

Comparison of Sensitivity to Methyl Methanesulphonate Among Tadpole Developmental Stages Using the Alkaline Single-Cell Gel Electrophoresis (Comet) Assay

Steven Ralph and Michael Petras*

Department of Biological Sciences, University of Windsor, Windsor, Ontario, Canada

In a previous study, we demonstrated that tadpoles are suitable organisms for monitoring small bodies of water (e.g., creeks, ponds, and drainage ditches) for genotoxicity using the alkaline single-cell gel DNA electrophoresis (SCG) or "comet" assay [Ralph and Petras, 1997]. This approach involves detection, under alkaline conditions, of cell DNA fragments which on electrophoresis migrate from the nuclear core, resulting in a "comet with tail" formation. In this initial study, most of the tadpoles collected were in the early stages of larval development, but this is not always possible. The present study evaluated the sensitivity of tadpoles, at different stages of larval development, to a range of concentrations of the genotoxicant methyl methanesulphonate (MMS). Four specific phases of *Rana clamitans* (green frog) larval development were examined: first-year limbless tadpoles (Stage I as defined by Taylor and Kollros [1946]), second-year limbless tadpoles (Stages II–III), second-year tadpoles with only hindlimbs (Stages X–XVIII), and second-year tadpoles with all four limbs evident and a tail undergoing resorption (Stages XXII–XXIII). Twenty-four hour exposures to MMS of tadpoles in the three earliest phases produced a significant ($P <$

0.01) added variance component among tadpoles for DNA damage and there were significant increases ($P < 0.05$) in the length:width ratios of the DNA patterns at concentrations as low as 1.56 mg/l. However, tadpoles in the last phase studied (both pairs of limbs present) showed no significant ($P > 0.05$) added variance component and no significant increases ($P > 0.05$) in DNA damage upon exposure to any of the MMS doses tested. A nested ANOVA indicated that, for each of the tested concentrations of MMS, but not the dechlorinated water control, there was significant heterogeneity ($P < 0.05$) in DNA damage when tadpoles of all four phases studied were compared. However, when tadpoles of the last phase of development were removed from the comparison, there was no significant heterogeneity ($P > 0.05$) among tadpoles of the remaining three phases. Possible reasons for this insensitivity to MMS as animals enter the metamorphic climax were considered. The results indicate that pooling of the early tadpole phases of *R. clamitans* for SCG environmental genotoxicity biomonitoring is acceptable. Environ. Mol. Mutagen. 31:374–382, 1998. © 1988 Wiley-Liss, Inc.

Key words: DNA damage; aquatic monitoring; alkaline comet assay; *Rana clamitans*; tadpoles; amphibians; genotoxicity; developmental stage

INTRODUCTION

The continued release of genotoxic agents into the environment has resulted in a need for sensitive assays to monitor their accumulation and impact. We recently reported on an in situ assay used to quantify genotoxicity of small bodies of water (e.g., creeks, ponds, and drainage ditches) in southwestern Ontario involving tadpoles and the alkaline single-cell gel DNA electrophoresis (SCG) or "comet" assay [Ralph and Petras, 1997]. In this study, the vast majority of tadpoles collected were in the early stages of larval development (Stages I–III as defined by Taylor and Kollros [1946]), but this is not always the case. Hence, an important consideration in any in situ assay for environmental biomonitoring is the impact of contaminants on the sentinel organism at different stages of development.

Tadpoles are useful organisms for environmental bio-

monitoring for several reasons, including availability in sufficient numbers, ease of collection, localization to particular bodies of water, and direct exposure to contaminants in the water, sediment, and vegetation (diet). As mentioned, we successfully used several species of tad-

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Current address for Steven Ralph: The British Columbia Cancer Research Centre, and the Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC.

*Correspondence to: Dr. Michael Petras, Department of Biological Sciences, University of Windsor, Windsor, ON N9B 3P4, Canada. E-mail: bioptrs@uwindsor.ca

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poles to study genotoxicity in small bodies of water that are subject to contaminant inputs from industrial and agricultural sources [Ralph and Petras, 1997]. This is consistent with a number of laboratory studies which have shown amphibians to be highly sensitive to genotoxic agents [Hart and Armstrong, 1984; Siboulet et al., 1984; Jaylet et al., 1986, 1987; Zoll et al., 1988; Krauter, 1992; LeCurieux et al., 1992; Rudek and Rozek, 1992; Djomo et al., 1995; Ralph et al., 1996; Clements et al., 1997].

Based on preliminary observations in earlier studies, we became concerned about possible differences in sensitivity to contaminants among the various tadpole stages. We chose to examine this under laboratory conditions, since in nature a number of factors which could be responsible for such differences cannot be controlled. For instance, under natural conditions (in the wild) differences in sensitivity could be due to variable exposure to contaminants as a result of changes in habitat selection among stages and stage-specific feeding behaviour. In addition, the observed DNA damage in tadpoles at late stages of development could well be due to exposure much earlier in life, rather than during the stage being studied.

Rana clamitans, the green frog, is one of the most common amphibians in southern Ontario and is ideal for biomonitoring because of its long tadpole lifespan. These animals typically inhabit permanent ponds. They are large in size (length 55–80 mm at transformation), appear in early spring, and overwinter before transforming [Walker, 1946]. After completing the short embryonic stage there is a long period of relatively little change in size or physiology (larval Stage I, length of up to 40 mm). This is followed by a rapid increase in size (Stages II and III, length approximately 70 mm). Then, beginning in mid-spring of their second year, first the hind legs appear followed by the forelimbs (Stages IV to XIX), and finally as the limbs increase in size the gradual resorption of the tail begins (Stage XX), and by Stage XXV it is completely resorbed [Taylor and Kollros, 1946]. Associated with Stages XX to XXV, major physiological changes take place which eventually culminate in the completion of metamorphosis.

As with our field studies, we used a modified alkaline SCG assay [Ralph et al., 1996] to quantify the levels of DNA damage in tadpoles. This technique was originally developed by Rydberg and Johanson [1978] who used isolated cells in a microgel to determine DNA damage. Modifications by Ostling and Johanson [1984] involving electrophoresis under neutral conditions permitted the detection of double-stranded DNA breaks. Subsequently, detection of single-stranded breaks and alkali-labile damage was made possible by Singh et al. [1988], who performed the electrophoresis under alkaline conditions [see McKelvey-Martin et al., 1993, and Fairbairn et al., 1995, for comprehensive reviews of this assay].

This study reports on the levels of DNA damage in

four larval phases of *R. clamitans* tadpoles after 24-hr exposure to three concentrations of methyl methanesulphonate (MMS) under laboratory conditions.

MATERIALS AND METHODS

Chemical Reagents

Low-melting agarose (electrophoresis purity quality) was obtained from BioRad (Mississauga, Ontario, Canada). Calcium- and magnesium-free phosphate-buffered saline (PBS) was purchased from Gibco-BRL (Grand Island, NY). Hank's balanced salt solution and the chemicals for the lysing solution, the electrophoresis buffer, and DNA staining came from Sigma (St. Louis, MO). Fully frosted microscope slides and number one cover glasses were supplied by Fisher (Toronto, Ontario, Canada). Methyl methanesulphonate (MMS) was obtained from Eastman Kodak (Rochester, NY). See Ralph et al. [1996] for further details.

Animals

Approximately 1,000 *R. clamitans* tadpoles were obtained in a single collection from a pond 5 km east-southeast of Duart, Ontario, Canada (Donker farm). The pond is used by livestock and is surrounded by a pasture and cultivated fields. Tadpoles were transported to the laboratory in water from the collection site. They were maintained in this water in polypropylene containers (29 × 19 × 13 cm) until they were bled, which, for the analysis of freshly caught samples, occurred within 4 days of their capture. Plant material from the collection site was added to containers as a food source. The animals were staged according to Taylor and Kollros [1946] and grouped into four categories or phases: 1) first-year limbless (limb bud Stage I, length approximately 40 mm); 2) second-year limbless (limb bud Stages II–III, length approximately 70 mm); 3) second year with only hindlimbs (paddle Stage X–metamorphic Stage XVIII, length approximately 70 mm); 4) second year with fore- and hindlimbs and a tail undergoing resorption (metamorphic Stages XXII–XXIII, length approximately 50–60 mm). To reduce any overlap between these categories, animals at stages intermediate to those listed above were not used.

Alkaline SCG Assay

The procedure used was basically that described by Singh et al. [1988, 1989] with modifications by Ralph et al. [1996]. Blood samples were collected from *R. clamitans* tadpoles by decapitation followed by immediately placing the animals in a 10% solution of Hank's balanced salt solution for 2 min. All animals were treated individually. Microscopic examination of preparations resulting from this procedure showed that the vast majority of cells collected were erythrocytes. After appropriate dilution, no nonerythrocytes were observed. Erythrocytes had been chosen because they are nucleated in amphibians. Serial dilutions were made so that three or four cells would be seen, without crowding, in a single field at 400× magnification. The diluted erythrocyte suspension was then mixed with 0.5% low-melting agarose and this suspension was pipetted onto fully frosted slides and covered with coverslips. The slides were stored at 3°C for 20 min to allow complete polymerization of the agarose.

After the agarose polymerized, the suspended cells were lysed (2 hr at room temperature) and then the slides were placed in an alkaline electrophoresis buffer for 15 min to unwind the DNA. The slides were then subjected to 25 V at 265–270 mA in the dark at 3°C for 20 min. After electrophoresis, the slides were placed in neutralizing buffer in the dark for 10 min, then drained and overlaid with ethidium bromide and covered with coverslips. The slides were examined the next day at 400× using an Olympus BH-2 epifluorescent microscope. All slides

were coded and examined randomly. Routinely, 25 cells per animal were examined. The length and width of the DNA masses were measured using an ocular micrometer disk. The length:width ratios were used in all comparisons. Under these conditions, a DNA pattern with a ratio of 1 has a DNA length of approximately 40 μm , and with a ratio of 3, a DNA length of approximately 120 μm . DNA masses that appeared as fine fluorescent granules with no distinct nuclear core were classified as unscorable cells. The percentage of unscorable cells was determined by examining an additional 25 cells per animal. In all cases, cells were selected using a straight-line scan of a slide beginning at an arbitrary point.

Dosage Studies

The tadpoles were housed in the laboratory for a minimum of two weeks before the onset of testing. In exposing tadpoles of the three early phases (developmental Stages I, II–III, and X–XVIII) to MMS, they were placed in 1-litre beakers in the dark. Second-year tadpoles with fully developed fore- and hindlimbs (Stages XXII–XXIII) were placed in glass baking dishes (27 \times 18 \times 4 cm) in the dark. Each beaker or dish contained either 500 ml of dechlorinated water or 500 ml of a dilution of MMS in water. The baking dishes were used for the oldest tadpoles because at this phase the animals have lungs and would drown if placed in 1 litre beakers. The 500 ml gave a depth of 1.0 cm in the baking dishes, which was sufficient to cover almost the entire animal. In all cases, the tadpoles were removed and bled after 24 hr of exposure. Each electrophoretic run included animals of at least two developmental phases and both treatment and control groups. Three of the 144 animals died during the 24-hr exposure period.

The levels of MMS used were based on the results obtained for concentrations ranging from 0 to 50 mg MMS per litre of water [Ralph et al., 1996].

Cell Viability Studies

Cell viability was determined using the trypan blue exclusion technique on aliquots of the erythrocyte suspensions (i.e., blood + 10% Hank's balanced salt solution) used in the alkaline SCG assay. Samples from each tadpole were processed simultaneously for both assays. Routinely, 100 erythrocytes per animal were examined.

Statistical Analysis

An alpha level of 0.05 was used to determine significance in all statistical analyses. Different groups of animals were compared using either a nested analysis of variance or a single-classification analysis of variance followed by pairwise comparisons [see Sokal and Rohlf, 1995]. The extent of intercellular heterogeneity within each of the data sets was determined from the range of the DNA length:width ratios to the standard deviation of these ratios. Values below 2 or above 6 indicated the data to be extremely homogeneous or extremely heterogeneous [see Vijayalaxmi et al., 1992]. Multiple linear regression analyses were carried out to establish the correlation coefficient between MMS dosage and induced DNA damage or percentage of unscorable cells. A test of the significance of the correlation coefficient was performed. An estimate of the normality of the distribution of the DNA length:width ratios was determined using the method of ranked normal deviates [see Sokal and Rohlf, 1995].

RESULTS

A summary of the levels of DNA damage per treatment group and developmental phase is given in Table I. First-

year *R. clamitans* tadpoles (Stage I) exposed to various concentrations of MMS had a significant ($P < 0.01$) added variance component for DNA damage compared to the controls (Table II). Subsequent pairwise comparisons between each treatment group and the control group showed significant increases ($P < 0.05$) in DNA damage for all concentrations of MMS (Table II). Similar patterns were seen in both second-year tadpoles without limbs (Stages II and III) and second-year tadpoles with only hindlimbs (Stages X–XVIII) (Table II). In contrast, second-year tadpoles with distinct hind- and forelimbs and a tail undergoing resorption (Stages XXII–XXIII) exposed to various concentrations of MMS did not show a significant ($P > 0.05$) added variance component for DNA damage relative to the control (Table II). Subsequent pairwise comparisons between each treatment group and the control group for animals in this phase showed no significant increases ($P > 0.05$) in DNA damage for all of the concentrations of MMS (Table II). The cellular distribution of DNA damage in tadpoles of each phase of development are shown in Figure 1.

The viability of cells from animals in both the control and treatment groups was $>90\%$, indicating there is no apparent reduction in viability after MMS exposure (Table I). Also, in all cases the range to standard deviation ratios indicated that the intercellular distribution of DNA damage was neither extremely homogeneous nor extremely heterogeneous (Table I). The DNA length:width ratios of the pooled controls ($n = 48$) were found to be normally distributed using the method of ranked normal deviates.

There were moderate linear correlations between DNA damage and dosage of MMS for second-year tadpoles, in both those with only hindlimbs and those without limbs ($r = 0.745 \pm 0.116$ SE of estimate, $P < 0.01$ and $r = 0.665 \pm 0.130$, $P < 0.01$, respectively). The linear correlation between DNA damage and dosage of MMS was weak for first-year tadpoles ($r = 0.530 \pm 0.145$, $P < 0.01$) and there was no correlation for second-year tadpoles with fully developed hind- and forelimbs ($r = 0.054 \pm 0.174$, $P > 0.05$).

All phases of tadpoles responded to increasing doses of MMS, with an increasing percentage of unscorable cells (Table I). The percentage of unscorable cells in all control animals was near zero. There was a strong linear correlation between percentage of unscorable cells and dosage of MMS for second-year tadpoles without limbs ($r = 0.839 \pm 0.095$ SE of estimate, $P < 0.01$). The linear correlation for second-year tadpoles with fully developed hind- and forelimbs was moderate ($r = 0.758 \pm 0.114$, $P < 0.01$), for second-year animals with only hindlimbs moderate ($r = 0.707 \pm 0.123$, $P < 0.01$), and for first-year tadpoles also moderate ($r = 0.584 \pm 0.139$, $P < 0.01$).

Tadpoles of each of the four phases of larval develop-

TABLE I. Detection of DNA Damage and Cell Viability in Erythrocytes of *R. clamitans* Tadpoles, at Various Phases of Larval Development, After a 24-hr Exposure to Different Concentrations of MMS in Water

Development stage and dosage (mg/l)	No. of tadpoles	DNA length:width ratio \pm SEM ^a	Cell viability (%) ^b	Unscorable cells (%) ^a	Range: SD ratio
First year (Stage I)					
Control (dechlorinated water)	12	1.815 \pm 0.171	92.0	4.0	3.04
1.56	8	2.485 \pm 0.096	92.6	15.0	3.04
3.125	7	2.378 \pm 0.221	90.8	11.7	2.62
6.25	9	2.720 \pm 0.158	92.7	44.0	2.85
Second year—limbless (Stages II–III)					
Control (dechlorinated water)	12	1.652 \pm 0.083	98.5	0.0	3.30
1.56	8	2.360 \pm 0.178	96.3	6.5	2.73
3.125	7	2.663 \pm 0.152	97.4	11.4	2.81
6.25	8	2.904 \pm 0.288	98.2	62.5	2.08
Second year—only hindlimbs (Stages X–XVIII)					
Control (dechlorinated water)	12	1.415 \pm 0.090	95.0	0.0	3.19
1.56	8	2.299 \pm 0.166	95.3	4.0	2.53
3.125	7	2.722 \pm 0.259	97.8	17.7	2.60
6.25	8	2.959 \pm 0.174	95.7	39.0	2.63
Second year—all limbs and partial resorption of tail (Stages XXII–XXIII)					
Control (dechlorinated water)	12	1.814 \pm 0.124	98.2	3.0	3.31
1.56	8	1.706 \pm 0.210	97.8	6.5	2.91
3.125	7	1.948 \pm 0.073	98.2	17.1	2.70
6.25	8	1.831 \pm 0.160	99.2	40.0	3.11

^aValues based on 25 cells/tadpole.^bValues based on at least 400 cells.

ment were grouped according to treatment to examine the impact at each phase. The DNA length:width ratios of tadpoles of each of the four phases exposed to only dechlorinated water were not significantly different ($P > 0.05$) (Table III). The variance among phases was greater than the variance among tadpoles within each phase. Tadpoles of each of the four phases that were exposed to 1.56 mg/l of MMS had significantly different levels of DNA damage ($P < 0.05$), but when second-year tadpoles with fully developed hind- and forelimbs were excluded from the comparison there was no significant difference ($P > 0.05$) among the remaining groups (Table III). The variance among phases was greater than the variance among tadpoles within each phase when all four phases were considered. If, however, animals with fully developed hind- and forelimbs were omitted from the analysis, the variance among tadpoles within phases was greater than the variance among phases. A similar pattern was seen among tadpoles exposed to 3.125 mg/l or 6.25 mg/l of MMS (Table III).

DISCUSSION

The alkaline SCG assay is ideal for use on sentinel organisms in situ because: 1) this assay permits the visualization of DNA damage in individual cells; 2) relatively few cells are required (i.e., a few hundred); 3) any cells

that have a nucleus can be used; and 4) the assay has been shown to be sensitive to a number of mutagens using various tissues [Olive et al., 1990; Tice, 1995]. Furthermore, the ability of this procedure to identify "sensitive" cells in an otherwise normal population of cells permits analysis of low dose–response relationships [Tice, 1995]. The simplicity and relatively low cost of the assay makes it suitable for use in large-scale studies. However, because the alkaline SCG detects single-strand breaks, alkali-labile damages, and cross-linkage, it can miss DNA alterations involving processes such as nucleotide substitution and adduct formation. In contrast to the effects of the other two damages, cross-linkage induction will result in an underestimate of DNA fragmentation. [For a broad overview, see McKelvey-Martin et al., 1993, and Fairbairn et al., 1995.]

The use of the SCG assay in situ using sentinel organisms has been limited. [See Tice, 1995, and Ralph and Petras, 1997, for a discussion of the in situ use of this assay for environmental biomonitoring.] Recently, we described the adaptation of the alkaline SCG assay for amphibian tadpoles [Ralph et al., 1996] and the application of this assay for in situ studies using two species of amphibians, *R. clamitans* and *Rana pipiens* (leopard frog). In that study, the vast majority of tadpoles collected were in the early stages of development (Stages I–III) since most of the larval lifespan is spent in these stages. How-

TABLE II. Single-classification ANOVA and Pairwise Comparisons of DNA Damage in Erythrocytes of *R. clamitans* Tadpoles, Grouped by Phase of Larval Development, After a 24-hr Exposure to Different Concentrations of MMS in Water

Source of Variation	DF ^a	MS ^b	F _s ^c	P ^d
First year (Stage I)				
Among treatments	3	1.577	6.144	<0.01
1.56 mg/l vs. control ^e	1	2.157	8.391	<0.01
3.125 mg/l vs. control	1	1.403	5.461	<0.05
6.25 mg/l vs. control	1	4.216	16.404	<0.001
Among tadpoles within treatments	32	0.257		
Second year—limbless (Stages II–III)				
Among treatments	3	2.955	11.057	<0.001
1.56 mg/l vs. control	1	2.384	8.930	<0.01
3.125 mg/l vs. control	1	4.493	16.828	<0.001
6.25 mg/l vs. control	1	7.490	28.052	<0.001
Among tadpoles within treatments	31	0.267		
Second year—only hindlimbs (Stages X–XVIII)				
Among treatments	3	4.649	20.351	<0.001
1.56 mg/l vs. control	1	3.747	16.433	<0.001
3.125 mg/l vs. control	1	7.544	33.089	<0.001
6.25 mg/l vs. control	1	11.443	50.188	<0.001
Among tadpoles within treatments	31	0.228		
Second year—all limbs and partial resorption of tail (Stages XXII–XXIII)				
Among treatments	3	0.074	0.371	>0.05
1.56 mg/l vs. control	1	0.056	0.284	>0.05
3.125 mg/l vs. control	1	0.079	0.401	>0.05
6.25 mg/l vs. control	1	0.001	0.007	>0.05
Among tadpoles within treatments	31	0.198		

^aDegrees of freedom.^bMean squares.^cSample variance ratio.^dBased on single-classification analysis of variance and pairwise comparisons.^eTadpoles in dechlorinated water only.

ever, this is not always the case and, therefore, it is essential to examine the sensitivity to genotoxicants of tadpoles at different stages of development.

From the beginning of larval development to stages approaching but not including the metamorphic climax (Stages XX–XXIV), *R. clamitans* tadpoles responded to MMS exposure with increasing DNA damage in a dose-dependent manner (Tables I and II). Tadpoles in Stages XXII and XXIII, that is, tadpoles in their second year with distinct fore- and hindlimbs and a tail undergoing resorption, did not demonstrate any increased DNA damage even after exposure to the high doses of MMS (Tables I and II). ANOVA indicated that this phase differed significantly ($P < 0.05$) from the rest in response to MMS treatment (Table III). The remaining three phases responded similarly to the various doses of MMS used. They showed DNA damage that increased with increases in MMS concentrations. The pattern did not differ significantly ($P > 0.05$) among the phases.

Very little information is reported in the literature on the sensitivity of different stages of amphibians to geno-

toxicants. Where comparisons are feasible, our results are consistent with those of other studies. For instance, Krauter et al. [1987] examined micronucleus frequencies in “late-stage” (median stage XVIII [Taylor and Kollros, 1946]) and “early-stage” (median Stage XIV) *Rana catesbeiana* (bull frog) tadpoles exposed to X-rays. The spontaneous micronucleus frequencies in early-stage tadpoles were higher than in late-stage tadpoles. At the higher dosages of X-rays (1.6, 2.1, and 3.3 Gy), the younger tadpoles had significantly increased ($P < 0.05$) micronucleus frequencies, whereas the older tadpoles only showed a slight, nonsignificant increase in micronucleus frequencies relative to the controls at the highest dose. Cooke [1972] exposed *Rana temporaria* (common frog) and *Bufo bufo* (common toad) tadpoles at different developmental stages to a range of concentrations of dichlorodiphenyltrichloroethane (DDT) in water and looked at behaviour and mortality. Tadpoles of both species seemed more resistant when they had external gills (embryonic stage), then became more susceptible as they developed, and finally became more resistant again as

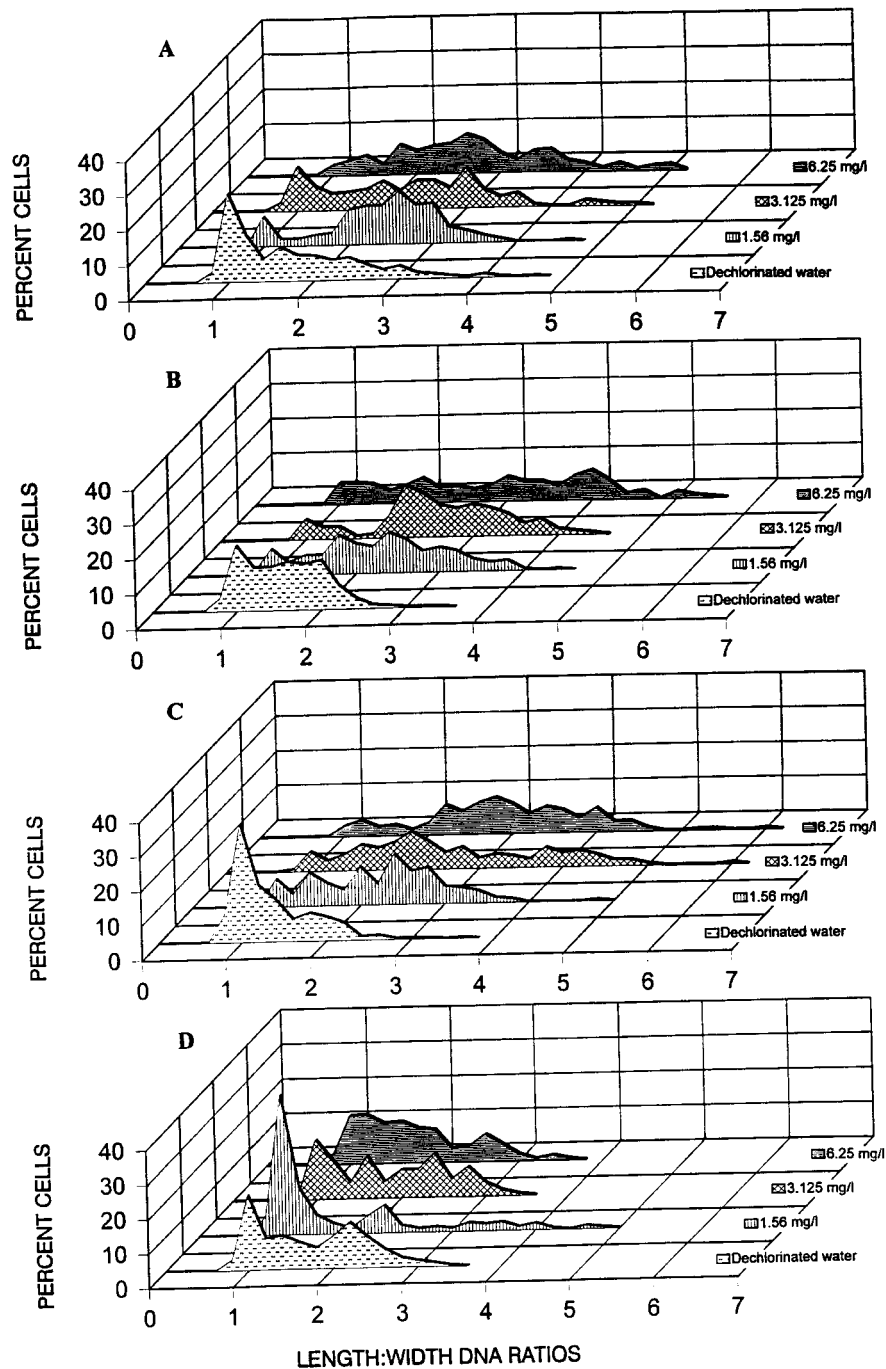


Fig. 1. Distribution of DNA damage (based on length:width ratios of DNA patterns) observed at the cellular level in *R. clamitans* tadpoles of varying larval developmental phases exposed to a range of MMS concentrations. Blood samples were collected from tadpoles 24 hr after

exposure began. (A) First-year tadpoles (Stage I); (B) second-year limbless tadpoles (Stages II-III); (C) second-year tadpoles with only hindlimbs (Stages X-XVIII); (D) second-year tadpoles with fore- and hindlimbs and a tail undergoing resorption (Stages XXII-XXIII).

they approached the metamorphic climax (Stage XX). Sanders [1970] examined *Bufo woodhousii fowleri* (fowler's toad) tadpoles exposed to DDT diluted in water. Measuring mortality, he found the highest sensitivity within the first 3 weeks of development, followed by dramatic

reductions in mortality at 4-5 weeks and further reduction in mortality at 7 weeks. This species typically transforms 40-60 days after hatching [Wright and Wright, 1949].

In another study (manuscript in preparation) we ex-

TABLE III. ANOVA Comparison of DNA Damage in Erythrocytes of *R. clamitans* Tadpoles, at Various Phases of Larval Development, Grouped by Treatment (Concentration of MMS in a 24-hr Exposure)

Source of Variation	DF ^a	MS ^b	F _s ^c	P ^d
Control – dechlorinated water				
All four phases				
Among phases	3	0.427	2.39	>0.05
Among tadpoles within phases	44	0.179		
1.56 mg/l MMS				
All four phases				
Among phases	3	0.960	4.277	<0.05
Among tadpoles within phases	28	0.225		
Without second-year tadpoles—all limbs and partial resorption of tail				
Among phases	2	0.072	0.395	>0.05
Among tadpoles within phases	21	0.182		
3.125 mg/l MMS				
All four phases				
Among phases	3	0.873	3.470	<0.05
Among tadpoles within phases	24	0.252		
Without second-year tadpoles—all limbs and partial resorption of tail				
Among phases	2	0.237	0.733	>0.05
Among tadpoles within phases	18	0.323		
6.25 mg/l MMS				
All four phases				
Among phases	3	2.211	6.715	<0.01
Among tadpoles within phases	29	0.329		
Without second-year tadpoles—all limbs and partial resorption of tail				
Among phases	2	0.135	0.367	>0.05
Among tadpoles within phases	22	0.369		

^aDegrees of freedom.

^bMean squares.

^cSample variance ratio.

^dBased on analysis of variance.

posed adult *R. pipiens*, after they had been maintained in the laboratory in dechlorinated water for 10 months, to MMS dissolved in water. Each animal was bled before the MMS exposure and several times afterwards over a period of 23 days. Blood samples were analysed immediately after collection using the comet assay. Adult frogs exposed for 48 hr to 6.25 mg/l, 1.56 mg/l, or 0.39 mg/l MMS had significantly increased levels of DNA damage relative to the animals before exposure and to the untreated controls. These animals were exposed to MMS in the same way as the four-limbed tadpoles in the current experiment; that is, partial submersion in an MMS solution. Adult *R. clamitans* collected from the wild and injected with 100 mg/kg of MMS also gave significantly increased levels of DNA damage relative to saline-injected controls. Moreover, both adult *R. clamitans* and *R. pipiens* collected from some sites in the wild gave DNA ratios significantly greater than those of adults maintained in the laboratory over the extended period of time. The latter gave values which were considered “baseline.” These results suggest that adult frogs, at least those belonging to *R. clamitans* and *R. pipiens*, are sensitive to

genotoxicants, unlike *R. clamitans* tadpoles in the last stages of metamorphosis.

In the past, authors have suggested that differences in sensitivity among larval stages may be due to changes in surface area:volume ratios, permeability of the skin, and metabolism. Other physiological changes at metamorphosis include: rapid apoptosis in various cell types, including those of the tail, gills, notochord, intestine, nervous system, and craniofacial structures; changes in hormone regulation; and structural rearrangements within tissues. It has also been shown that at metamorphosis there is a differentiation of a new adult erythrocyte population with unique hemoglobin compared to the larval form [Dorn and Broyles, 1982]. Benbassat [1970] measured in vitro DNA synthesis in circulating erythrocytes of *R. pipiens* and *R. catesbeiana* of different larval stages and found an increase in tritiated thymidine uptake in Taylor and Kollros Stages V–XII, a decline in Stages XII–XIX, and an increase again in Stages XX–XXV. Thus, not only does the population of erythrocytes change during this short time span, but the number of erythrocytes also increases [Benbassat, 1970]. The lifespan of erythrocytes

also differs between the adult and larvae. Forman and Just [1976] reported the larval red blood cell lifespan in *R. catesbeiana* to be 89 days, whereas in adults of the same species it has been reported to be 24 days [Baca Saravia, 1961, in Altland and Brace, 1962]. Several of these factors may contribute to the insensitivity of tadpoles at the metamorphic climax to genotoxicants, but we have not tried to directly implicate any of these in this study.

The moderate correlations between percentage of unscorable cells and dosage of MMS among tadpoles at each phase of development are consistent with the amount of DNA damage measured using the comet assay except in the case of second-year tadpoles with fore- and hindlimbs (Table I). In this case, the fact that there is an increase in the number of unscorable cells with increasing MMS dosage, without any increase in length:width ratios, suggests that at least some erythrocytes exposed to the mutagen die rather than undergo repair of any of the damage. The larval erythrocytes may be particularly prone to cell death at the metamorphic climax since there is a rapid shift from larval to adult red blood cells at this point in development.

The consistently high cell viability (>90%, Table I) indicates that neither the assay itself nor exposure to a genotoxicant, in this case MMS, induced a significant amount of cell death.

In conclusion, our results indicate that for most of the larval lifespan there is no difference in sensitivity to genotoxicants, at least in the case of the direct-acting MMS. However, at the climax of metamorphosis (Stages XXII–XXIII), tadpoles appear less sensitive to MMS exposure. Thus, when conducting environmental biomonitoring it is essential to select larval tadpoles that are not entering the metamorphic climax. This should pose no problem since most of the samples collected are in the early stages. Indirect-acting genotoxicants should also be examined. In addition, we have shown that even after exposure to MMS, there is no reduction in cell viability compared to untreated controls.

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E. Zeiger