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PREVALENCE OF SKELETAL AND EYE MALFORMATIONS IN FROGS FROM NORTH-CENTRAL UNITED STATES: ESTIMATIONS BASED ON COLLECTIONS FROM RANDOMLY SELECTED SITES

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ABSTRACT: Skeletal malformation rates for several frog species were determined in a set of randomly selected wetlands in the north-central USA over three consecutive years. In 1998, 62 sites yielded 389 metamorphic frogs, nine (2.3%) of which had skeletal or eye malformations. A subset of the original sites was surveyed in the following 2 yr. In 1999, 1,085 metamorphic frogs were collected from 36 sites and 17 (1.6%) had skeletal or eye malformations, while in 2000, examination of 1,131 metamorphs yielded 16 (1.4%) with skeletal or eye malformations. Hindlimb malformations predominated in all three years, but other abnormalities, involving forelimb, eye, and pelvis were also found. Northern leopard frogs (*Rana pipiens*) constituted the majority of collected metamorphs as well as most of the malformed specimens. However, malformations were also noted in mink frogs (*R. septentrionalis*), wood frogs (*R. sylvatica*), and gray tree frogs (*Hyla* spp.). The malformed specimens were found in clustered sites in all three years but the cluster locations were not the same in any year. The malformation rates reported here are higher than the 0.3% rate determined for metamorphic frogs collected from similar sites in Minnesota in the 1960s, and thus, appear to represent an elevation of an earlier baseline malformation rate.

Key words: Amphibian, anuran, broadleaf forest ecoregion, malformation, survey.

INTRODUCTION

Over the past several years, a number of malformed frogs and toads have been discovered in the USA and Canada; malformations have been reported in specimens of 54 amphibian species in 44 USA states and four Canadian provinces since 1995 (Johnson et al., 2000; and see also North American Reporting Center for Amphibian Malformations, 2002). Normal amphibian populations, like those of other vertebrates (Gilbert, 2000), appear to accommodate a low number of malformed individuals. Reports in the literature support the concept of a baseline malformation rate, which has been documented in several large-scale population studies (Merrell, 1969; Meyer-Rochow and Asashima, 1988; Gray, 2000), as well as in numerous reports detailing spectacular anomalies such as ectopic or supernumerary legs (reviewed by Van Valen, 1974 and Ouellet, 2000). Occasionally, acute malformation outbreaks occur in which an isolated population has a very high malformation rate (Reynolds and Stephens, 1984; Volpe and Rosenbaum, 2000). These usually involve a single species and last for just one generation (Van Valen, 1974; Ouellet, 2000). Many of the current reports fall within the acute category. However, in contrast to historical reports, the current observations are characterized by malformed animals among multiple species at a single site, multiple sites with high malformation prevalence spread over wide areas, and recurrence of malformed animals in the same site in successive years (Ouellet et al., 1997; Gardiner and Hoppe, 1999; Johnson et al., 1999; Converse et al., 2000; Helgen et al., 2000; Hoppe, 2000). Malformation rates at some sites can be very high. Johnson et al. (1999) reported an average of 12.3% malformed *Hyla regilla* from 13 sites and Helgen et al. (2000) sampled several sites that yielded 40–65% malformed *Rana pipiens*, while matching

reference sites yielded no greater than 1% malformed specimens. Ouellet et al. (1997) matched 14 agricultural sites, in which an average of 12% of the frogs had malformations, with 12 non-agricultural sites, in which they found only 0.7% malformed specimens. Unfortunately, little is known about the causes of the malformations or whether acute malformation outbreaks represent transient elevations of the baseline rate or whether the two have different etiologies.

While malformation rates at a single site might be estimated using a well designed sampling study, valid baseline malformation rates for populations on local or regional scales can be difficult to glean from studies not specifically designed for that purpose. For instance, abnormalities generated through injury or disease must be distinguished from true developmental errors. Also, since relatively few malformed adults are seen (Helgen et al., 2000), studies that do not distinguish between adults and metamorphs may underestimate the true malformation rate. Despite these limitations, some information on historic malformation rates is available. By close examination of preserved specimens, Hoppe (2000) determined a 0.3% malformation rate for 2,166 northern leopard frog (*Rana pipiens*) metamorphs collected from known sites in central Minnesota, USA in the 1960s. Malformations in this group included ectrodactyly (missing digits), syndactyly (fused digits), polydactyly (multiple digits), and ectromely (missing limbs or limb segments) all of which are common in the current phenomenon. The study reported here addresses the issue of the current background malformation rates using random site selection and a systematic collection protocol in wetlands in the north-central region of the US.

MATERIALS AND METHODS

Study area and site selection

The study area is encompassed by the broadleaf forest ecoregion (Bailey, 1983) which spans northeastern Illinois, southern Wisconsin, and southern Minnesota (USA; Fig. 1). Current land cover within this region ranges from forest to intensive agricultural or urban uses. Site selection was conducted using a stratified random design. We randomly selected fourth order watersheds from the broadleaf forest ecoregion. Wetlands within the selected watersheds were mapped using National Wetland Inventory data. To capture the available gradient of conditions available within each watershed, we selected individual wetlands from regions representing a spectrum of land use intensity (e.g., less disturbed to most disturbed). Field reconnaissance was conducted to select four to eight wetlands in each watershed. Individual sample sites were also subject to the following criteria: 1) classification as a palustrine aquatic bed or emergent wetland (Cowardin et al., 1979), 2) landowner permission, and 3) evidence that the site would remain under a consistent management regime for the duration of the study. We refer to groups of sites within watersheds as wetland clusters (WC), which are identified by wetland cluster and site numbers (e.g., WC 1, site 1=1.1; see Fig. 1 and Table 1). A total of 62 sites in 13 wetland clusters were selected and sampled in 1998. In 1999 and 2000 the objectives of the larger project that encompassed this malformation assessment required the selection of 36 sites within a smaller region. This set was constructed using 18 of the existing sites and 18 additional sites, 10 of which were associated with the existing wetland clusters (WC 9–13), and eight of which were additional sites adjacent to, but not contiguous with the original watersheds. This set of eight additional sites was designated as WC 14.

Field methods

Each site was visited three times during the spring and summer (March–April, May–June, and late July–August) to gather physical, chemical, and biological data. During the July–August visit, attempts were made to capture metamorphosing frogs at all sites. A minimum effort of two person-hr was expended searching for frogs at each site that was not completely dry. If no frogs were captured during that time, the site was abandoned. At each site where frogs were found, an effort of approximately 16 person-hr was expended for collection and analysis. During 1998, the animals were measured and examined in the field, and representative specimens, including malformed individuals and randomly selected reference specimens, were collected for laboratory analyses. Frogs were euthanized by immersion in 1% MS 222 (3-amino benzoic acid ethyl ester) and stored in 10% neutral buffered formalin. Only meta-

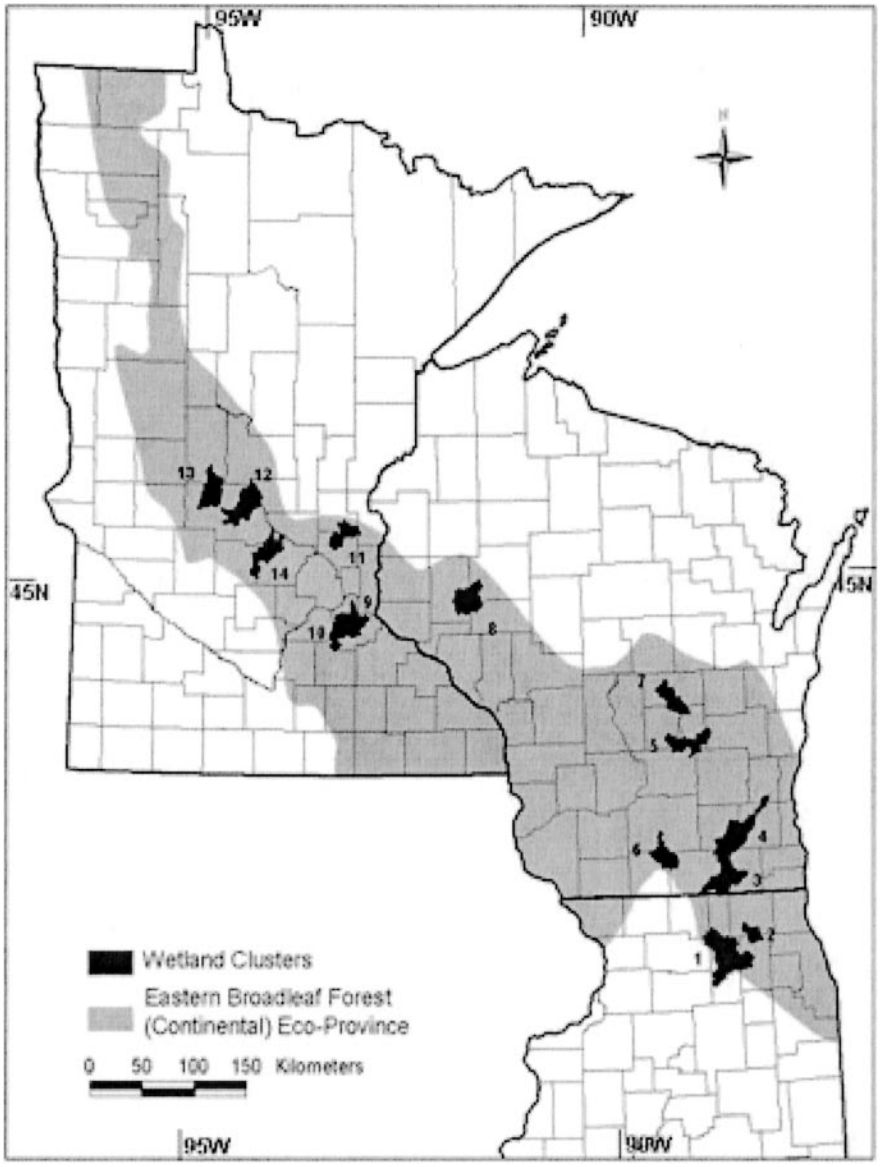

FIGURE 1. Amphibian malformation study area. The study area included 78 wetland sites contained in 14 spatial clusters distributed through southern Minnesota, Wisconsin, and northern Illinois.

morphic animals with emerged forelimbs (Gosner stage 42–46 and beyond; Gosner, 1960) were included in the malformation survey and collection. Adults were not included because of the possibility that they could have migrated from other wetlands.

During the 1999 and 2000 collections, the field researchers endeavored to capture 100 metamorphic frogs; the first 40 frogs collected from each site were distributed between the National Wildlife Health Center (Madison, Wisconsin, USA) and the University of Illinois College of Veterinary Medicine (Urbana, Illinois, USA), for malformation assessment, as well as gross and histologic pathology and parasitology studies (to be reported elsewhere). Any additional frogs captured were examined in the field. Malformed frogs were kept for laboratory analysis and the remaining specimens were released.

Staining

Specimens were fixed in neutral buffered formalin or 70% ethanol. Cartilage and bone

were stained with alcian blue and alizarin red (Kelly and Bryden, 1983) with enzymatic tissue clearing (Dingerkus and Uhler, 1977). Cleared specimens were stored in glycerin.

RESULTS

1998 collections

A total of 389 metamorphic anurans from seven species were examined in the field (Table 1). The majority were *R. pipiens* and *R. clamitans,* with *R. septentrionalis, R. sylvatica, R. catesbeiana, Hyla* spp. (the gray tree frog, *H. versicolor,* and Cope's gray tree frog, *H. chrysoscelis* are distinguishable in the field only by their call), and *Bufo americanus* represented in much lower numbers (Table 2). Fifty-four frogs, including malformed and reference specimens, were euthanized and returned to the lab for skeletal analysis. After clearing and staining, nine (2.3%) frogs were identified with malformations (Table 2), which included anophthalmia, ectromely, ectrodactyly, taumely (a bony triangle, in which a long bone, such as the femur or tibiafibula, is bent in the middle, such that the heads of the bone are apposed), polymely, and polydactyly (Table 2). Two frogs that were considered normal in the field (i.e., reference specimens) were found by bone staining to have asymmetric pelvises, in which the left ilial shaft was longer than the right, resulting in torsion of the lower trunk. Both of these animals came from the same wetland cluster (WC 4; Table 1), although not from the same site. This subtle malformation was not seen in cleared and necropsied specimens in the 1999 or 2000 collections (Tables 3 and 4).

1998 distribution

Metamorphic frogs were collected in 45 of the 62 sites sampled in 1998 (Table 1). Six sites contained only adult or larval forms and 11 sites yielded no frogs during the collection period. The nine malformed specimens were distributed among seven sites, with six sites having one malformed specimen and one site having three malformed specimens. Four of the nine malformed specimens came from one wetland cluster (WC 11), and another wetland cluster contained two malformed specimens (WC 4; Table 1).

1999 collections

Metamorphic frogs were collected from 30 of the 36 sites sampled in 1999 (Fig. 1, Table 1). A total of 1,085 metamorphic ranid frogs were examined in the field or lab for skeletal malformations (Table 3). Of these, 267 were examined only in the field, 306 were cleared and stained, and 512 were subjected to necropsy, which involved histopathologic examination of soft tissue (to be reported elsewhere). Skeletal malformations were found in 17 (1.6%) frogs, including abnormalities in the limbs, mandible, pelvis, and spine (Table 3). Bone staining revealed several instances of unique or rarely reported skeletal malformations. One *R. pipiens* had a severely malformed coccyx, which appeared to be in several separate pieces at the articulation with the spine. Several (13) other *R. pipiens* had less severe variations in normal coccygeal form and position, but, since these did not appear to produce torsion or mispositioning of the pelvis or legs, they were considered to be variations in normal growth and not malformations. Several specimens also had asymmetric vertebrae and ribs, which, in one case, was severe enough to produce scoliosis.

1999 distribution

Malformed frogs were found at 11 sites, with rates ranging from 0.8–8.8% by site (Table 1). Seven sites produced a single malformed specimen; two sites produced two malformed specimens; and two sites produced three malformed specimens. Malformed frogs tended to be grouped in particular sites and wetland clusters. Specifically, sites 64–68, which are all within WC 13, produced 53% of the specimens with skeletal malformations. Only one other site (site 44, WC 10) yielded multiple malformed specimens. These two geo-

TABLE 1. Amphibian metamorph collections and prevalence of malformations by site and year; 1998–2000.

Site[a] number	Identification	1998		1999		2000	
		Prev[b]	(Malf[c]/total[d])	Prev[b]	(Malf[c]/total[d])	Prev[b]	(Malf[c]/total[d])
1	1.1	0	(0/2)	—	—	—	—
2	1.2	0	(0/3)	—	—	—	—
3	1.3	—	(0*)	—	—	—	—
4	1.4	—	(0*)	—	—	—	—
5	1.5	0	(0/8)	—	—	—	—
6	2.1	—	(0*)	—	—	—	—
7	2.2	0	(0/2)	—	—	—	—
8	2.3	—	(0*)	—	—	—	—
9	2.4	0	(0/1)	—	—	—	—
10	3.1	—	(0**)	—	—	—	—
11	3.3	0	(0/3)	—	—	—	—
12	3.4	0	(0/1)	—	—	—	—
13	3.5	0	(0/4)	—	—	—	—
14	4.1	25	(1/4)	—	—	—	—
15	4.2	0	(0/1)	—	—	—	—
16	4.3	13	(1/8)	—	—	—	—
17	4.4	0	(0/6)	—	—	—	—
18	4.5	0	(0/27)	—	—	—	—
19	4.6	0	(0/16)	—	—	—	—
20	4.7	0	(0/12)	—	—	—	—
21	4.8	0	(0/16)	—	—	—	—
22	5.1	17	(1/6)	—	—	—	—
23	5.2	0	(0/39)	—	—	—	—
24	5.3	—	(0*)	—	—	—	—
25	5.4	0	(0/14)	—	—	—	—
26	6.1	—	(0*)	—	—	—	—
27	6.2	0	(0/15)	—	—	—	—
28	6.3	0	(0/7)	—	—	—	—
29	6.4	—	(0**)	—	—	—	—
30	7.1	0	(0/4)	—	—	—	—
31	7.2	—	(0*)	—	—	—	—
32	7.3	—	(0*)	—	—	—	—
33	7.4	0	(0/2)	—	—	—	—
34	8.1	0	(0/1)	—	—	—	—
35	8.2	9	(1/11)	—	—	—	—
36	8.3	0	(0/8)	—	—	—	—
37	8.4	0	(0/1)	—	—	—	—
38	8.5	—	(0**)	—	—	—	—
39	9.1	—	(0*)	—	(0*)	—	(0*)
40	9.2	0	(0/5)	0	(0/5)	1.7	(2/117)
41	9.3	0	(0/5)	—	—	—	—
42	9.4	—	(0*)	0	(0/9)	—	(0*)
43	9.5	0	(0/6)	4	(1/23)	0	(0/7)
44	10.1	—	(0**)	6	(3/47)	0	(0/104)
45	10.2	0	(0/3)	—	—	—	—
46	10.3	0	(0/6)	0	(0/8)	—	(0*)
47	10.4	0	(0/13)	0	(0/4)	0	(0/1)
48	11.1	—	(0*)	—	—	—	—
49	11.2	25	(1/4)	0	(0/1)	0	(0/10)
50	11.3	0	(0/1)	—	(0*)	0	(0/2)
51	11.4	27	(3/11)	0	(0/13)	—	(0*)
52	11.5	0	(0/16)	—	(0*)	0	(0/33)

TABLE 1. Continued.

Site[a] number	Identification	1998		1999		2000	
		Prev[b]	(Malf[c]/total[d])	Prev[b]	(Malf[c]/total[d])	Prev[b]	(Malf[c]/total[d])
53	11.6	—	(0**)	—	(0*)	—	(0*)
54	11.7	0	(0/16)	0	(0/2)	0	(0/4)
55	11.8	—	—	0	(0/9)	—	(0*)
56	11.9	—	—	0	(0/41)	2	(2/92)
57	12.2	0	(0/11)	—	—	—	—
58	12.3	0	(0/27)	0	(0/51)	0	(0/142)
59	12.4	—	(0**)	0	(0/1)	—	(0*
60	12.5	—	—	0	(0/54)	1.8	(2/113)
61	12.6	—	—	0	(0/48)	3.3	(7/210)
62	12.7	—	—	0	(0/45)	0	(0/39)
63	12.8	—	—	—	(0*)	—	(0*)
64	13.1	0	(0/1)	7	(1/14)	100	(1/1)
65	13.2	0	(0/6)	2	(2/86)	0	(0/1)
66	13.3	0	(0/4)	1.7	(2/118)	0	(0/1)
67	13.4	0	(0/12)	0.8	(1/123)	—	(0*)
68	13.5	5	(1/20)	9	(3/34)	2	(1/42)
69	13.6	—	—	—	(0*)	—	(0*)
79	13.7	—	—	0	(0/2)	—	(0*)
71	13.8	—	—	0	(0/29)	0	(0/2)
72	14.1	—	—	2	(1/65)	0	(0/1)
73	14.2	—	—	0	(0/18)	0	(0/14)
74	14.4	—	—	2	(1/42)	—	(0*)
75	14.5	—	—	2	(1/47)	0.9	(1/111)
76	14.6	—	—	1	(1/86)	0	(0/15)
77	14.7	—	—	0	(0/48)	0	(0/60)
78	14.8	—	—	0	(0/12)	0	(0/9)
Totals		2.3	(9/389)	1.6	(17/1,085)	1.4	(16/1,131)

[a] Site identification refers to wetland cluster (WC 1–14), followed by the individual site within that cluster. A total of 78 sites in 14 wetland clusters were sampled during the 3 yr of the study. 1998: 62 sites (1.1–13.5); 1999 and 2000: 36 sites (9.1–14.8).

[b] Prevalence; percent of collected individuals with skeletal malformations.

[c] Number of malformed metamorphic frogs collected/total number collected; 0* = collection attempted but no frogs captured; 0** = only larvae and adults collected; — = no collection attempted.

[d] Skeletal and eye malformations only.

graphically separated clusters accounted for 70% of the malformed frogs.

2000 collections

The 36 sites that were sampled in 1999 were sampled again in 2000. A total of 1,131 metamorphic frogs were collected from 24 sites (Table 1). Of these, 478 were examined for malformations in the field and returned and 653 were subjected to necropsy. Sixteen (1.4%) were determined to be malformed. As observed in previous years, the majority of malformations occurred in the hindlimbs (Table 4), however, five specimens with eye malformations were also collected.

2000 distribution

Seven of the 24 sites that yielded frogs had at least one malformed frog (Table 1). As observed in previous years, the malformations were clustered, with two sites (60 and 61, WC 12) contributing nine of the 16 malformed specimens.

Annual variation

Frog captures were highly variable among sites within the same year. Of the 62 sites sampled in 1998, 11 yielded no frogs and 35 yielded fewer than 10 metamorphic frogs during the collection period. In 1999, 15 of the 36 sites yielded zero to nine metamorphic frogs, and in 2000,

TABLE 2. Summary of malformations in anurans collected in 1998.

Species	n[a]	Malf[b]	Prev[c] (%)	Malformation type[d] and (number)[e]
Rana pipiens	232	6	2.6	pelvic asymmetry (2), polydactyly (2), anophthalmia (1) ectromely (1), polymely (1), taumely (1)
R. clamitans	95	1	1	brachydactyly and brachymely
R. septentrionalis	22	0	0	
R. sylvatica	10	0	0	
R. catesbeiana	1	0	0	
Hyla spp.	13	2	10	bilateral amely (1), brachydactyly and brachymely (1)
Bufo americanus	4	0	0	
unknown	12	0	0	
Total	389	9	2.3	

[a] Number of metamorphic frogs collected.
[b] Skeletal and eye malformations.
[c] Prevalence; percentage of specimens malformed in sample.
[d] All limb malformations occurred in the hindlimbs, unless otherwise noted; pelvic asymmetry-ilial shaft longer on one side; polydactyly-multiple digit; anophthalmia-missing eye; ectromely-missing limb or segment; polymely-multiple limbs; taumely-bony triangle, where long bones are bent such that the proximal and distal ends are apposed and the middle forms the apex of a triangle; brachydactyly-reduced metatarsal or phalanges; brachymely-reduced limb segment; amely-absence of hindlimb and acetabulum.
[e] Some specimens had multiple anomalies.

22 of 36 sites yielded zero to nine frogs (Table 1). Also, there appeared to be little consistency in the number of metamorphic frogs captured in the same site from year to year, even though the collecting team was similar in all years. Although some sites produced relatively high numbers of frogs in 1999 and 2000 (e.g., WC 12), most others showed no pattern. In addition, the distribution of malformed frogs was not consistent in the three collection years. Of the 36 sites that were surveyed in both 1999 and 2000, 15 contained malformed specimens (Table 1). However, only three sites contained malformed frogs in both years, and of these, only one had multiple malformed frogs in a single year (site 13.5, 1999). Twenty sites were sampled in all 3 yr. Two of these sites yielded no frogs and three sites yielded fewer than 10 total frogs across all 3 yr. Even though the 22 malformations from these sites represented over half of the total malformations observed during the 3 yr, only one site (13.5)

TABLE 3. Summary of malformations in anurans collected in 1999.

Species	n[a]	Malf[b]	Prev[c] (%)	Malformation type[d] and (number)[e]
Rana pipiens	931	13	1.4	brachydactyly (3), forelimb brachydactyly (1), brachygnathia (2), brachymely (1), craniofacial (1), ectrodactyly (1), ectromely (1), polymely (1) pelvic (1), polydactyly (1)
R. septentrionalis	119	3	2.5	brachydactyly (1), ectromely (1), spinal (1)
R. clamitans	23	1	4	brachymely with cutaneous fusion
R. sylvatica	12	0	0	
Total	1,085	17	1.6	

[a] Number of metamorphic frogs collected.
[b] Skeletal and eye malformations.
[c] Prevalence; percentage of specimens malformed in sample.
[d] All limb malformations occurred in the hindlimbs, unless otherwise noted; brachydactyly-reduced metatarsal or phalanges; brachygnathia-reduced mandible; brachymely-shortened limb; craniofacial-anomalous zygomatic arch; ectrodactyly-missing digit, including metatarsal; ectromely-missing limb or segment; polymely-multiple limbs; pelvic anomalous pelvic development; polydactyly-multiple toes; spinal-abnormality causing scoliosis; cutaneous fusion-skin joined over two limb segments.
[e] Some specimens had multiple anomalies.

TABLE 4. Summary of malformations in anurans collected in 2000.

Species	n[a]	Malf[b]	Prev[c] (%)	Type[d] and (number)[e]
Rana pipiens	1,107	15	1.4	anophthalmia (5), brachymely (2), ectromely (2), forelimb ectromely (1), bony projection (1), brachydactyly (1), ectrodactyly (1), lateral asymmetry (1), pelvic (1), polymely (1)
R. clamitans	21	0	0	
R. septentrionalis	3	1	33	brachydactyly (1)
Total	1,131	16	1.4	

[a] Number of metamorphic frogs collected.
[b] Skeletal and eye malformations.
[c] Prevalence; percentage of specimens malformed in sample.
[d] All limb malformations occurred in the hindlimbs, unless otherwise noted; anophthalmia-missing eye; brachymely-reduced limb; ectromely-missing limb or segment; bony projection-anomalous appendage, in this case originating in the pelvic area; brachydactyly-reduced metatarsal or phalanges; ectrodactyly-missing digit, including metatarsal; lateral asymmetry-one half of body smaller than the other; pelvic-anomalous pelvic development; polymely, multiple limbs.
[e] Some specimens had multiple anomalies.

produced malformed specimens in each of the 3 yr.

Severity

Malformations were categorized as "severe" (Table 5) if they were estimated to cause a major reduction in fitness. On this basis, minor skeletal malformations, such as missing or reduced phalanges, were not considered severe. Similarly, abnormal coloration noted on the hindlimb skin would probably not impair fitness, although it is recognized that skin color pattern could, under certain circumstances, be relevant to mate selection and susceptibility to predation.

TABLE 5. Summary of severe malformations found in anurans collected in 1998–2000.

		Malformations			
Year	Number of frogs	Total malf[a]	Prev (%)[b]	Severe[c]	Prev[b] (%)
1998	389	9	2.3	6	1.5
1999	1,085	17	1.6	11	1.0
2000	1,131	16	1.1	13	1.1
Total	2,605	42	1.6	30	1.2

[a] Skeletal and eye malformations.
[b] Prevalence; percentage of specimens malformed in sample.
[c] Severity was based on an estimation of the effect of the malformation on fitness.

DISCUSSION

Comparisons of current and historical malformation rates

Gray (2000) reported that in a set of over 10,000 cricket frogs (*Acris crepitans*) collected in Illinois between 1968 and 1971, 0.4% were abnormal. The majority of these were missing limbs or limb segments which could have resulted from either developmental malformations or traumatic amputations. A survey of Illinois cricket frogs in the early 1990s also identified only one skeletal malformation in 341 animals (Reeder et al., 1998). These studies suggest that, either *A. crepitans* is resistant to etiologic agents causing malformations among other anurans or that the causative factor(s) were not widely distributed during that time. Hoppe (2000) conducted a retrospective analysis of preserved *R. pipiens* metamorphs collected in central Minnesota between 1958 and 1963 in which malformations were distinguished from traumatic deformations on the basis of scar tissue formation. Six of 2,166 frogs (0.3%) had malformations, including hindlimb ectrodactyly, syndactyly, and ectromely, as well as three cases of forelimb ectromely, syndactyly, and polydactyly. Notably absent from these studies were cutaneous fusions and eye and jaw

malformations which were common malformations in more recent observations of metamorphic *R. pipiens* from the same area (this study; Hoppe, 2000), as well as the bony triangles and ectopic bones found in this and other recent studies (Gardiner and Hoppe, 1999). Thus, if these 1960s collections were unbiased, the available data suggest that baseline malformation rates for *R. pipiens* and *A. crepitans* in the north-central region of the USA was 0.3 to 0.4% 30 yr ago and that there may be both qualitative and quantitative differences in the malformation types currently observed. Of 2,605 metamorphic frogs collected over 3 yr in the current study, 42 (1.6%) had skeletal or eye malformations, representing a four-to five-fold increase over the historic background prevalence reported for this region.

Causes of malformation

Although no single cause has been found to explain all amphibian malformations, three major hypotheses, each with limited theoretical and empirical support, have emerged. These are: trematode parasites, chemical mimics of retinoic acid, and ultraviolet-B radiation (UV-B). Ankley et al. (1998, 2000) showed that *R. pipiens* embryos and larvae exposed to artificial or natural UV-B developed extensive limb malformations, consisting almost exclusively of hindlimb deletions or reductions. Militating against the UV hypothesis is the fact that only limb deletions and truncations were produced which does not reflect the current situation in the field where *R. pipiens* and other ranids are found with a wide variety of malformations in limb and other tissue.

A second hypothetical cause of malformations is exposure to natural or anthropogenic chemicals that act as retinoids which are potent regulators of development in the vertebrate brain, eye, mandible, and limbs. Although essential for proper development, retinoids can be teratogenic if applied at inappropriate times or in abnormal concentrations. For example, retinoic acid produced duplicate limbs when applied directly to developing frog limb buds (Bryant and Gardiner, 1992), or regenerating tails (Maden, 1993), and produced deletions when applied to regenerating axolotl limbs (Scadding and Maden, 1986a). Few studies have been performed with native frogs, but Degitz et al. (2000) established that retinoic acid caused hindlimb malformations in *R. sylvatica* that were similar to those seen in field specimens of *R. septentrionalis* (Gardiner and Hoppe, 1999). Significantly, these malformations included bony triangle malformations of the long bones of the leg which have also been observed in retinoic acid-treated *Xenopus* (Scadding and Maden, 1986b) and other vertebrates (Summerbell, 1983; Tickle et al., 1985). Although Ouellet et al. (1997) observed a correlation between malformed amphibians, agricultural land use, and implied exposure to chemical pesticides, no direct evidence for the presence of active retinoids has been established in the current malformation phenomenon.

The remaining hypothesis is that trematode parasites cause malformations when they physically or chemically disrupt the developing limb, jaw, or eye fields (Sessions et al., 1999). Experimental infections of *Hyla regilla* (Johnson et al., 1999) and *Bufo boreas* (Johnson et al., 2001) with cercariae from the trematode parasite *Ribeiroia* sp. produced multiple types of hindlimb malformations, including missing or shortened limbs as well as multiple digits and limbs. In addition, these infections caused the bony triangle formation in hindlimbs that has been noted as a retinoid effect (Gardiner and Hoppe, 1999) suggesting that the parasites may produce retinoids or stimulate retinoid-regulated pathways. Malformation induction was dependent on the parasite species because infection with *Alaria mustelae* cercariae did not result in abnormal limb development (Johnson et al., 1999). These experimental infections produced only hindlimb

and forelimb malformations and not the other forms that have been consistently found in wild populations. Also, even though *Ribeiroia* sp. has been found in some of our Minnesota study sites, none of the malformed frogs identified in our study harbored these metacercariae (Schotthoefer, data not shown). The apparent lack of *Ribeiroia* sp. in malformed individuals indicates that either trematode parasites other than *Ribeiroia* sp. can cause malformations, that *Ribeiroia* cercariae are cleared from the frogs, or that other mechanisms are at work.

Malformation and declining amphibian populations

A recent analysis documented the decline in amphibian populations over the last several decades and also showed that the phenomenon exists on a global scale (Houlahan et al., 2000). Despite the fact that malformations appear to occur at scales that would not contribute significantly to population declines (Carey, 2000) the increased malformation prevalence and declining populations are occurring in the same time frame, which may be more than coincidental. Many population reductions can be associated with habitat destruction, degradation, fragmentation, and contamination (Reeder et al., 1998; Beasley et al., 2002) but others appear to be occurring in areas less affected by human influences (Berger et al., 1998). Infectious diseases have been implicated as the proximate cause of declines or extinctions in some areas (for review, see Dazsak et al., 1999). Diseases caused by a chytrid fungus (Berger et al., 1998) and by *Saprolegnia ferax* (Kiesecker et al., 2001) have contributed to widespread amphibian deaths in areas described as ecologically pristine. Other diseases implicated in recent decimation of amphibian populations include *Aeromonas hydrophila,* the bacterium responsible for red leg disease (Cunningham et al., 1996) and iridoviruses (Bollinger et al., 1999). Additional stressors include introduced predators or competitors (Corn, 2000), increased temperatures and other deleterious aspects of climate change (Pounds et al., 1999), and increased exposure to UV-B, which has been suggested to reduce reproductive success and survival of amphibians at high altitudes (Blaustein et al., 1994).

Biotic stressors may cause amphibian malformations and declining populations directly (Blaustein et al., 1994; Ankley et al., 2000), or they may contribute indirectly by suppressing immune competence (Carey et al., 1999), or by increasing susceptibility to pathogens. Thus, the apparent concurrence of amphibian population declines and elevated malformation rates in the preceding 40 yr may be coincidental or the two phenomena may have responded to changing environmental conditions. Anthropogenic factors, such as elevated temperature and pH, widespread exposure to chemical toxicants, and increased exposure to ambient UV radiation, may combine to reduce reproductive success, increase susceptibility to disease, and suppress behavioral and immunologic defenses against pathogens. Long-term studies of amphibian population dynamics and the occurrence and causes of malformations will be necessary to determine whether the factors that contribute to high malformations rates also contribute significantly to health problems of amphibians that, in turn, influence population sustainability on local, regional, and global scales.

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