Journal of Wildlife Diseases, 33(1), 1997, pp. 95–104

HINDLIMB DEFORMITIES (ECTROMELIA, ECTRODACTYLY) IN FREE-LIVING ANURANS FROM AGRICULTURAL HABITATS

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ABSTRACT: High prevalences of hindlimb deformities were recorded in wild-caught green frogs (*Rana clamitans*), northern leopard frogs (*Rana pipiens*), American toads (*Bufo americanus*), and bullfrogs (*Rana catesbeiana*) from agricultural sites exposed to pesticide runoff in the St. Lawrence River Valley of Québec, Canada, between July and September 1992 and 1993. Of 853 metamorphosing anurans examined in 14 farmland habitats, 106 (12%; range 0 to 69%) had severe degrees of ectromelia and ectrodactyly, compared to only two (0.7%; range 0 to 7.7%) of 271 in 12 control sites. However, the variation in the proportion of deformities among sites was too large to conclude that there was a significant difference between control and pesticide-exposed habitats. Clinical signs varied and were characterized by segmental hypoplasia or agenesis of affected limbs. Conspicuous abnormalities interfered with swimming and hopping, and likely constituted a survival handicap. Because of circumstances and the frequency of these malformations in nine distinct habitats, and in three different species from one of our study sites, we propose a teratogenic action of exogenous factors. Despite the fact that many biotic and abiotic agents are potentially harmful to limb development, agricultural contaminants were suspected as primary aggressors. Thus, clinical examination and frequency of deformities in anurans might be an economical screening tool to assess ecosystem health and the presence of environmental contaminants.

Key words: Anurans, amphibians, ectromelia, ectrodactyly, hindlimb deformities, pesticides, agricultural contaminants, Québec.

INTRODUCTION

Limb deformities and developmental abnormalities have been encountered occasionally in natural populations of amphibians for years by many authors. Most previous reports are related to the occurrence of supernumerary limbs or digits among anurans and salamanders (Bishop, 1947; Rostand, 1958; Woitkewitch, 1961; Hebard and Brunson, 1963; Van Valen, 1974; Worthington, 1974; Reynolds and Stephens, 1984; Borkin and Pikulik, 1986; Sessions and Ruth, 1990). A general review of other external anomalies and mutations in European populations of the *Rana esculenta* complex is presented by Dubois (1979). However, the absence of all or part of a limb (ectromelia) or digit (ectrodactyly) in anurans has been observed quite rarely. These defects have been detected sporadically in Europe (Rostand, 1958) and in Russia (Mizgireuv et al., 1984; Vershinin, 1989). Most records of leg deformities and other malformations are often anecdotal throughout North America and their prevalence in the wild is virtually unknown.

Growing concern about conservation and monitoring of amphibian populations as well as the evaluation of whether pesticides have effects on these species has also been stressed recently (Harfenist et al., 1989; Hall and Henry, 1992). Amphibians may potentially be subject to more environmental stressors and toxic exposure due to their biphasic life cycles and skin permeability. In southern Québec, Canada, pesticides contaminate watercourses and underground waters in agricultural regions (Giroux and Morin, 1992; Berryman and Giroux, 1994). Intensive sweet corn and potato farming are common in this area and are often pesticide-consuming. Pesticide concentrations in watercourses frequently exceed the recommended values to protect aquatic life (Berryman and Giroux, 1994). The intensification of agriculture is also reducing the number and the quality of breeding habitats, contributing to the general impoverishment of anuran communities (Bonin et al., 1997a).

To assess the health status of some anuran populations and to develop a health monitoring strategy, a multidisciplinary study was carried out in agricultural habitats from the St. Lawrence River Valley of Québec, Canada (Bonin et al., 1997b). Health assessment included physical, postmortem, and histopathological examinations; complete hematology counts, blood biochemical profiles, and rates of erythrocyte micronuclei; parasitology; DNA profiles, content, and genome size variability of erythrocyte nuclei; brain cholinesterase activity; and concentrations of trace metals in many specimens. Water samples were also analyzed for cytotoxicity and genotoxicity as described in Gagné and Blaise (1995). During the course of this survey, a high frequency of hindlimb deformities in anurans occupying ponds or ditches exposed to agricultural pesticides was observed. Severe degrees of ectromelia and ectrodactyly were recorded in different populations of green frogs (*Rana clamitans*), northern leopard frogs (*Rana pipiens*), American toads (*Bufo americanus*), and bullfrogs (*Rana catesbeiana*). Our objective was to describe these anomalies and determine their prevalence in agricultural habitats of Québec, Canada. A comprehensive review of different agents potentially harmful to leg development is also presented in the discussion.

MATERIALS AND METHODS

Populations of *Rana clamitans*, *Rana pipiens*, *Bufo americanus*, and *Rana catesbeiana* were examined in 14 agricultural and in 12 pesticide-free environments from southern Québec, Canada (Tables 1 and 2). We collected 727 metamorphosing individuals (tadpoles with fully developed hind legs to recently metamorphosed juveniles) from 19 different sites between July and September 1992. In 1993, 397 metamorphosing specimens from one known site (VE32) and from seven additional locations were also examined between July and August. A third collection in the site VE32 was also attempted in 1994. All stations were within a region of 200 × 400 km around the St. Lawrence River Valley between a latitude of 45°00′ and 46°55′N and a longitude of 71°50′ and 76°30′W. All sites occurred within similar agroclimatic zones (Massin, 1971), and were situated in open habitats fully exposed to solar radiation. The pH of water, measured with a meter at each station during the sampling, ranged from 6.0 to 8.5.

Metamorphosing individuals were caught by dip-net in ponds or ditches subjected to pesticide applications and in similar habitats free from agricultural contaminants. In agricultural areas, the aquatic habitat was always at risk from toxic chemical runoff, being adjacent to plots of vegetable (potato, sweet corn) or cereal (barley, corn, soya, wheat). Through a survey of farmers, a wide range of products was proven to be used in these sites up to three times during a given season. Prior to our collection, various herbicides (including atrazine, bentazon, bromoxynil, butylate, dicamba, diquat dibromide, glyphosate, linuron, MCPA, metobromuron, metolachlor, metribuzin and/or trifluralin), insecticides (especially azinphos-methyl, carbofuran, cypermethrin, oxamyl, and/or phorate), and occasionally fungicides (largely chlorothalonil, or mancozeb) had been used on vegetable crops (Bonin et al., 1997b), while only herbicides had been applied to cereal crops. Pesticide-free areas were usually localized either in pastures or old fields without history of chemical applications or pollution during previous years.

Anurans were examined alive immediately after capture and any macroscopic abnormalities were noted. Deformities were diagnosed with the naked eye, and under a dissecting microscope for smaller specimens. Weights, snout-vent, and tibia lengths were recorded for each specimen. After euthanasia by a rapid decapitation at the first cervical vertebra (Canadian Council on Animal Care, 1984), a postmortem examination was conducted on all deformed individuals to evaluate the presence of any internal lesions. Thirteen *R. pipiens* and one *R. clamitans* with representative leg anomalies were selected for complete histological examination. Freshly killed frogs were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Serial sections of each hindlimb were cut at 4 μm and stained with hematoxylin-phloxine-saffron (Luna, 1968).

The pond VE32 was the only site surveyed for three consecutive years due to the high prevalence of deformities encountered during the first year. Between May and July 1993, tadpoles of *B. americanus* and *Rana sylvatica* were also reared in captivity in this pond for a period

TABLE 1. Prevalence of hindlimb deformities in metamorphosing anurans[a] from 14 agricultural sites subjected to pesticides in 1992 and 1993.

Species	Prevalence %	Prevalence Number	Malformations	Station/Year	Habitat/Crop	Pesticides[b]
Rana clamitans	7.4	(15/203)[c]	Ectromelia and ectrodactyly	2/1993	Pond/potato	H, I, F
	7.4	(5/68)	Ectromelia and ectrodactyly	1/1993	Pond/potato	H, I, F
	6.7	(1/15)	Ectromelia	SA37/1992	Stagnant stream/barley	H
	5.9	(1/17)	Ectromelia and ectrodactyly	EA16/1992	Stagnant stream/corn	H
	—	(1/1)	Ectromelia	VE32/1992	Pond/corn	H
	0	(0/21)		4/1993	Pond/sweet corn	H, I
	0	(0/14)		VE20/1992	Ditch/corn, soya, wheat	H
	0	(0/10)		LA46/1992	Pond, ditch/corn, soya	H
	0	(0/6)		SC38/1992	Pond/corn, sweet corn	H, I
	0	(0/3)		LA15/1992	Ditch/corn	H
	0	(0/3)		VE32/1993	Pond/corn	H
	0	(0/3)		3/1993	Ditch/sweet corn	H, I
	0	(0/2)		LA50/1992	Ditch/corn	H
Rana pipiens	66	(40/61)	Ectromelia and ectrodactyly	VE32/1992	Pond/corn	H
	0	(0/64)		VE32/1993	Pond/corn	H
	0	(0/38)		VE20/1992	Ditch/corn, soya, wheat	H
	0	(0/34)		VE12/1992	Ditch/corn, soya, wheat	H
	0	(0/15)		LA15/1992	Ditch/corn	H
	0	(0/8)		EA16/1992	Stagnant stream/corn	H
	0	(0/4)		SC38/1992	Pond/corn, sweet corn	H, I
	0	(0/1)		VE31/1992	Pond/barley	H
Bufo americanus	69	(37/54)	Ectromelia and ectrodactyly	VE32/1992	Pond/corn	H
	5.7	(3/53)	Ectromelia	LA46/1992	Ditch/corn, soya	H
	1.4	(2/145)	Ectromelia	VE31/1992	Pond/barley	H
Rana catesbeiana	10	(1/10)	Ectromelia	SC38/1992	Pond/corn, sweet corn	H, I

[a] Tadpoles with fully developed hindlimbs to recently metamorphosed juveniles.
[b] H = herbicides, I = insecticides, and F = fungicides.
[c] Number of individuals deformed/number of individuals examined.

of 31 to 41 days (stages 25 to 46 according to Gosner, 1960). One hundred larvae of *B. americanus* from the site were caged up in a similar way as proposed by Cooke (1981). Another 100 larvae of *B. americanus* and 200 larvae of *R. sylvatica* from control areas were likewise caged in this habitat. According to species, hand-made hoop nets containing 25 tadpoles each were kept half submerged in the water to monitor the larval development.

Statistical analyses were conducted to test whether the proportion of deformed individuals was greater in sites subjected to pesticides than in pesticide-free habitats. Because of clustering of metamorphosing anurans in various sites, the hypothesis of independence of individuals required by the Chi-square test was not valid (McCullagh and Nelder, 1989). In fact, since the proportion of deformed individuals varied among stations in the same group (pesticide or pesticide-free), we would encounter extra-binomial variation which is common in the case of clustered samples. The Pearson chi-square statistic for testing homogeneity of proportion of deformed individuals among stations can be used to evaluate the importance of ex-

TABLE 2. Prevalence of hindlimb deformities in metamorphosing anurans[a] from 12 pesticide-free habitats in 1992 and 1993.

Species	Prevalence % Number	Malformations	Station/Year	Habitat
Rana clamitans	7.7 (1/13)[b]	Ectromelia	LA01/1992	Marsh
	0 (0/33)		8/1993	Ditch
	0 (0/17)		EA01/1992	Stagnant stream
	0 (0/16)		CO39/1992	Marsh
	0 (0/6)		CO38/1992	Lake
	0 (0/4)		SA40/1992	Pond
	0 (0/3)		SC01/1992	Pond
	0 (0/1)		LC01/1992	Lake
	0 (0/1)		6/1993	Pond
	0 (0/1)		7/1993	Pond
Rana pipiens	7.7 (1/13)	Ectromelia	LA01/1992	Marsh
	0 (0/1)		EA01/1992	Stagnant stream
Bufo americanus	0 (0/149)		VE01/1992	Pond
Rana catesbeiana	0 (0/9)		LC01/1992	Lake
	0 (0/4)		HU14/1992	Marsh

[a] Tadpoles with fully developed hindlimbs to recently metamorphosed juveniles.
[b] Number of individuals deformed/number of individuals examined.

tra-binomial variation. When the ratio of this statistic chi-square to its degree of freedom ($n - 2$, n being the total number of stations) is greater than 1, there is an indication of overdispersion. The ratio $\chi^2/(n - 2)$ was used to correct the usual Wald statistic (Hosmer and Lemeshow, 1989) used to compare groups in a logistic regression model where the dependant variable is the presence or absence of malformation and the independent variable is a dummy variable taking value 0 for control sites and 1 for pesticide-exposed sites (McCullagh and Nelder, 1989). The corrected statistic is $Z_c = Z/(\chi^2/n - 2)^{1/2}$, where Z is the usual Wald statistic. Under the hypothesis of no difference between groups, this statistic is approximately normally distributed. We tested the hypothesis of no difference between groups versus the unilateral hypothesis of a greater proportion of malformation in pesticide-exposed sites. Statistical analysis was performed using SAS/INSIGHT (SAS Institute Inc., 1993).

RESULTS

Developmental anomalies in metamorphosing anurans were detected from nine different stations. Of 853 individuals examined in 14 farmland habitats, 106 (12%; range 0 to 69%) were malformed (Table 1), while two (0.7%; range 0 to 7.7%) of 271 were abnormal in 12 pesticide-free habitats (Table 2). A great variation in the proportion of deformed individuals among sites from the same group and even within the same pond (VE32) between two consecutive years was noticeable (Table 1). Taking together all anurans of all species, the proportion of deformities in control sites was not significantly smaller than in those subjected to pesticides ($Z_c = 1.03$, $P = 0.15$). *Rana clamitans* alone, the species with the largest sample size, had little extra-binomial variation and the corrected Wald test approached significance ($Z_c = 1.61$, $P = 0.054$). For this species, the prevalence of malformations was 6.3% in pesticide-exposed stations compared to 1.1% in control sites.

Except for two specimens, abnormalities observed were always confined to the pelvic limbs. Of the hindlimb deformities, ectromelia and ectrodactyly were the only lesions diagnosed. The two other types of malformations, encountered solely in *R. clamitans*, were one case of forelimb ectromelia associated with a contralateral hindlimb ectromelia (station 2) and one case of unilateral anophthalmia (VE32,

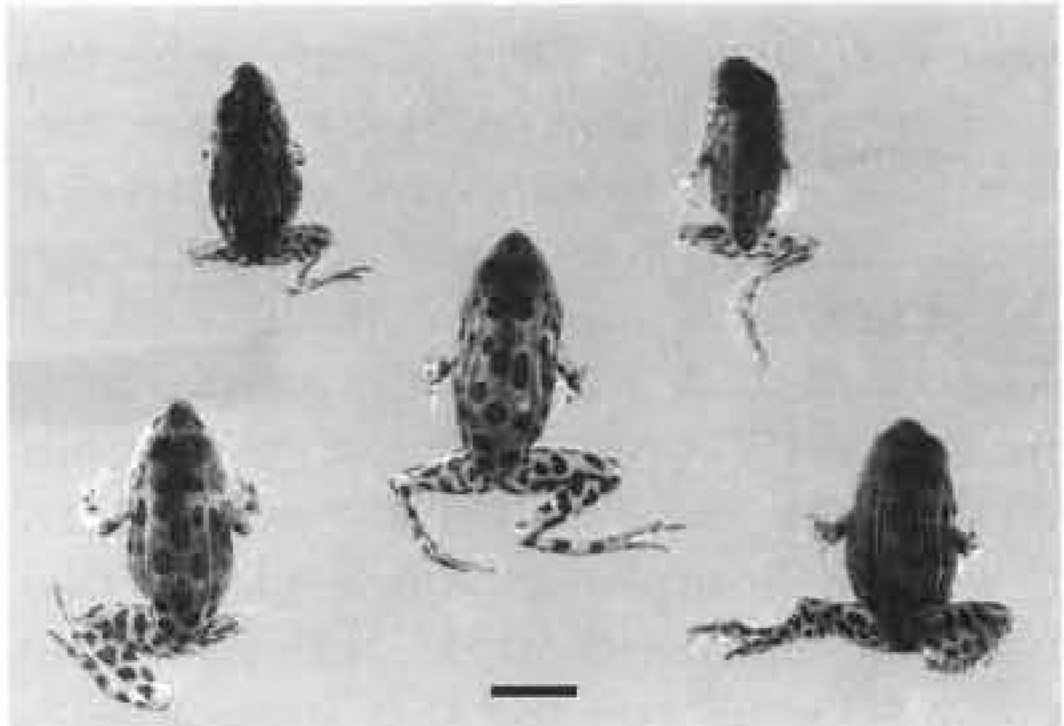

FIGURE 1. Different degrees of ectromelia and ectrodactyly in newly metamorphosed *Rana pipiens*. Bar = 1 cm.

FIGURE 2. Recently metamorphosed *Rana pipiens* with ectromelia, ectrodactyly, and a knee anteversion of the right hindlimb. Bar = 1 cm.

1993). In addition to metamorphosing individuals, 146 adults of various ages were examined during the same period. Only one (0.7%) had a comparable hindlimb deformity (*R. clamitans*, station 3).

Ectromelic individuals were characterized by the absence of one or more parts of the limb (Figs. 1 and 2). The tarsal bones, tibiofibula, and femur were sometimes partially or completely missing. The affected leg was usually marked by muscular atrophy and occasionally by bony excrescences. Ectrodactyly (Figs. 1 and 2) was represented by the lack of one or more toes or parts of toes (phalanges, metatarsal bones). Missing digits varied from one to five on the affected foot. Both conditions were polymorphic and occurred on the right or the left side of the body. Bilateral deformities without symmetrical pattern were also observed among some anurans. In general, the proximal part of the remaining leg was clinically functional. On microscopic examination, the affected limbs had epiphysial hypoplasia and segmental hypoplasia of the diaphysis of the femur and tibiofibula bones. Partial or complete agenesis of the tarsus, metatarsal bones and phalanges was also usually present. Epiphyseal cartilage proliferation was observed in one individual affected by a knee anteversion (Fig. 2). Inflammatory changes, parasites, and neoplastic alterations were not encountered in relation to limb structures.

Limb development in pond VE32 was totally normal after stage 46 in caged *B. americanus* (n = 44, of which 24 were from VE32) and in *R. sylvatica* (n = 76). The mortality rate was, however, quite high in these cages (78% and 62%, respectively). In this particular habitat, delayed metamorphosis was noticeable in the case of *B. americanus* during the summer of 1992. In fact, tadpoles were still abundant in September, while none persisted in two nearby sites (VE31, VE01). Though egg masses were numerous in VE32 during the spring of 1993, *B. americanus* tadpoles were not present at the sampling time. No living frogs were found in the habitat in 1994, visibly polluted by a rust-like foam of unknown origin.

DISCUSSION

Unexpectedly high prevalences of hindlimb deformities were detected in anurans during the course of this study. To our knowledge, this constitutes the first published report of mass ectromelia and ectrodactyly in North America. These malformations were not difficult to distinguish from other hindlimb mutilations because of their awkward forms. As a matter of fact, traumatic lesions and injuries did not occur frequently in our investigated stations. Hindlimb deformities similar to our findings (ectromelia) and cases of unilateral anophthalmia have been encountered among Russian populations of *Rana chensinensis* (Mizgireuv et al., 1984). However, these authors mentioned the simultaneous presence of tumor-like dysplasias of osteochondrous tissue of hindlimbs, which was not found in our histologically examined specimens.

The highest prevalence of ectromelia and ectrodactyly was found in the metamorphosing-anuran age group. These deformities were inconspicuous in younger stages because distal bone segments and digits were not yet developed. Many tadpoles with abnormal hindlimb buds (unequal sizes) were thus excluded from the analysis. Furthermore, the low prevalence observed in adults was evidence of a survival disadvantage for anurans with these anomalies. In fact, these defects appeared to conflict with swimming and hopping in the surrounding habitat. A survival handicap after the metamorphic stage was similarly proposed for other types of malformations (Rostand, 1971; Sessions and Ruth, 1990). Mizgireuv et al. (1984), by contrast, concluded that the distribution of limb abnormalities was independent of age. Further population level studies are therefore required to grasp the impact of such abnormalities on anuran survivorship.

As with different studies (Rostand, 1958; Woitkewitch, 1961; Mizgireuv et al., 1984; Sessions and Ruth, 1990), hindlimbs were almost the only appendage affected by developmental defects in our different populations. In anuran larvae encountered in our study, the forelimbs develop protected in peribranchial sacs within the branchial chamber and emerge fully developed at stage 42 (Gosner, 1960; Duellman and Trueb, 1986). Conversely, the hindlimbs develop in contact with the surrounding environment and are exposed much earlier to the aquatic medium (stage 26). Therefore, and as hypothesized by Sessions and Ruth (1990), pelvic limb buds would be prone to greater exogenous insult than their anterior counterparts. This possible explanation is evidence that hindlimb development in anuran larvae would be more vulnerable to environmental hazards during critical cell-division stages and morphogenesis.

Though we observed some difference in the prevalence of deformities between pesticide-exposed and control populations, the variation in the proportion of deformities among sites was too important for us to conclude, with so few sites, that there was a real difference. Therefore, we emphasize the need to sample a larger number of anurans from a larger number of sites in order to reach a significant conclusion on the possible teratogenic effects of agricultural pesticides. In the same way, it is also conceivable that the systematic use of radiographic and histological examinations could have increased the prevalence of affected individuals in our problematic populations.

We propose that environmental factors such as pesticides were responsible for the high prevalence of deformities in some of our stations for several reasons. Firstly, the simultaneous occurrence of these abnormalities in more than one species at a single site (VE32, 1992) was evidence for the action of exogenous factors. Secondly, their overall presence in distinct habitats supported the fact that these manifestations were not isolated and unique events. Thirdly, the prevalence of deformities varied between years at a given site; for example, in VE32, malformations were encountered in 1992 but not in 1993. The acting factor may be accidental, and therefore not a permanent local characteristic of the environment or an hereditary mechanism of the breeding population. Fourthly, the action of environmental mutagens was supported by the finding of genetic damage in frogs and by the measurement of correspondingly high levels of genotoxicity in water samples from the cropland sites surveyed in 1993 (Bonin et al., 1997b). Of metamorphosing individuals from station 2 with ectromelia and ectrodactyly for which genetic data were available (n = 3), coefficients of variation of DNA content (indicative of genomic damage) were significantly higher than those of non-deformed individuals from the same pond (n = 18). The correlation between DNA damage and hindlimb deformities does not necessarily indicate a causal link, although this remains a possibility. This correlation is evidence, however, that both phenomena are related to a common factor which could be the exposure to contaminants.

Agricultural contaminants can cause developmental anomalies in amphibians. High incidence of curvature and tail deformities has been observed in caged tadpoles of *Rana temporaria* after application of oxamyl, a carbamate insecticide and nematocide, on potato fields (Cooke, 1981). Similarly, experimental exposures to different herbicides (Dial and Bauer, 1984), insecticides (Rzehak et al., 1977; Marchal-Segault and Ramade, 1981; Pawar et al., 1983; Pawar and Katdare, 1984; Schuytema et al., 1991), fungicides (Bancroft and Prahlad, 1973), and fertilizers (Hecnar, 1995) have caused teratogenic effects such as axial skeleton and tail malformations, and mortalities in anuran embryos and tadpoles. These previous works dealt with legless animals. Consequently, it is difficult to make comparisons with our findings.

Further environmental pollutants such as fuel compounds (Greenhouse, 1976) and methylmercury (Dial, 1976) produce tail anomalies in anurans exposed at early stages of development in laboratory experiments. In Russia, greater percentages of morphological aberrations in *Rana arvalis* were observed in heavily urbanized areas (1.5 to 15%), while only 0 to 2.2% were encountered in suburban forests and natural populations (Vershinin, 1989). Juveniles of *R. arvalis* and *Rana temporaria* were more affected as the degree of human activities increased. Mizgireuv et al. (1984) found that between 31 and 42% of individuals were malformed in three regions contaminated with sewage effluent from paper factories and municipal gutters.

Other exogenous abiotic factors might lead to the emergence of limb malformations. Anomalies in hindlimb skeletons (ectrodactyly) have been induced artificially in larvae of *Bufo vulgaris formosus* reared at 30 C (Muto, 1969). The skeletal element which differentiates at the earlier stages would be more resistant to be involved in defective changes than the element which differentiates at the later stages. In the field, the teratogenic action of high temperature has been sometimes postulated in isolated ponds (Worthington, 1974), but additional scientific evidence is required. In laboratory experiments, limb deformities in *Rana temporaria* have been induced in individuals raised at pH 4 (Cummins, 1987). Developmental anomalies (lordosis) have also been obtained at an early stage in *Bufo boreas boreas* tadpoles following prolonged exposure to UV-B radiation (Worrest and Kimeldorf, 1975). Further environmental agents like chemical composition of the water (e.g., osmotic pressure, calcium ion content), and radioactive pollution are also conceivable. As deformed individuals originated from a total of nine different stations in our study, however, it is unlikely that these factors are involved.

A severe form of polydactyly ("anomalie P") in *Rana esculenta* is induced by coexistence with certain fish (*Tinca* sp., *Anguilla* sp.) during the first days of the larval life; larvae were considered to be sensitive to a factor present in the digestive tracts of these fish (Rostand, 1971). No case of polydactyly was found at our study sites, although different freshwater fish were sometimes observed. Another biotic association was described by Sessions and

Ruth (1990) for both *Hyla regilla* and *Ambystoma macrodactylum*. In this case, parasitic cysts (trematode) interfered mechanically with normal leg development to produce supernumerary hindlimbs.

Hereditary mechanisms resulting in genetic mutants have been demonstrated in abnormally legged amphibians (Ponse, 1941; Rostand, 1958; Uehlinger, 1969). In mice, a hereditary ectromelia (lack of tibia) was maintained for 5 yr in one breeding population (Hovelacque, 1920). Other etiologic agents have also been suggested for limb abnormalities including hyperregeneration after injury (Dubois, 1979), teratogenic virus (Rostand, 1971; Dubois, 1979), excessive density of tadpoles reared together (Berger, 1971), and nutritional deficiencies or diseases in captive specimens (Martínez et al., 1992; Crawshaw, 1993). Considerable effort should be made in the future in order to prove the exact mechanisms of several of these stressors.

In conclusion, the difficulty of separating natural or stochastic events from anthropogenic factors emphasizes the need for further studies under field conditions. The cumulative and synergistic action of several of these potential elements do not make this task simpler. Moreover, it is difficult to predict the sensitivity of different amphibian species to potential environmental hazards. Nevertheless, the occurrence of morphological abnormalities in anurans might prove to be useful in assessing the health of aquatic ecosystems. Clinical examination constitutes a non-invasive approach and teratogenesis might be a suitable indicator of external aggressors. This physical monitoring is not expensive and easily applicable in the field to evaluate impacts of agricultural practices.

ACKNOWLEDGMENTS

We thank D. Fontaine, Y. Bachand, R. Dauphin, and J. Boulé for their assistance in the field. We are grateful to N. Plante (Canadian Wildlife Service) for assistance with statistical analyses, G. J. Crawshaw (Metropolitan Toronto Zoo) and D. Martineau (Université de Montréal)for helpful comments, and to J. A. Bantle (Oklahoma State University) for useful information. We also thank D. M. Green (McGill University) for the use of his laboratory. This study was supported by the Canadian Wildlife Service, Québec Region.

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Received for publication 29 January 1996.