AMPHIBIANS IN DECLINE

CANADIAN STUDIES OF A GLOBAL PROBLEM

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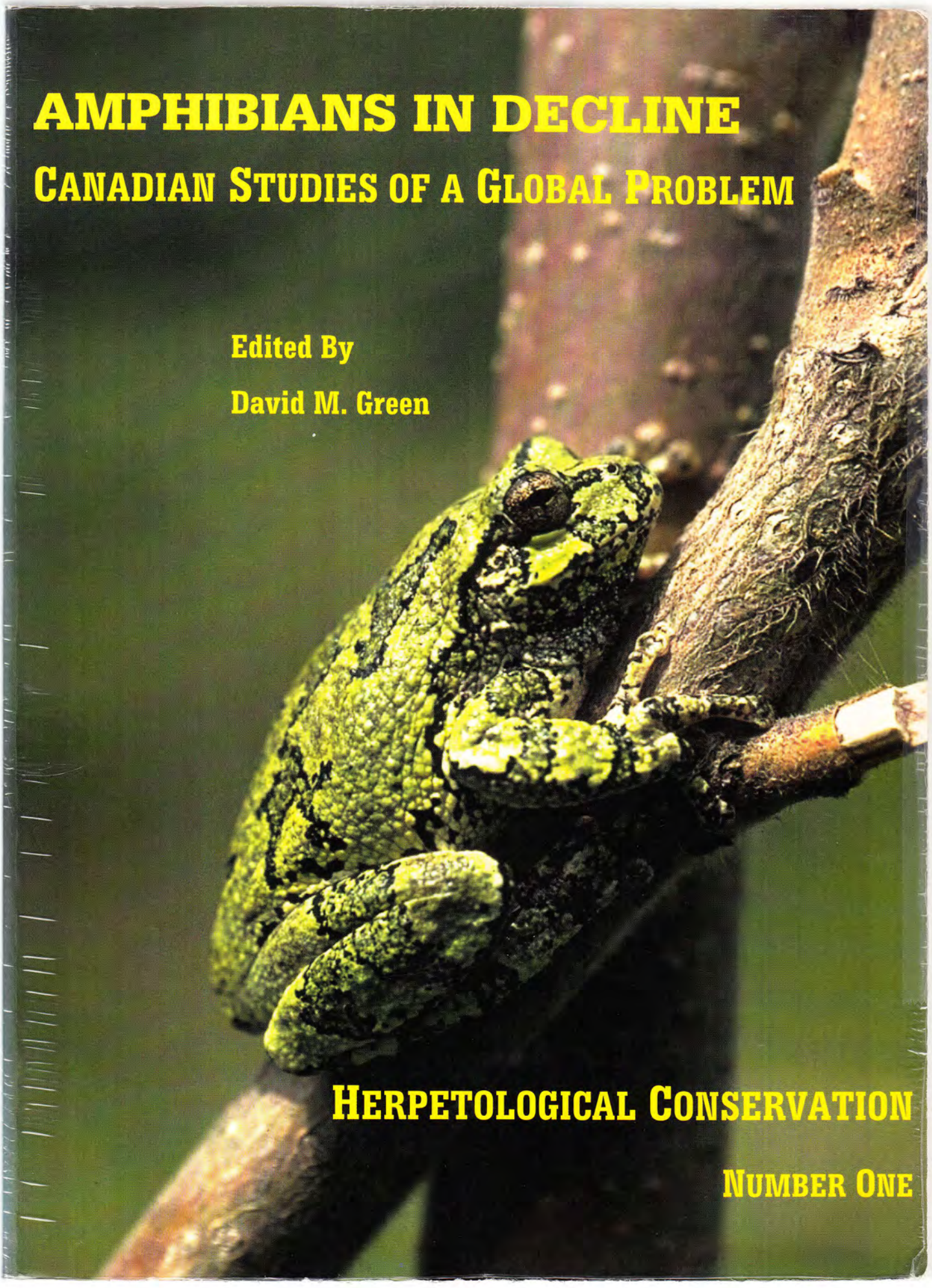

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Chapter 25

MEASURING THE HEALTH OF FROGS IN AGRICULTURAL HABITATS SUBJECTED TO PESTICIDES

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ABSTRACT.—A number of clinical methods were used to determine the health of anurans living in agricultural habitats in southern Québec to identify the most useful methods for detecting sublethal effects of agricultural pollutants. In 1993, metamorphosed and metamorphosing *Rana clamitans* were collected from ponds and ditches in 2 potato fields, 3 sweet corn fields, and 3 uncultivated areas having similar habitats. Physical and postmortem examinations revealed diseases and hindlimb deformities mainly in metamorphosing individuals from potato fields. Blood smears indicated comparable white cell counts, hemoparasite presence, and micronucleus frequencies in all sites. Hematological and blood biochemical analyses, for adults only, appeared consistent with normal health at all locations. Brain acetylcholinesterase activity levels were also normal. Flow cytometry revealed genomic disruption in adult and metamorphosing individuals from all cultivated areas. Deformed individuals from the potato field had increased genome size variability. Similar genomic effects were also found in pesticide-exposed individuals showing no apparent physical or physiological changes. Analyses showed correspondingly high genotoxicity values for water samples taken from the cultivated sites. Clinical examination and flow cytometry are practical techniques for examining the sublethal effects of environmental contaminants on frogs. This investigation indicates a general mutagenic effect of agricultural pollutants on anurans, in addition to potential teratogenic and pathogenic effects in some cases.

RÉSUMÉ.—Différentes méthodes cliniques ont été employées afin de vérifier l'état de santé d'anoures vivants dans les milieux agricoles du sud du Québec. L'objectif était de reconnaître les méthodes les plus utiles pour détecter les effets subléthaux de polluants agricoles. En 1993, des individus métamorphosés et en métamorphose de *Rana clamitans* ont été échantillonnés dans des étangs et fossés de 2 champs de pommes de terre, 3 champs de maïs sucré et 3 sites non-cultivés présentant des habitats similaires. Les examens physique et post-mortem ont révélé des maladies principalement chez les in-

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dividus en métamorphose provenant d'un champ de pommes de terre. L'analyse des frottis sanguins indiquait des valeurs comparables dans tous les sites pour le différentiel des globules blancs, la prévalence des hémoparasites et la fréquence des micro-noyaux. Les valeurs hématologiques et biochimiques obtenues chez les adultes seulement, représentaient probablement des valeurs normales dans toutes les stations. Les niveaux d'activité de l'acétylcholinestérase étaient également normaux. La cytométrie en flux a révélé des modifications génétiques chez les adultes et les individus en métamorphose dans tous les sites cultivés. Les individus malformés du champ de pommes de terre avait une variabilité génétique accrue. Des effets génétiques similaires ont été également trouvés chez des individus exposés aux pesticides qui ne présentaient aucunes anormalités physiques ou physiologiques. Les analyses ont aussi démontré une génotoxicité élevé de l'eau dans les sites cultivés. L'examen clinique et la cytométrie en flux sont donc des méthodes utiles pour étudier les effets subléthaux des contaminants environnementaux sur les anoures. Cette première investigation indique un effet mutagénique général des polluants agricoles sur les anoures, en plus d'effets tératogènes et pathogènes possibles dans certains cas.

Amphibians may be especially sensitive to environmental contaminants due to their biphasic life histories and semi-permeable skin (Hall and Henry 1992). In farming areas, where wetlands adjacent to crops become contaminated with pesticides (Wauchope 1978; Giroux and Morin 1992; Berryman and Giroux 1994; Giroux 1994), anuran breeding populations may be affected (Hazelwood 1970; Cooke 1981). There exist few comprehensive field studies of the effects of environmental contaminants on amphibian populations (Bishop 1992; Hall and Henry 1992), and little comparative information for the symptomatic diagnosis of frog population health is available (Crawshaw 1992a, this volume). Most toxicological tests examine the lethal effects of various compounds on amphibians under controlled laboratory conditions but virtually no light is thereby shed upon their transient or permanent sublethal effects in the natural milieu (Harfenist et al. 1989; Hall and Henry 1992).

Diverse degrees of mutagenic, teratogenic, and physiological effects on animals have been reported for agricultural pesticides (Hayes and Laws 1991). Documented effects on amphibians include increased mortality, lowered hatching success of eggs, retarded growth, abnormal development, behavioural changes, alteration in thermal resistance, and hematological variation (Harfenist et al. 1989; Bishop 1992; Berrill et al., this volume). The inhibition of brain acetylcholinesterase activity has been seen as indicative of poisoning by organophosphate and some carbamate pesticides (Martin et al. 1981), but amphibians were found to be very resistant (Wang and Murphy 1982). However, a common occurrence after chemical exposure in amphibians is the clastogenic generation of micronuclei (Fernandez et al. 1993; Krauter 1993).

The objective of this study was to identify health assessment techniques that could be useful for detecting sublethal effects of agricultural pollutants on frog populations in the wild. A number of clinical methods were used to determine the health of green frogs, *Rana clamitans*, living in aquatic habitats adjacent to potato and sweet corn fields. These crops are farmed extensively in southern Québec, and the numerous pesticides applied during their cultivation contaminate watercourses (Giroux and Morin 1992; Giroux 1993, 1994; Berryman and Giroux 1994). From the exposure to agricultural pollutants, we expected differences in physical and postmortem conditions of frogs, including hematology and blood biochemistry profiles, brain acetylcholinesterase activity, and genomic characteristics (Lowcock et al. 1997; Ouellet et. al. 1997).

Materials and Methods

We concentrated our study on *R. clamitans* because of its sedentary lifestyle (Martof 1953) and omnipresence in southern Québec. Frog populations from 2 potato fields (sites 1 and 2), 3 sweet corn fields (sites 3, 4 and 5) and 3 control sites (sites 6, 7 and 8) from uncultivated open areas were sampled in southern Québec. The corn fields were located within 2 km of each other, while the 2 potato fields were 150 km apart and separated by the St. Lawrence River. The control sites were spread over a distance of 150 km, in the vicinity of the corn and potato fields. Frogs were collected from ponds and ditches exposed to pesticide contamination through direct application and runoff (Table 1) and from similar habitats at the control sites. Land use at all sites had been the same for > 10 yr. Adult frogs (those ≥ 1-yr post-metamorphosis) were caught by hand or dipnet in July 1993, 1 to 5 days after

application of the insecticides carbofuran (carbamate) on the sweet corn and azinphos-methyl (organophosphate) on the potatoes. Metamorphosing or newly metamorphosed juveniles were collected by dipnet in late August 1993. Field examination of the general appearance and behaviour of each frog was conducted immediately after capture. The frogs were then placed in separate plastic jars containing pond water and stored in a cool dark room pending laboratory analysis.

Within 12 hr of capture, physical examination and tissue collection were completed in the laboratory. After external examination, each frog was decerebrated following a rapid decapitation at the first cervical vertebra (Canadian Council on Animal Care 1984) and its brain was placed in liquid nitrogen for cholinesterase activity analysis. A necropsy was performed and a blood sample (0.5 or 1 ml) was collected from the exposed heart using a syringe. A few drops of blood were taken for blood smears and for flow cytometric DNA analysis (Sharbel et al., this volume). If possible, a greater volume of blood was also collected for hematology and for biochemical profiles. Each frog was then measured (snout to vent length, and length of the right tibia), weighed, sexed and given a complete physical and postmortem examination during which evident parasites and/or parasitic cysts were collected from the body surface, the oral cavity, beneath the skin, or from the surfaces of the viscera. In selected cases, tissues were collected and preserved in 10% buffered formalin for histopathological examination.

Blood smears were made from both juveniles and adults and were immediately fixed with absolute methanol. Smears were then stained using a modified Wright-Giemsa colorant ("Diff-Quik", Baxter). Differential white cell counts, thrombocyte estimates, mitotic indices and screenings for hemoparasites, micronuclei, and other cellular abnormalities were performed manually on 82 apparently healthy adults and 7 juve-

Table 1. *Pesticides applied to potato and sweet corn fields in southern Québec in 1993. For potato fields, the 1st application of pesticides was in mid-May, the 2nd was from late June to mid-August, and the 3rd was at the end of August. For sweet corn fields, the 1st application of pesticides was during May and the 2nd (twice) was in July through early August. Common names of pesticides are from Worthing and Hance (1991).*

		Pesticide applications		
Site	Crop	1st	2nd	3rd
1	potato	linuron[H]	azinphos-methyl[I] cypermethrin[I] oxamyl[I] mancozeb[F] chlorothalonil[I]	diquat[H]
2	potato	metribuzin[H] phorate[I]	same as site 1	diquat[H]
3	sweet corn	atrazine[H]	carbofuran[I]	
4	sweet corn	atrazine	carbofuran	
5	sweet corn	atrazine glyphosate[H] butylate[H]	carbofuran	

		Crop rotation and adjacent crops		
		Period	Crop	Pesticide applications
1	potato	3 yr	potato	same as other potato fields
2	potato	2 yr	potato	same as other potato fields
3	sweet corn	2 yr	cucumber cantaloupe	endosulfan[I] chlorothalonil maneb[F]
4	sweet corn	2–3 yr	cabbage raspberry strawberry	endosulfan cypermethrin captan[F] metamidophos[I] benomyl[F] mancozeb
5	sweet corn	none	cabbage	metamidophos mancozeb

[H]herbicide, [I]insecticide, [F]fungicide

niles in various states of health as determined through physical and postmortem examination. The number of micronuclei found in 1000 mature erythrocytes was recorded twice for each slide (Krauter et al. 1987). Only micronuclei clearly separated from the nucleus were recorded. Before the blood smears were scored, all slides were coded and then scored blind by a single observer.

Eight hematological values and 23 blood biochemical parameters were measured in samples from adults that had a sufficient volume of blood (0.6 ml for hematology and > 0.6 ml for biochemistry). Hematology was performed on 78 adults (> 19 g). Fresh blood samples kept in Microtainer (Becton Dickinson) with lithium heparin were analyzed within 24 hr at Vita-tech Canada Inc., Markham, Ontario, using a Technicon H*1 Hematology analyzer. Complete biochemical analysis was performed on 32 adults (> 30 g). Plasma samples kept frozen (−80°C) were analyzed at the Université de Montréal, Faculty of Veterinary Medicine, St. Hyacinthe, Québec, using a Synchron Cx Systems apparatus (Beckman Instruments).

Acetylcholinesterase activity was measured on brain samples from 104 frogs to determine if this enzyme was inhibited in frogs exposed to pesticides. Analyses were performed at the National Wildlife Research Centre, Hull, Canada. Activity was estimated by the rate of formation of thionitrobenzoic acid (measured spectrophotometrically at 405 nm), a product of the reaction between dithiodinitrobenzoic acid and thiocholine, which originates from the hydrolyzation of acetylthiocholine iodine by the cholinesterase present in the brain sample (Ellman et al. 1961; Hill and Fleming 1982). To verify inhibition by organophosphorus pesticides, samples having a low enzymatic activity were reactivated chemically using pyridine 2-aldoxime methiodide (2-PAM; Martin et al. 1981).

The DNA content of erythrocyte nuclei in samples of 45 juveniles and 53 adults was measured using flow cytometry. For each individual, more than 15,000 cells were plotted. Abnormal profiles (aneuploid mosaic, polyploid or other) were identified and, in the case of a normal profile, the c-value (in pg DNA / haploid nucleus) was determined and the coefficient of variation (CV) was calculated as a measure of intra-individual genome size variability (Bickham et al. 1988; Licht and Lowcock 1991).

Water samples for toxicity analyses were collected from sites 1 to 6 at the same time as the adult frogs were collected (Table 2). In order to evaluate the spatiotemporal variability of toxicity, the samples were collected from different areas of ponds 1 and 2, and pond 2 was resampled 3 days later. Two *in vitro* genotoxicity bioassays using rainbow trout (*Oncorhynchus mykiss*) hepatocytes were performed (Gagné and Blaise 1995). The 1st test was a nick translation assay (Snyder and Matheson 1985), which estimates small-scale chromatin damage by the relative amounts of labelled nucleotide uptake following DNA repair. The 2nd test was a DNA alkaline precipitation assay (Olive et al. 1988), an estimate of large-scale DNA damage via fluorometric detection of DNA strands. In addition, cellular viability was estimated by the propidium iodide exclusion test (Gagné and Blaise 1995). The results of 4 replicates of 5 different dilutions (0.1, 1, 10, 25, and 50% v/v) were compared by analysis of variance (ANOVA or the non-parametric Kruskal-Wallis test), followed by a comparison test (Dunnett test or the non-parametric Dunns test).

Results

Disease was found mostly in juveniles from site 2, a potato field which had the greatest density of metamorphosing frogs (Table 2). It was also the only site where dead frogs were found; 7 dead metamorphosing individuals were collected, all showing evidence of red leg (Crawshaw 1992b; this volume). Most of the sick frogs showed red leg symptoms such as erythema and cutaneous hemorrhages (n = 9), ocular lesions (panophthalmitis; n = 8), cutaneous ulcerations (n = 4), and subcutaneous edema (n = 1). Hepatic lipidosis, a degenerative change in the liver characterized by a fatty and yellowish appearance, was detected in 11 juveniles with red leg and in 5 others with no external signs of illness. Kidneys sometimes showed an abnormal yellowish coloration in individuals with hepatic lipidosis. Histologically, hepatocytes were pale and swollen with lipid vacuoles, and their nuclei were displaced to the periphery of the cells. At the other locations, only 1 juvenile (at control site 8) had an ocular lesion (possibly traumatic), and 1 adult (at sweet corn site 3) had a cutane-

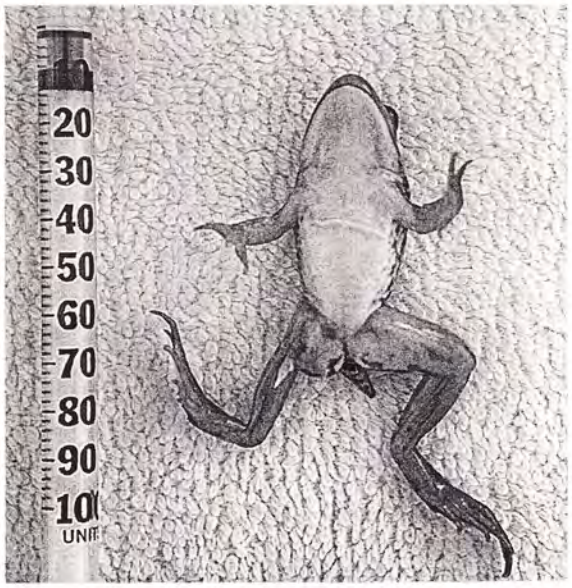
Table 2. *Study sites description and number of* Rana clamitans *with disease or deformity collected from farmlands in southern Québec in 1993. Juveniles = metamorphosing individuals (mean mass = 4.9 ± 2.5 g, range 1.7 to 16.3 g). Adults were ≥ 1-yr post-metamorphosis (mass = 43.5 ± 15.7 g, 18.9 to 113.2 g).*

			Juveniles			Adults		
Site	Habitat	Crop	n	Disease	Deformity	n	Disease	Deformity
1	pond	potato	68		5	8		
2	pond	potato	203	27	15	9		
3	large ditch	sweet corn	3			12	1	1
4	pond	sweet corn	21			14		
5	ditch	sweet corn	0			10		
6	pond	none	1			21		1
7	pond	none	1			6		
8	ditch	none	33	1		0		
Total			330	28	20	80	1	2

ous mycosis (chromomycosis). Scars were found on 4 healthy adults from potato and sweet corn fields. No parasites were found during the physical examination except for 1 adult (at control site 6) with a leech on a hindlimb.

Deformities were found in 20 juveniles (7.4%) from the 2 potato sites (Table 2). In general, the deformities consisted of conspicuous skeletal and muscular defects of the hindlimbs which did not appear to be caused by mechanical amputation. One individual also had a deformed forelimb. Noticeable lesions included agenesis or segmental hypoplasia of 1 or more long bones (ectromelia) and absence of all or part of a digit (ectrodactyly). No individuals with similar deformities were found at the other sites but 1 adult male had abnormal muscular tissue on 1 hindlimb (sweet corn site 3) and another had a single enlarged testis (control site 6).

All adult frogs providing blood samples were diagnosed as normal based on the physical and postmortem examinations. Hematological and biochemical values varied considerably among individuals from a given population. Differential white cell counts and about ¼ of the biochemical parameters had coefficients of variation > 50%. Part of the variation was related to sex and body size: hematocrit values, red blood cell counts, hemoglobin, and mean corpuscular hemoglobin concentrations differed by sex (Mann Whitney $P < 0.05$); hematocrit values, Na^+, Cl^- and CO_2 were correlated to body weight (Kendall's τ, $P < 0.05$). Small differences (Kruskal-Wallis and Mann-Whitney, $P < 0.05$) between sites, whether for the same or different crops, were obtained for a few parameters. However, these variations were often related to differences in body size, and thus seemed to fall within the range of normal health status. Differential white cell counts did not vary among crops or sites (ANOVA, n = 3 replicates of 82 adults, $P > 0.05$ for each cell type), and were



Metamorphosing green frog, Rana clamitans, *with a deformed right hind leg. Photo by Martin Ouellet.*

comparable between sick juveniles (red leg and/or hepatic lipidosis, n = 6) and other healthy individuals.

Most frogs (n = 89) carried intraerythrocytic parasites. Thirty-seven percent had *Lankesterella minima*, 27% had the rickettsia *Aegyptianella ranarum*, and 22% had *Hepatozoon* sp. (= *Haemogregarina* sp.). Three species of *Trypanosoma* sp., including *T. rotatorium* in 21% of the individuals and *T. ranarum* in 11%, were frequent in the plasma. These frequencies were similar between crop and control sites. Microfilariae were found in the blood of 2 individuals.

Although micronuclei were seen in some erythrocytes, cellular abnormalities were infrequent. The mean number of micronuclei in erythrocytes from the 82 adults and 7 juveniles was 0.6 ± 1.0‰ and 0.4 ± 0.5‰ respectively. Frequencies were also similar among crop and control sites. The mitotic index was 0.2 ± 0.5‰ for both juveniles and adults.

Acetylcholinesterase activity levels were similar regardless of crops (Kruskall-Wallis $P > 0.05$). The mean levels (in μmole/min/g ± SD) were 22.2 ± 5.3 for potato (n = 41), 21.2 ± 6.0 for sweet corn (n = 36), and 22.4 ± 3.6 for the controls (n = 27). Activity levels were not correlated to brain or body mass (Kendall's τ, $P > 0.05$ in both cases), and were thus similar in juveniles and adults (Mann-Whitney, $P > 0.05$). Lower acetylcholinesterase activity levels were measured in some of the samples for all crop scenarios. A test for enzyme reactivation was possible with only a few samples (4 for potato, 5 for sweet corn, and 1 for the controls) because the other brains were too small. Only 1 sample from potato site 1 gave a positive response with acetylcholinesterase activity increasing from 14.9 to 31.7 μmole/min/g after chemical reactivation by 2-PAM, suggesting prior inhibition by an organophosphate pesticide.

DNA content analysis indicated significant differences between the crop and control sites. Abnormal profiles (including aneuploids) were more frequent at the sweet corn than at the control sites (Fisher's exact test, $P = 0.03$). DNA content values were consistent with those known for *R. clamitans* (6.0 to 6.6 pg: L. Lowcock, pers. data), but there were differences in intra-individual genome size variability (CV values) between the crop and control sites as well as between juveniles and adults. In juveniles, CVs at the sweet corn sites were greater than at the control sites (Mann-Whitney, $P < 0.05$). In adults, CVs at both the potato and the sweet corn sites were greater than at the control sites (Mann-Whitney, $P < 0.01$ in both tests). Deformed juveniles (n = 3) from site 2 had greater CVs than normal ones (n = 18) from the same pond (Mann-Whitney, $P < 0.05$). There was no difference in CVs between healthy and sick juveniles (Mann-Whitney, $P > 0.05$) and the prevalence of abnormal DNA profiles and high CVs was not related to higher micronuclei and intraerythrocytic parasite frequencies.

A significant genotoxic effect was detectable in all the water samples. The samples from croplands appeared more genotoxic than that those from control site 6 (Fig. 1). The highest values of cellular toxicity were measured in water samples from potato fields. Large spatial and temporal variability in water sample toxicity was revealed by our tests (sites 1 and 2 in Fig. 1). The highest values of genotoxicity were comparable with industrial effluents of intermediate toxicity (Gagné and Blaise 1995).

Discussion

Physical and postmortem examinations provide an immediate assessment of frog health. However, diagnosis of disease often requires extensive use of microbiology and histopathology (Ouellet et al. 1994). Histopathology will also identify neoplasms (Rose and Harshbarger 1977; Mizgireuv et al. 1984). Although tumors were not diagnosed in this study, they are known to occur sporadically in frogs and would be expected to develop more frequently under the influence of certain chemicals (Rose and Harshbarger 1977; Mizgireuv et al. 1984; Crawshaw 1992a). Die-offs are particularly difficult to study because remaining bodies are often rotten.

Hepatic lipidosis has been linked with chemical poisoning in mammals (Jones and Hunt 1983), and red leg in amphibians is known to break out following environmental stressors (Crawshaw 1992a, this volume). However, causative factors for these diseases in our study still remain unknown. Hepatic lipidosis, red leg, and mortality encountered at potato site 2 has been also observed in remote habitats (Ouellet et al. 1994), which suggests that these diseases are not specific to cases of pesticide intoxication. Site 2 had a relatively high density of metamorphosing frogs. Three species of fish (*Culaea inconstans, Notemigonus crysoleucas*, and *Pimephales promelas*) and a leech (*Macrobdella decora*) were also found dying in this pond. High biological oxygen demand, water temperature increases, inputs of organic matter from cropland runoff, and lowered water levels resulting from pumping for irrigation are possible environmental stressors, independent of pesticide contamination, that might have contributed to this disease outbreak. Such conditions may be less than optimal for the frogs and may change the pond's microbial balance in favour of pathogenic organisms (Schotts et al. 1972).

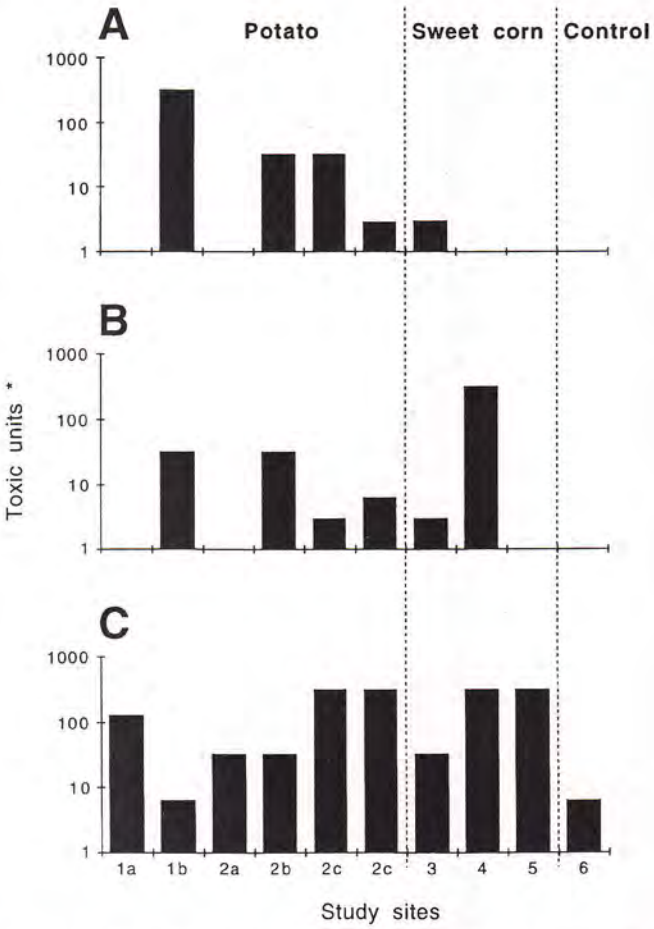


Figure 1. *Relative water toxicity values of 10 samples taken from potato fields, sweet corn fields, and a control site using 3 different bioassays: a) cellular viability, b) genotoxicity - DNA alkaline precipitation assay, c) genotoxicity - nick translation assay. Toxic unit = 100% / geometric mean of the no-observable-effect concentration and the lowest-observable-effect concentration in % v/v (Costan et al., 1993). Study sites are listed in Table 2. Samples "a" and "b" were from 2 different areas in the same pond while samples "c" were collected 3 days later.*

Limb deformities in metamorphosing frogs are uncommon events that have only rarely been studied in North America (Ouellet et al. 1997). The types of limb deformities encountered here contrast with those most commonly reported in the literature (polymely and polydactyly), but their frequency and unpredictable occurrence were comparable to what has been reported elsewhere (Rostand 1971; Dubois 1979; Mizgireuv et al. 1984; Borkin and Pikulik 1986; Vershinin 1989). Developmental defects could originate from genetic, abiotic (xenobiotic pollutants or environmental conditions), biotic (biological products or parasites [Sessions and Ruth 1990]) and nutritional factors. At the potato sites, putative genetic damage in the frogs, together with the genotoxicity of the water samples, suggest the action of environmental mutagens. The known teratogenic effect of various pesticides and fertilizers on eggs and tadpoles of frogs (Hazelwood 1970; Cooke 1981; Harfenist et al. 1989; Bishop 1992) raises a serious possibility that agricultural pollutants are the primary cause of the observed developmental defects.

Disease and deformity were not observed in all situations. It may be that they occurred only when toxic events were synchronous with specific sensitive stages of anuran development. Temporal variation in toxicity was suggested by repeated water sampling at site 2, and is reported in more detailed surveys (Berryman and Giroux 1994). Moreover, the prevalence of deformities in hindlimbs

over other organs suggests a peculiar sensitivity of the hindlimb buds during their early development stage. Our results also indicate the susceptibility of frogs to disease and mortality during metamorphosis. During the resorption of the tail at metamorphosis, bioaccumulated pesticides are mobilized and can cause the death of young frogs (Cooke 1970).

The prevalence of disease and deformity may also be related to a greater contamination risk at some sites. Improper disposal of pesticide containers is a risk factor and the location of a pond may be important as well. For example, site 2, where diseases and deformities were frequent, is an isolated pond located downhill from a field, with few plants along the shore to stop runoff. A large amount of sediment was reaching this pond, and white foam were seen floating along the shore after a rain. This site was considered to be the most exposed to contamination and water tests indicated a correspondingly high toxicity.

Blood sampling in the field proved to be very difficult with tadpoles and moribund juveniles. In these debilitated animals, blood samples were often clotted and contaminated by lymph fluid, and as a result, hematological values were inaccurate. Complete hematology and biochemistry data were thus obtained only from apparently healthy adults, which limits the usefulness of the blood parameters in detecting the sublethal effects of agricultural pollutants.

Kaplan and Glaczenski (1965) observed decreased white blood cell counts and variations in the differential white cell counts following laboratory exposure to pesticides in the northern leopard frog, *Rana pipiens*. Our results do not confirm such clinical findings. The variation in many of the hematological and biochemical parameters with sex and body size in our data, and with other factors such as reproductive state, season, body temperature, nutritional state, desiccation, disease, and stress from recent capture (Hutchisson and Szarski 1965; Harris 1972; Crawshaw 1992b) make it difficult to interpret the results. In addition, there are few reference data for comparative purposes. A reliable diagnosis cannot be obtained from hematological data if sample size is small, and if the normal variability for a given species is unknown (Hutchisson and Szarski 1965). Consequently, controlled

Improper disposal of empty pesticide containers. Photo by Martin Ouellet.

laboratory tests or analyses of large samples from populations in the wild are necessary to determine whether pesticide treatments affect hematological and biochemical values.

Blood smears enabled evaluation of parasitic load. Hemoparasites were frequent in aquatic frogs and seemed to be well tolerated in the individuals examined. This appears to be a normal condition since Barta and Desser (1984) found similar frequencies of *Haemogregarina* sp. (in 49% of the individuals, n = 57), *Lankesterella minima* (14%), *Trypanosoma rotatorium* (44%), and *T. ranarum* (11%) in a population of *R. clamitans* from an unpolluted lake in Ontario.

Consistent acetylcholinesterase activity levels in our results probably represent normal activity levels for the frogs during the summer. The values were comparable for all crop sites and evidence of inhibition was rare. This is in agreement with previous studies (Andersen and Mikalsen 1978; Wang and Murphy 1982), suggesting that anurans are very resistant to acetylcholinesterase inhibition associated with pesticide exposure. However, for this assessment, the collection of frogs immediately after intoxication remains an essential condition which was hardly verifiable in the field. Furthermore, enzyme reactivation could not be performed on brains of juveniles because they were too small (50 mg) for sub-sampling.

The micronucleus test has been proposed as a simple and reliable method for evaluating genotoxic effects of freshwater pollutants (Fernandez et al. 1993). In the wild animals studied here, the frequency of micronuclei was low (< 1‰) compared to what has been observed in laboratory exposition tests with larvae of ranid frogs (Krauter et al. 1987; Krauter 1993) and other amphibians (Fernandez et al. 1993). Frequencies were not higher in the pesticide-exposed populations than in the controls. This suggests that these scores represent spontaneous frequencies of micronuclei. The spontaneous frequency of micronuclei in circulating erythrocytes of metamorphosing tadpoles of bullfrogs, *Rana catesbeiana*, (Krauter et al. 1987) was comparable to our scores in juvenile and adult *R. clamitans*. We also obtained comparable low values for adult *R. catesbeiana* collected from our study sites (unpubl. data). Changes from tadpole to adult erythropoietic centers and the possibility of selective removal of micronucleated erythrocytes from the peripheral blood of frogs are important consideration for the micronucleus assay (Krauter et al. 1987). Investigation of these aspects is required before we decide on the value of this test for wild metamorphosed anurans.

Flow cytometric analysis of DNA provided evidence of hidden damage which could not be evaluated using the other techniques in our study. Comparison of micronuclei scores and flow cytometric results indicated no relations between the 2 assessments of genotoxic effects. The correlation between putative DNA damage (high CVs) and hindlimb deformity does not necessarily indicate a causal link, although this remains a possibility. The use of flow cytometry in the study of environmental mutagens is in its infancy and ours has been its 1st application in assessing genomic disruption in wild amphibian populations (Lowcock et al. 1997; Sharbel et al., this volume). Comprehensive interpretation of the flow cytometric results will require further controlled investigations of the mechanisms and cells (somatic vs gonadal) which are involved.

The prevalence of genetic damage and the genotoxicity of water samples indicate that agricultural practices are affecting habitat quality and frog health. However, contamination of habitats or carcasses by pesticides was not determined and we cannot incriminate specific pesticides or crops. Both the potato and the sweet corn sites, which were exposed to different pesticides, had frog populations with similar genetic defects. Diverse degrees of mutagenicity have been reported for some of the pesticides used at the test sites, including azinphos-methyl, carbofuran, cypermethrin, endosulfan and diquat (Hayes and Laws 1991). Controlled *in vivo* toxicological studies will be required to identify their specific effects. Though pesticide residues in watercourses (Wauchope 1978; Giroux et Morin 1992) are usually at lower concentrations than those used in laboratory tests (Harfenist et al. 1989; Bishop 1992), actual contamination processes under field conditions (i.e. cumulative exposure, multiplicity of products, synergistic effects, etc.) might increase the risk of genotoxicity.

In evaluating the value of health assessment methods in detecting effects of agricultural pollutants, consideration should be given to the fact that pesticides and other agricultural pollutants might produce a broad spectrum of sublethal and lethal effects that are still poorly understood. The various methods can supplement each other for this task. Physical examination is easily applied in the field, and provides valuable information on disease and deformity. Blood samples for DNA, blood smears, and selected hematological or biochemical parameters can also be obtained without sacrificing the animal. Flow cytometry has proven to be a potentially useful tool for the measurement of the effects of environmental mutagens (Sharbel et al., this volume). Other approaches used here require sacrificing the animal. In this study, cholinesterase analysis, complete hematology, and blood biochemistry profiles were not feasible on juveniles, and gave few additional indications of health problems associated with agriculture. On the other hand, postmortem examination was crucial in the diagnosis of disease, and should include histopathological examination and bacterial cultures. A complete physical and postmortem examination, including blood, tissue and parasite collection, took a veterinarian and a technician approximately 45 min for an adult frog.

Health assessment proved to be a practical approach to detecting the effects of environmental stressors on amphibians in the wild. This approach is attractive in its simplicity of sampling (frogs are caught at random in their habitat), as compared to population studies where survivorship, recruitment and density need to be determined. However, population level studies are required to understand the ultimate impact of diseases on populations in the wild. Health is usually determined from survivors, and it is not known whether sick frogs are easier or harder to find than healthy ones. If sublethal responses to some contaminants are skewed toward lethality, and dead eggs, tadpoles, or frogs are not readily found, the impact of agricultural pollutants might be underestimated.

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