoria basin form the breeding grounds for the clawed frogs. Validation studies using compounds with known mammalian or human developmental toxicity, suggest that the predictive accuracy of the FETAX approximates 85%, so it can be used to screen potential human developmental health toxicants (Courchesne and Bantle 1985; Dawson and Bantle 1987; Sabourin and Faulk 1987; Bantle et al. 1989). *Xenopus laevis* is relatively easy to breed under laboratory conditions. Moreover, a standard protocol for conducting teratogenicity tests with the species is available (ASTM 1994). The species is therefore a relevant organism in teratogenicity and toxicity tests with pesticides applied on the farmlands in the Lake Victoria basin.

**Materials and Methods**

Female and male *Xenopus laevis* used for this study were collected from the ponds at the shores of Lake Victoria, Kisumu district, Kenya. The adult frogs were kept in laboratory glass aquaria in dechlorinated tap water. Dechlorination was achieved by addition of 1 ml saturated sodium thiosulfate solution to 75 litres of tap water.

Amitraz (analytical grade 97.5%), 2,4-dimethylaniline (98%), paraquat (analytical grade 99%) and analytical grade solvent dimethylsulfoxide (DMSO) were obtained from Fluka Riedel-de Haën®. FETAX solution that comprised of 625 mg NaCl, 96 mg NaHCO₃, 30 mg KCl, 15 mg CaCl₂, 60 mg CaSO₄·2H₂O and 5 mg MgSO₄ per litre of distilled water was prepared and used as a medium in the assays (ASTM 1994). All the salts were analytical grade quality.

Three pairs (one per cage) of mating *X. laevis* were placed in 0.3 × 0.3 × 0.3 m cages half-filled with FETAX solution. The bottoms of the cages were separated about 3 cm from the frogs by use of a perspex floor with 1 cm-diameter holes. The holes allowed the eggs to sink below, out of reach of the adult frogs, who would otherwise quickly consume them. The medium was aerated and maintained at 23°C. The frogs were induced to mate by injection of 500 (male) and 1000 (female) i.u. human chorionic gonadotropin hormone (Pregnil®) into the dorsal lymph sac at 6.00 PM on the eve of the start of the tests using a tuberculin syringe fitted with a 26-gauge needle. Fourteen hours later the eggs from each pair were collected and de-jellied by use of 2% w/v L-cystein prepared in FETAX solution. IN
NaOH was used to adjust the pH of the cysteine solution to 8.1. The eggs were swirled in the L-cysteine for 1 - 3 minutes. They were then washed in copious amounts of FETAX solution. Embryos of stage 8 (midblastula) to stage 11 (early gastrula) were selected for the assay using the ‘Normal Tables’ (Nieuwkoop and Faber 1975).

A 96-h static-renewal whole embryo assay was conducted using the standard protocol for the FETAX (ASTM 1994) as a guideline. The experimental runs were conducted in a random block design with embryos from each pair forming a block (n = 3). Each block comprised duplicate tests and in each test 25 embryos were exposed to 10 ml of nominal concentrations (0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0), (1.56, 3.25, 6.25, 12.5, 25, 50, 100), and (0, 0.05, 0.1, 0.2, 0.5, 1, 2, 5) mg/L of amitraz, 2,4-dimethylaniline, and paraquat, respectively, in 60 mm diameter petri dishes. DMSO was used to facilitate dissolution of the chemicals and the final concentration of the DMSO was 1% (v/v) in all the test concentrations. The control group comprised 2 dishes of 25 embryos in 10 ml FETAX solution and 2 dishes with FETAX solution plus DMSO (1% v/v). Dishes were placed in random positions in an incubator set at 24°C (range of 23 - 25°C). At 24 h, 48 h, and 72 h the test solutions were renewed and the dead embryos were discarded. After 96 h the experiment was terminated and the live larvae were counted and fixed in 3% formalin. The 96-h LC50 and 96-h EC50 (malformation) were determined by probit analysis (Wardlaw 1985) after adjustment for mortalities and malformations in the control as follows: $Y = 100\% \times (C - T)/C$, where $Y$ = % response; $C$ = % not responding in the DMSO control; $T$ = % not responding in the test dilution and the response was considered as percent growth retardation, mortality or malformation accordingly (Abbott 1925). The teratogenic index (TI) was deduced by dividing the 96-h LC50 by 96-h EC50 and was used to quantify the degree of teratogenicity of compounds (Dumont et al. 1983).

The effects of the pesticides and the degradation product of amitraz, 2,4-dimethylaniline, on survival, growth, and malformation were observed with a binocular-dissecting microscope. Head (mouth) to tail lengths (end of the tail) were measured at the end of each run by use of an ocular micrometer at a magnification of $\times 20$. Separately, the tail length (end of the tail to the region of the hind limb bud) and body length (the difference between the tail length and the whole tadpole body) were recorded for each larva. For the flexed larvae, measurements were taken along the curvature of
the notochord. The tadpoles were examined for developmental abnormalities under the binocular-dissecting microscope. The "Atlas of Abnormalities" was consulted as a guide to distinguish between normal and abnormal embryos and to determine the different kinds of abnormalities (Bantle et al. 1990). An embryo was considered abnormal if it exhibited at least one type of malformation. In this investigation an abnormality was scored regardless of its seriousness. For example, a slight facial edema, large gut edema, or extensive edemas were all scored as edema. Tadpoles were preserved in rubber-sealed vials with 3% formalin.

Statistical analyses were done with the Student's 't'-test and ANOVA (with Bonferroni adjustment matrix of pairwise comparison probabilities, \( \alpha = 0.05 \) level) in the SYSTAT® (1996) statistical program to determine any significant difference in growth of the tadpoles.

Results

Embryolethal effects

The survival in the DMSO control was 93.3% after 96 h. The 96-h LC50 were 3.27 (95% CI 2.56 - 4.16) and 0.67 (95% CI 0.57 - 0.81) mg/L for amitraz and paraquat, respectively. There was no mortality for the highest tested concentration (100 mg/L) of 2,4-dimethylaniline. The embryos in media that contained \( \geq 0.5 \) mg/L paraquat appeared moribund at 96 h, as they were unable to swim, but could be seen to be alive from the pulsation of their hearts. For paraquat drastic increased lethal effects from 24 h to 96 h of incubation were observed (Fig 1, Table 1), but not for amitraz or 2,4-dimethylaniline in this study, nor for the chloroacetanilides; alachlor, metolachlor and their aniline degradation products in our previous study (Osano et al. 2002a). Table 1 gives the LC50 values of the compounds after different periods during the test.

Growth effects

The mean size of the tadpoles in the DMSO control after 96h incubation was 7.51 ± 0.048 mm (SE) \((n = 144)\). The minimum concentration to inhibit growth (MCIG) was 0.1 mg/L \((p<0.05)\) for both amitraz and paraquat. 2,4-Dimethylaniline caused subtoxic stimulation of
**Teratogenicity of amitraz, 2,4-dimethylaniline and paraquat**

Table 1: 24-h, 48-h, 72-h, and 96-h LC50(s); 96-h EC50 (malformation); and TI(s) of the test chemicals for *Xenopus laevis* embryos.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amitraz</th>
<th>2,4-Dimethylaniline</th>
<th>Paraquat</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-h LC50</td>
<td>5&lt;LC50&lt;10 mg/L</td>
<td>&gt;100 mg/L</td>
<td>&gt;10 mg/L</td>
</tr>
<tr>
<td>48-h LC50</td>
<td>5&lt;LC50&lt;10 mg/L</td>
<td>&gt;100 mg/L</td>
<td>14.55 mg/L (95% CI 9.80-21.60)</td>
</tr>
<tr>
<td>72-h LC50</td>
<td>5&lt;LC50&lt;10 mg/L</td>
<td>&gt;100 mg/L</td>
<td>3.30 mg/L (95% CI 2.30-4.55)</td>
</tr>
<tr>
<td>96-h LC50</td>
<td>3.27 mg/L (95% CI 2.56-4.16)</td>
<td>&gt;100 mg/L</td>
<td>0.67 mg/L (95% CI 0.57-0.81)</td>
</tr>
<tr>
<td>96-h EC50</td>
<td>1.21 mg/L (95% CI 0.48-3.03)</td>
<td>*20 mg/L</td>
<td>0.18 mg/L (95% CI 0.16-0.20)</td>
</tr>
<tr>
<td>TI</td>
<td>2.70 (95% CI 0.84-8.67)</td>
<td>&gt;5</td>
<td>3.72 (95% CI 2.26-6.40)</td>
</tr>
</tbody>
</table>

* Estimated

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Fig 1. The % survival of the *Xenopus laevis* tadpoles against concentrations of paraquat after incubation for different periods.
growth between 3.13 - 100 mg/L (p<0.001) concentrations (Fig 2). Amitraz caused a small but significant growth retardation of up to 10% in the maximum tolerated concentration (5.0 mg/L) (Fig 2). Paraquat caused substantial growth retardation with the tadpoles mean length of 5.27 (± 0.067 SE, n = 74) mm in 1 mg/L medium. This represents a 30% reduction of the growth relative to the control. There was no significant difference (p = 0.31) between the lengths at this concentration and the lengths at 2 mg/L (5.16 ±0.094 SE) mm. Relative to the full lengths of the tadpoles the tail lengths were proportionally shorter in the 4-day-old tadpoles incubated in the higher concentration of paraquat than those in the control (p = 0.027). In 1.0 mg/L the tail was 68.9% (±0.61 SE, n = 19) of the total lengths of the tadpole compared to 70.8% (±0.59 SE, n = 20) in the control. Paraquat induced a general reduction of the lengths of both the deformed and the non-deformed tadpoles in this study. However, the tadpoles that showed other deformities (other than stunting) exhibited more severe growth retardation than those that were not deformed (Fig 3).

Teratogenic effects

The 96-h EC50 malformation in the embryos of X. laevis amounted to 1.21 (95% CI 0.48 - 3.03) and 0.18 (95% CI 0.16 - 0.20) mg/L for amitraz and paraquat, respectively (Table 1). The TI were 2.70 (95% CI 0.84 - 8.67), 3.72 (95% CI 2.26 - 6.40) and >5 for amitraz, paraquat, and 2,4-dimethylaniline, respectively (Table 1).

Amitraz caused edema and axial flexures as the main types of abnormalities (Fig 4). At 5 mg/L all the surviving embryos were edematous. Axial flexures were identified as curvature of the notochord or bending of the tail (Plate B). Edemas comprised edema of the face, heart and/or abdomen (Plate C). The significant effect of 2,4-dimethylaniline was a progressive loss of pigment together with encephalomegaly that was observable from 25-mg/L test media. At 100 mg/L there was total loss of the pigment leading to loss of colour contrast between the eyes and the rest of the body, and the bifurcation at the forebrain was indistinguishable as a result of swelling of the brain (Plate D). For paraquat medial flexures of the notochord and stunting were the main types of abnormalities observed on the embryos (Fig 4). The frequency of deformities increased with increase in concentration of paraquat. At 0.5 mg/L, 98% of the embryos were deformed, out of which 45% were stunted and 50% had their notochords
flexed compared to 11, 0, and 7%, respectively, for the same types of deformities in the control. Plates A & E show a normal (control) tadpole, and a flexed and stunted specimen, respectively. Table 1 summarizes the LC50s, EC50s and TIs in the present study.

**Discussion**

Paraquat was highly toxic to the embryos of *X. laevis* (LC50 = 0.67 (95% CI 0.57 - 0.81) mg/L; MCIG = 0.1 mg/L (p = 0.05)). It is persistent in the soil environment with a reported half-life of >1000 days (Wauchope *et al.* 1992; Weed Science Society of America 1994). It is however degraded by UV light to 4-carboxy-1-methylpyridinium chloride and methylamine hydrochloride as the major decomposition products (Slade 1965, 1966). Amitraz is quickly (half-life < 1 day) degraded to 2,4-dimethylaniline and other metabolites (Kimmel *et al.* 1986; Kidd and James 1991). It was found to be relatively less toxic than paraquat to the embryos of *X. laevis* (3.27 [95% CI 2.56 - 4.16]). Moreover, its major use as an acaricide on livestock restricts its contamination to focal points such as around the spray races, cattle plunge dips, and veterinary clinics. However, careless disposal of the used mixtures of the acaricide could expose the environment to the risks of the degradation products. The environmentally stable aniline degradation product of amitraz (2,4-dimethylaniline) proved non-lethal to the embryos of *X. laevis* at our tested concentrations of 0 - 100 mg/L.

The impairment of growth of *Xenopus* in the present study was proportional to the paraquat concentration. This corroborates earlier findings of growth retardation in tadpoles of *Rana pipiens* and fingerlings of *Oreochromis niloticus* (Dial and Bauer 1984; Babatunde 1997). Also the tail abnormalities, flexure and shortness, have been observed in *Rana* (Dial and Dial 1995), but the present study demonstrates that this aberration is induced by very low paraquat concentrations. *X. laevis* embryos are potentially more sensitive test organisms than the previously used vertebrates, although in the present study, the tests were initiated with eggs and not older larvae like in the tests using fish (Babatunde 1997) and other frog species (Dial and Dial 1995). Shortening of the embryos were more apparent in the assay with paraquat than those with amitraz, in spite of the latter compound being an α2-adrenergic agonist that causes contraction of muscles in pigs (uterus).
and rats, (Hsu and Kaku 1984; Shin and Hsu 1994). Paraquat causes loss of actin bundles thus affecting the spatial organization of the muscle cytoskeleton actin structures (Vismara et al. 2000). Apparently this leads to a marked reduction in the lengths of the tadpoles and more especially the tail that comprises mostly of the notochord and tail muscle (Dial and Bauer 1984; Dial and Dial 1987; Vismara et al. 2000). In this study the prominent effect of amitraz of generalised edema may have been due to a disruption of osmoregulation resulting from cell membrane lipid bilayer disruption. Similarly, related aniline based compounds alachlor and metolachlor exert their toxicity to the chironomid larvae through narcosis (Osano et al. 2002b).

The exposure of the embryos in the present study was through the water medium rather than through the food as by Dial and Dial (1987). The apparent drastic increase in toxicity of paraquat to the tadpoles from 24 h to 96 h post hatch suggests that the toxicity in the early stages of embryogenesis may be decreased through physical protection of any remaining jelly or a different physiological process. A similar observation was made in amphibians and crayfish (Leung et al. 1980; Dial and Bauer 1984). Paraquat and amitraz have short half-lives in aquatic environment, the former being adsorbed and concentrated by sediment, suspended solids or aquatic plants while the latter breaks down to BTS27271 and subsequently to 2,4-dimethylaniline and other metabolites (Way et al. 1971; Calderbank 1972; Kosinki and Merkle 1984; Bernal et al. 1997). Paraquat is not easily degraded chemically or microbiologically and demonstrates a long half-life (>56 d) in river water medium (Wang et al. 1994). In the wild the larvae that survive paraquat contamination at the early stages would be additionally challenged by oral route of exposure at the later stages when they feed on contaminated algae. A significant number of tadpoles fed three days on paraquat treated Myriophyllum were observed to have abnormal tails (Dial and Dial 1995). In the present experiment the fertilised Xenopus eggs were kept in dilute media without organic additions and nourishment, other than the embryonic yolk sac, was absent. Such an effect could be enhanced by the degradation of paraquat in association with plants (Slade 1965, 1966). The resulting degradation products (4-carboxy-1-methylpyridinium chloride and methylamine hydrochloride) have not been tested for teratogenic effects.
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Fig 2. Growth expressed as % of DMSO control mean length of the *Xenopus laevis* embryos after 96-h incubation in different concentrations of amitraz, 2,4-dimethylaniline and paraquat. The error bars represent standard errors of mean.

Fig 3. Growth expressed as % of DMSO control mean length of the *Xenopus laevis* non deformed tadpoles and tadpoles with deformities other than stunting of *Xenopus laevis* after 96-h incubation in paraquat. The error bars represent standard errors of mean.
Fig 4. The % occurrence of normal and abnormal tadpoles of *Xenopus laevis* after 96-h incubation in different concentration of amitraz (top) and paraquat (bottom).

Effects of amitraz on developing amphibians have not been reported in literature. Our TI of 2.7 indicates teratogenicity of the compound, which is increased after its degradation into 2,4-dimethylaniline (TI>5). All the embryos grown in the media of 25 - 100 mg/L 2,4-dimethylaniline were deformed (showed loss of pigment) but survived the test concentrations (100%). The encephalomegaly observed in this study confers neural
Plates: Four-day-old tadpoles of *Xenopus laevis*, A: in the FETAX DMSO control solution, B: in 0.5 mg/L amitraz. The tadpole is flexed, C: in 0.5 mg/L amitraz. The tadpole is edematous; D: in 100 mg/L solution of 2,4-dimethylaniline. The tadpole has enlarged brain and is depigmented making it difficult to contrast between the eyes and the rest of the body. Notice the lack of pigmentation on the left eye, E: in 1 mg/L solution of paraquat. The tadpole is stunted and flexed.
deformity. Similarly, a high teratogenicity upon degradation was observed for other aniline based pesticides, alachlor and metolachlor, in our previous study (Osano et al. 2002a). Teratogenic effects of the degradation products of paraquat need study.

*X. laevis* used in our study are native species of Lake Victoria basin and inhabit the ponds that drain agricultural farmlands where paraquat is applied while amitraz is the current acaricide of choice in the same region. The wastes of the used acaricide are often drained into the environment without regard of their potential toxicity. We suggest that the sensitivity of *Xenopus* embryonal development to the environmentally stable 2,4-dimethylaniline and paraquat in conjunction with the observed effects of paraquat sorbed in plants is likely to cause detrimental effects on natural populations of this species in the Lake Victoria basin. Further studies on levels of the pesticides and their degradation products in water, sediment and biota of Lake Victoria basin water bodies are required as well as observations on teratogenic effects in nature.

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