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Hind Limb Malformations in Free-Living Northern Leopard Frogs (*Rana pipiens*) From Maine, Minnesota, and Vermont Suggest Multiple Etiologies

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ABSTRACT

Background: Reports of malformed frogs have increased throughout the North American continent in recent years. Most of the observed malformations have involved the hind limbs. The goal of this study was to accurately characterize the hind limb malformations in wild frogs as an important step toward understanding the possible etiologies.

Methods: During 1997 and 1998, 182 recently metamorphosed northern leopard frogs (*Rana pipiens*) were collected from Minnesota, Vermont, and Maine. Malformed hind limbs were present in 157 (86%) of these frogs, which underwent necropsy and radiographic evaluation at the National Wildlife Health Center. These malformations are described in detail and classified into four major categories: (1) no limb (amelia); (2) multiple limbs or limb elements (polymelia, polydactyly, polyphalangy); (3) reduced limb segments or elements (phocomelia, ectromelia, ectrodactyly, and brachydactyly; and (4) distally complete but malformed limb (bone rotations, bridging, skin webbing, and micromelia).

Results: Amelia and reduced segments and/or elements were the most common finding. Frogs with bilateral hind limb malformations were not common, and in only eight of these 22 frogs were the malformations symmetrical. Malformations of a given type tended to occur in frogs collected from the same site, but the types of malformations varied widely among all three states, and between study sites within Minnesota.

Conclusions: Clustering of malformation type suggests that developmental events may produce a variety of phenotypes depending on the timing, sequence, and severity of the environmental insult. Hind limb malformations in free-living frogs transcend current mechanistic explanations of tetrapod limb development.

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INTRODUCTION

The dramatic increase in reports of malformed frogs in recent years has drawn the attention of scientists and the public. Malformed frogs have been documented in 43 states, in 38 species of frogs, and 19 species of toads, with estimates as high as 60% in some local populations (NARCAM, '99). Some controversy exists over the extent to which these malformations occur in a healthy population, with most estimates at 2% or less (Ouellet et al., '97; Gardiner and Hoppe, '99; Sessions et al., '99). Scientists agree, however, that current numbers of reported malformations exceed any norm and that the situation warrants urgent attention.

Limb development is one of the most thoroughly studied aspects of vertebrate ontogeny. The molecular mechanisms guiding these processes in vertebrate limbs have been and are the subject of intense and

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illuminating research (Johnson and Tabin, '97; Zeller and Duboule, '97; Martin, '98; Pearse and Tabin, '98; Schwabe et al., '99). Three signaling centers have been described that direct development of the three limb axes. Very briefly, the proximal-distal axis is dependent on the apical ectodermal ridge (AER) through the actions of fibroblast growth factor (FGF) family members on the underlying bud mesoderm (reviewed in Martin, '98). The anteroposterior axis is controlled by the zone of polarizing activity (ZPA) through the activity of Sonic hedgehog (reviewed in Pearse and Tabin, '98). The limb bud ectoderm guides the dorsoventral axis through the activities of Wnt7a and Engrailed-1 (reviewed in Chen and Johnson, '99). The possibility of interdependence of these signaling centers for maintenance of gene expression and function is currently being explored.

There is every reason to believe that this general outline of the control of limb axial specification and realization is true for all reptiles, birds and mammals. Similar information for amphibians is now becoming available. The most information is available for *Xenopus laevis*, where it is clear that a ZPA synthesizing Sonic hedgehog exists (Endo et al., '97). Similarly, a group of cells at the apex of the *X. laevis* limb bud synthesizes FGF8 (Christen and Slack, '97). This is of importance because the morphological ridge in *X. laevis* is only transient (Tarin and Sturdee, '71, '74), but evidence is building that the apical epithelium is the functional correlate of the amniote AER in anurans and urodeles, regardless of its structure. The biology of the dorsoventral axis in amphibian limbs has not been reported.

A notable and important result of the molecular understanding of limb patterning is the elucidation of the molecular basis of limb mutations in birds and mammals (including humans). For example, polydactyly in the mouse is due to a mutation in the Gli3 gene that permits ectopic Sonic hedgehog expression in the preaxial limb bud mesoderm (Schimmang et al., '92; Hui and Joyner, '93). Teratogens can also be interpreted in terms of the molecular models of limb development (Bell et al., '99). However, the complexity of malformations in free-living frogs suggests that multiple factors may be involved and the mechanisms are unclear. Scientists are attempting currently to identify potential teratogenic agents in the environments where malformations occur and exploring their potential role in this challenging problem. Pivotal to understanding malformations in field populations is accurate information regarding species affected and the diversity and distribution of malformation phenotypes. Therefore, the present study focused on the northern leopard frog (*Rana pipiens*), which is the species most commonly reported with malformations (Helgen et al., '98; NARCAM, '99). A thorough radiographic analysis was performed to study the anatomy of malformed limbs of 157 leopard frogs collected over a 2-year period from a large study area that included 16 sites in three states in the eastern and midwestern United States. The limb malformations were classified into four major categories with 16 subcategories, such that patterns in their phenotypes could be discerned. The detailed descriptions and classification presented here will contribute to the foundation for approaches designed to provide insights into the mechanisms responsible for the malformations in free-living frogs.

MATERIALS AND METHODS

Specimen collection

Newly metamorphosed northern leopard frogs with hind limb malformations were collected during frog surveys at two sites in Maine, eight sites in Minnesota, and five sites in Vermont. Collection sites included both perennial and ephemeral ponds on private agricultural land as well as ponds on nonagricultural private, state, and federal lands. We selected 10 sites in which the occurrence of malformations in preceding years was greater than 7% (high occurrence sites) and six paired sites with a history of 2% frog malformations (low occurrence sites). Malformed frogs were included in this report regardless of the site in which they were found. As no malformed frogs were found at the low-occurrence site in Maine, only the malformation data from the high-occurrence site in Maine is included in this report. Frogs were shipped alive in hard-sided coolers via overnight delivery to the National Wildlife Health Center (NWHC). Table 2 summarizes the total number of frogs submitted from each state. Table 4 summarizes the field data from the Minnesota collection sites reported in this article.

Specimen examination

Within 36 hr of arrival, frogs were examined and anesthetized by partial submersion in a 1:2,000 dilution of 3-amino benzoic acid ethyl ester (MS 222, Sigma Chemical Co., St. Louis, MO) in saline, neutralized with sodium bicarbonate. Once the frogs were nonresponsive to external stimuli, they were photographed and examined externally and internally. Euthanasia was performed by removal of the heart while the frogs were in a deep plane of anesthesia. Internal organs were removed for histopathology and microbiology. The carcass was then taped to plastic petri dishes with limbs in uniform orientation and submerged in 10% neutral buffered formalin for overnight fixation. Once removed from the petri dish, they were held in formalin for subsequent radiography. A full description and classification of malformations were carried out even on unique malformations assuming that even rare malformations could yield important developmental information and potentially represent a larger set of malformations that were either not observed or caused poor survival.

Radiography

All radiographs were taken to provide a ventral-dorsal (VD) view, using a Faxitron Specimen Radiography System model MX-20. Radiographs were ex-

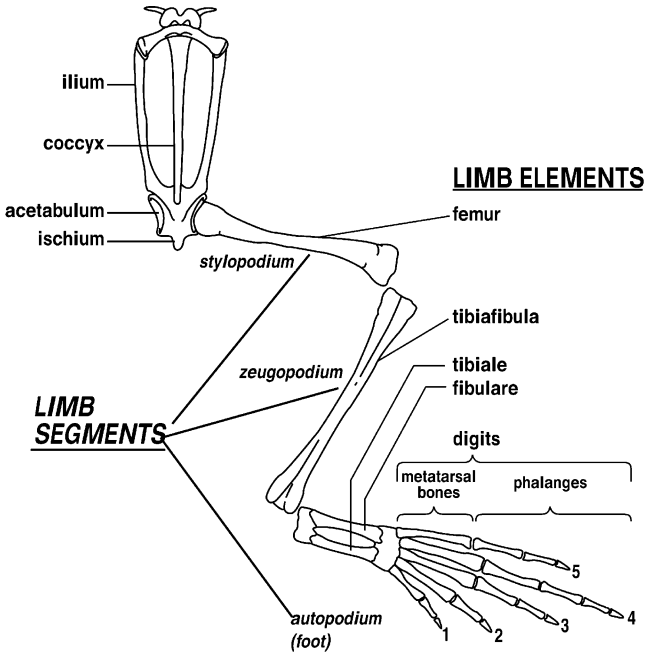

Fig. 1. Anatomy and terminology describing the frog limb. (Diagram adapted from Duellman and Trueb, '96.)

posed at 18 kV for 120 sec with 0.3 mA of continuous current. After initial radiographs without magnification, frogs with malformations of the pelvis and digits were radiographed at 4× magnification to define pelvic and digital structures more clearly. Oblique positioning was used to evaluate malformations that were obscured by overlying bone. The "R" on the radiographic images represents the right side of the body.

Definitions

Bone segments of the limb are the stylopodium, zeugopodium, and autopodium (Fig. 1). These terms are used interchangeably with femur, tibiafibula, and foot, respectively. Bone segments are composed of bone elements: the fused tibia and fibula (tibiafibula) make up the zeugopodium; the tibiale, fibulare, metatarsal bones, and phalanges comprise the autopodium. The limb pattern is defined as distally complete when at least one element of each segment (stylopodium, zeugopodium, autopodium) is present.

Where possible, the definitions and terms used in describing malformations are consistent with the terminology established by Wise et al. ('97). Amelia is defined as the absence of both limb and acetabulum. Polymelia refers to a supernumerary limb whether it originates near the pelvis or further distally on the stylopodium. The term "primary limb" refers to the normal limb in a frog with polymelia. Frogs with phocomelia have small, disorganized, unidentifiable, proximal bones to which are attached an abnormal foot which originates very close to the body. Ectromelia refers to reduced limb segments. Ectromelic limbs were classified on the basis of the affected limb segment. For example, ectromelia of the stylopodium refers to a limb that terminates anywhere along the femur, and ectromelia of the autopodium refers to termination of the tibiale and fibulare with no distal foot elements.

The digital patterns used in this report are based on anatomic features and relative position (Fig. 1). When the term digit is used to signify a specific toe, it is abbreviated D. When it refers to a specific bone, phalanx is abbreviated P. Digits 3 and 5 each have three phalanges of similar length and cannot be distinguished when they are duplicated or when the absence of adjacent digits precludes positional identification. Digits 1 and 2 each have two phalanges, but the bones in D2 are longer, providing criteria for identification. Digit 4 has four phalanges and can be identified even when other digits are abnormal. Where the reference points of digits were lost due to supernumerary or absent digits, the best-fitting patterns are presented. In a normal *R. pipiens* digit a small, curved terminal phalanx is the most distal bone. We therefore define a digit as distally complete when the terminal phalanx is present, regardless of the number of proximal phalanges. We define polydactyly as a supernumerary digit that has a duplicate metatarsal bone. Polyphalangy refers to a digit with one metatarsal bone at the tibiale/fibulare-metatarsal joint, but with duplicate sets of phalanges. Duplications resulting in polyphalangy may result from a mid-shaft split of the metatarsus, a mid-shaft split in the phalanx, or from duplication at a metatarsal-phalangeal or interphalangeal joint.

In this article, ectrodactyly and brachydactyly are distinguished. Ectrodactyly refers to the complete absence of a digit, including the metatarsal bone. Brachydactyly is defined as a normal number of metatarsal bones, but with reduced numbers of phalanges for that digit.

In a "complete but malformed limb," all segments and elements were present, but the limb was rotated, had a bone bridge, a skin web, or was micromelic. Although limbs with a skin web or bone bridge were rotated, a limb classified as "rotated" had severely twisted bone without bone bridging or skin webbing. A skin web is defined as band of skin crossing a joint; it does not include limbs with skin simply covering a bony abnormality such as a bone bridge. A limb with a skin web could also have other malformations, often of the autopodium. A bone bridge is the bone structure that spans the space between the adjacent cortices of a bent long bone (see Fig. 8).

Malformations did occur in both hind limbs in some frogs but were not always in the same category in our classification system. Bilateral malformations refer, for example, to amelia of the left limb and ectrodactyly of the right limb. Malformations were termed bilaterally symmetrical if the malformation in each hind limb was in the same category. An example of a bilaterally symmetrical malformation is ectromelia of both the left and right femurs.

Terms that refer to orientation of anatomical structures are consistent with classical definitions. The

TABLE 1. Hind limb malformations of *Rana pipiens* examined at the NWHC in 1997 and 1998

Malformation	Figures	Left	Right	Both	Total no. of limbs
I. No limb					
A. Amelia	2A–D	6	5	0	11
II. Multiple limb segments					
A. Polymelia	3A–F	2	4	2 (pairs)	10
III. Multiple limb elements					
A. Polydactyly (had multiple tibiale or fibulare)	5A	1	0	0	1
B. Polyphalangy	5B	2	2	0	4
C. Polydactyly with polyphalangy	5C–D	3	1	0	4
IV. Reduced limb segments					
A. Phocomelia	6	0	2	0	2
B. Ectromelia					
1. Femur (stylopodium)	7A–B	6	9	1	17
2. Tibiafibula (zeugopodium)	7C–E, 8C	11	14	1	27
3. Tibiale Fibulare (autopodium)	7E–F	3	8	1	13
V. Reduced limb elements					
A. Missing tibiale or fibulare	8D	5	2	0	7
B. Missing tibiale and fibulare	4D	1	2	0	3
C. Ectrodactyly		1	1	1	4
D. Brachydactyly	8F	5	4	1	11
E. Brachydactyly with ectrodactyly	4A–B, 8D	10	12	1	24
VI. Mixed digit patterns					
A. Brachydactyly with polyphalangy	4C	1	2	0	3
B. Ectrodactyly with brachydactyly and polyphalangy	4A, 5E	2	2	0	4
C. Brachydactyly with polydactyly and polyphalangy	4F	1	0	0	1
VII. Distally complete but malformed limbs.					
A. Rotation	9A	0	0	4	8
B. Bone bridging	8A–F	7	6	0	13
C. Skin web	9B	15	13	3	34
D. Micromelia		2	0	0	2
Total:		87	89	16	203

NWHC, National Wildlife Health Center.

terms preaxial and postaxial apply to the embryonic limb bud as it emerges from the lateral plate of the body wall and refer to either side of an axis that runs through the center of the limb in the proximal-distal direction. As the embryonic limb develops in the tadpole, the postaxial aspect of the limb bud is oriented dorsally and the axial aspect is ventral. The apex of the developing limb bud points caudally, toward the tail tip. During metamorphosis, the foot rotates so that toes are directed toward the head. After metamorphosis and limb rotation, the preaxial aspect of the limb comes to lie in the medial plane adjacent to the body and the postaxial aspect lies in the lateral plane most distant from the body. Thus, in the post-metamorphic frog, the clinical terms "medial" and "lateral" replace "preaxial" and "postaxial," respectively.

Classification

Each radiographed malformation was described and classified individually by limb structure (Table 1); distribution of malformed frogs by the state of origin (Table 2); and by malformations of digits and autopodia (Table 3). Malformations observed during 1997 and 1998 field surveys in eight Minnesota study sites are described in more general categories to accommodate the reduced level of visual assessment that is practical in the field without radiographic support (Table 4). If a frog or limb had more than one malformation, it was entered in more than one category in this table. Consequently, the numerical tallies in these categories reflect the number of malformations rather than the absolute number of frogs.

RESULTS

No limb elements: Amelia

Amelic frogs lacked both limb and coxofemoral joint (Fig. 2A–D). Of the 11 amelic frogs in this study, eight also presented with malformations of at least one ipsilateral pelvic element, which included absence of the ilium (Fig. 2B), absence of the ischium and pubis (Fig 2C), and absence of ilium, ischium and pubis (Fig. 2D) on the side with the missing limb. The remaining pelvic elements were often abnormal in shape with irregular cortices and proliferation of thick bone trabeculae traversing the medullary silhouette (Fig. 2C). Severe pelvic malformation was associated with lateral displacement of the distal coccyx in some of these frogs, which

TABLE 2. State from which *Rana pipiens* with hind limb malformations were collected in 1997 and 1998

Malformation	Minnesota 1997	Minnesota 1998	Vermont 1997	Maine 1998	Total no. of frogs
I. No limb					
A. Amelia	3	2	6	0	11
II. Multiple limb segments					
A. Polymelia	2	2	0	4	8
III. Multiple limb elements					
A. Polydactyly	0	1	0	0	1
B. Polyphalangy	0	1	0	3	4
C. Polydactyly with polyphalangy	0	2	0	2	4
IV. Reduced limb segments					
A. Phocomelia	2	0	0	0	2
B. Ectromelia	9	12	34	0	55
V. Reduced limb elements					
A. Ectrodactyly	0	2	1	0	3
B. Brachydactyly	0	7	4	0	11
C. Brachydactyly with ectrodactyly	2	7	15	0	24
VI. Mixed digit patterns					
A. Brachydactyly with polyphalangy	0	1	1	1	3
B. Ectrodactyly with brachydactyly and polyphalangy	1	2	1	0	4
C. Brachydactyly with polydactyly and polyphalangy	1	0	0	0	1
VII. Complete but malformed limb					
A. Rotation	4	0	0	0	4
B. Bone Bridge	1	8	1	3	13
C. Skin Web	2	13	4	12	31
D. Micromelia	1	0	0	1	2
Total malformations:	28	60	67	26	181
Total frogs:	26	44	65	21	157

NWHC, National Wildlife Health Center.

presented the appearance of scoliosis; however, the vertebrae were properly aligned when examined radiographically (Fig. 2A,B).

Minnesota field survey findings (Table 4). Amelic frogs comprised 3.9% (22 of 570) of all malformations documented during field surveys at Minnesota sites during 1997 and 1998. Frogs from five of eight Minnesota sites had amelia; at two of these sites, amelia was seen in both years.

Multiple limb segments

Polymelia: multiple limbs originating at the pelvis. Supernumerary limbs that originated from the pelvis or from bones resembling duplicate pelvic elements were found in seven frogs. Supernumerary limbs in six of these frogs were independent of a pair of complete and apparently normal primary limbs (Fig. 3C–E). Although all three limb segments (stylopodium, or femur; zeugopodium, or tibiafibula; autopodium, or foot) were represented in these supernumerary limbs, they often lacked specific elements (Fig. 3E,F). The extreme of this condition was found on a limb in which the autopodium had developed only the tibiale or fibulare and lacked digits (not shown). Duplication of an element in a supernumerary limb was seen in only one frog (not shown). The limb had a single bone in the zeugopodium, no tibiale or fibulare, and three digits. Two of the digits were D4s, one of which had a split P1 with two distal phalanges (polyphalangy).

Supernumerary limbs that arose as a pair of limbs from a point between the two normal primary limbs had opposite polarity or "handedness" (mirror images) (Fig. 3C,D). These supernumerary limbs were the appropriate symmetrical partner to the closest primary limb, such that the pattern was right-[left-right]-left (the brackets enclosing the supernumerary pair). In Figure 3C, this symmetrical relationship with the adjacent primary limbs applies even though the two supernumerary limbs are fused along their medial femoral interface. In Figure 3D, the relationship is similarly retained although the supernumerary limbs are oriented perpendicular to the primary pair. Paired supernumerary limbs moved independently of the primary limbs, indicating functional innervation.

The supernumerary limb of one of these polymelic frogs was fused with the primary limb (Fig. 3A,B). The duplicated femurs were fused distally and the single zeugopodial mass comprised five fused tibia/fibula and the autopodium had five fused tibiale/fibulare bones. The 12 digits of the autopodium were, however, independent of one another.

Six of the seven frogs with multiple limbs originating at the pelvis had abnormal pelves. The malformations included a missing pubis, missing ischium and pubis (Fig. 3D), multiple pelvic elements (Fig. 3C,D), and/or very thick pelvic elements that may represent unseparated multiple bones (Fig. 3B).

TABLE 3. Digit malformations in *Rana pipiens* examined at the NWHC in 1997 and 1998*

Malformation	Figures	n = Feet affected	Digit affected					Type of polyphalangy				Other limb malformations					
			1	2	3	4	5	Split metatarsus	Split phalanx	Duplicate phalanx	Duplicate terminal phalanx	Tibiale-fibulare dysplasia	Tibiale-fibulare absent	Tibia-fibula dysplasia	Bone bridge	Skin web	Rotation
Polydactyly	5A	1	1		1							SN[a]1		1	1		
Polyphalangy	5B	4			1	2	3	2	1	3				1	1	2	2
Ectrodactyly		3	3									3	1-A	1	1	1	1
Brachydactyly	8F	11	5	4	5	8	7					3		2	1	2	2
Polyphalangy and polydactyly	5D	4	1 1	1 3	1 1	1 1	1 1	1		1	2	2				1	1
Brachydactyly and polydactyly		0															
Ectrodactyly and polyphalangy		0															
Brachydactyly and polyphalangy	4C	3	2 0	1 0	2 1	3 2	2 0				3	2		1	1	2	2
Bracydactyly, ectrodactyly and polydactyly		0															
Total:			12	6	12	15	14										
Sequence of digits only; no identity																	
Brachydactyly, polyphalangy and polydactyly	4F	1	1 0 0	1 1 0	1 0 0	1 0 0	1 0 1				1						
Brachydactyly, polyphalangy and ectrodactyly	4A, 5E	4	3 0 0	3 2 0	3 1 1	0 1 2	0 0 3	2			2	2	1-A				1
Bracydactyly and ectrodactyly	8D, 4B	24	19 2[a]	18 4	14 8	5 19	3 22					11	5-A[a], 3-AA[a]	7	2	4	7
Total:		55	259 Total digit abnormalities involving 196 digits					5	1	4	8	24	7-A, 3-AA	13	7	12	16

NWHC, National Wildlife Health Center; SN, supernumerary; A, 1 bone absent; AA, both bones absent.
[a]Two frogs were missing only digit 1; no identity could be assigned other 21 feet.
*Numbers represent individual digits or feet. Limbs with polymelia and with phocomelia not included.

TABLE 4. Hind limb malformations of *Rana pipiens* documented in eight Minnesota study sites in 1997 and 1998*

Site	NEY 97	NEY 98	CWB[a] 97	CWB 98	ROI 97	ROI 98	SUN 97	SUN 98	LMS 97	LMS 98	CLE 97	BLO 98	BUR 98
Total limb malformations	83	19	60	68	90	129	27	14	11	13	35	8	13
Types of limb malformation													
I. No limb: amelia	7 (8)	1 (5)	0 (0)	0 (0)	2 (2)	5 (4)	3 (11)	0 (0)	0 (0)	3 (23)	1 (3)	0 (0)	0 (0)
II. Multiple limb segments: polymelia	12 (14)	2 (11)	11 (18)	6 (9)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
III. Multiple limb elements: polydactyly and polyphalangy	11 (13)	0 (0)	3 (5)	6 (9)	0 (0)	5 (4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
IV. Reduced limb segments													
A. Phocomelia	2 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
B. Ectromelia													
1. Femur	8 (10)	2 (11)	0 (0)	5 (7)	13 (14)	27 (21)	4 (20)	2 (14)	4 (36)	1 (8)	4 (11)	0 (0)	0 (0)
2. Tibiafibula	9 (11)	6 (32)	4 (7)	1 (1)	13 (14)	26 (20)	9 (20)	2 (14)	2 (18)	2 (15)	3 (9)	1 (13)	1 (8)
3. Tibiale fibulare	7 (8)	2 (11)	0 (0)	0 (0)	18 (20)	13 (10)	2 (10)	0 (0)	1 (9)	2 (15)	3 (9)	2 (25)	1 (8)
V. Reduced limb elements: ectrodactyly or brachydactyly	20 (24)	4 (21)	8 (13)	10 (15)	41 (46)	49 (38)	9 (37)	8 (57)	4 (36)	5 (38)	13 (37)	4 (50)	10 (77)
VI. Complete but malformed limb													
A. Rotation	2 (2)	0 (0)	3 (5)	0 (0)	2 (2)	2 (2)	0 (0)	0 (0)	0 (0)	0 (0)	1 (3)	0 (0)	0 (0)
B. Bone bridging	2 (2)	1 (5)	4 (7)	5 (7)	1 (1)	1 (1)	0 (0)	1 (7)	0 (0)	0 (0)	6 (17)	0 (0)	0 (0)
C. Skin web	0 (0)	0 (0)	27 (45)	35 (52)	0 (0)	0 (0)	0 (0)	1 (7)	0 (0)	0 (0)	4 (11)	1 (13)	1 (8)
D. Micromelia	3 (4)	1 (5)	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

[a]Data collected by Dr. David Hoppe.
*Numbers represent individual malformations and include field data that supplement the NWHC data discussed in detail in this article. Numbers in parentheses represent percentage.

Polymelia: multiple limbs originating at the stylopodium. A complete limb duplicated from a point distal to the hip was found in only one frog (Fig. 3F). This limb appeared to be "split" from the dorsolateral aspect of the primary femur. While the primary limb was normal, the distal segments of the extra limb contained only a single element of the zeugopodium and proximal autopodium, and only the three lateral digits (D3–D5).

Minnesota field survey findings (Table 4). Polymelia was found in frogs collected at two Minnesota sites during both years of the survey and represented 5.4% (31 of 570) of all malformations documented during field surveys at Minnesota sites.

Multiple limb elements

Tibia or fibula. The zeugopodia of two frogs flared distally (Fig. 4C,D). Although this might suggest an unseparated duplication, the abnormality could not be differentiated from bone dysplasia. These were the only specimens in which duplications may have originated at the level of the zeugopodium.

Tibiale or fibulare. In two frogs, the tibiale or fibulare were duplicated at the junction between the zeugopodium and autopodium. One frog had polymelia with fused proximal segments (Fig. 3B). In the limb of the other frog (Fig. 5A), the joint space was very wide and the distal half of the zeugopodium appeared as a broad silhouette of unfused tibia and fibula.

Polydactyly and polyphalangy. Multiple digits and digital elements were a common finding in frogs from certain study sites (Tables 2, 4). All frogs observed with polyphalangy and/or polydactyly in the absence of ectro- or brachydactyly came from one site in Minnesota and the site in Maine. The frog shown in Figure 5B is from the site in Maine which had three frogs with polyphalangy and two with polyphalangy and polydactyly. The autopodia of the four frogs from the Minnesota site looked very similar to one another. The tibiale and fibulare of affected limbs were dysplastic, curved in the dorsoventral plane, and the metatarsals were displaced in the same plane (Fig. 5A,C,D). In the frog shown in Figure 5D, this resulted in a digital pattern of D1 in the ventral plane; D2 and D3 in the dorsal plane; and D3, D4, D5 were in the normal plane and associated with more normal-appearing metatarsals.

In all, one frog was observed with polydactyly (Fig. 5A), four with polyphalangy (Fig. 5B), and four with polydactyly and polyphalangy (Fig. 5D). Polyphalangy

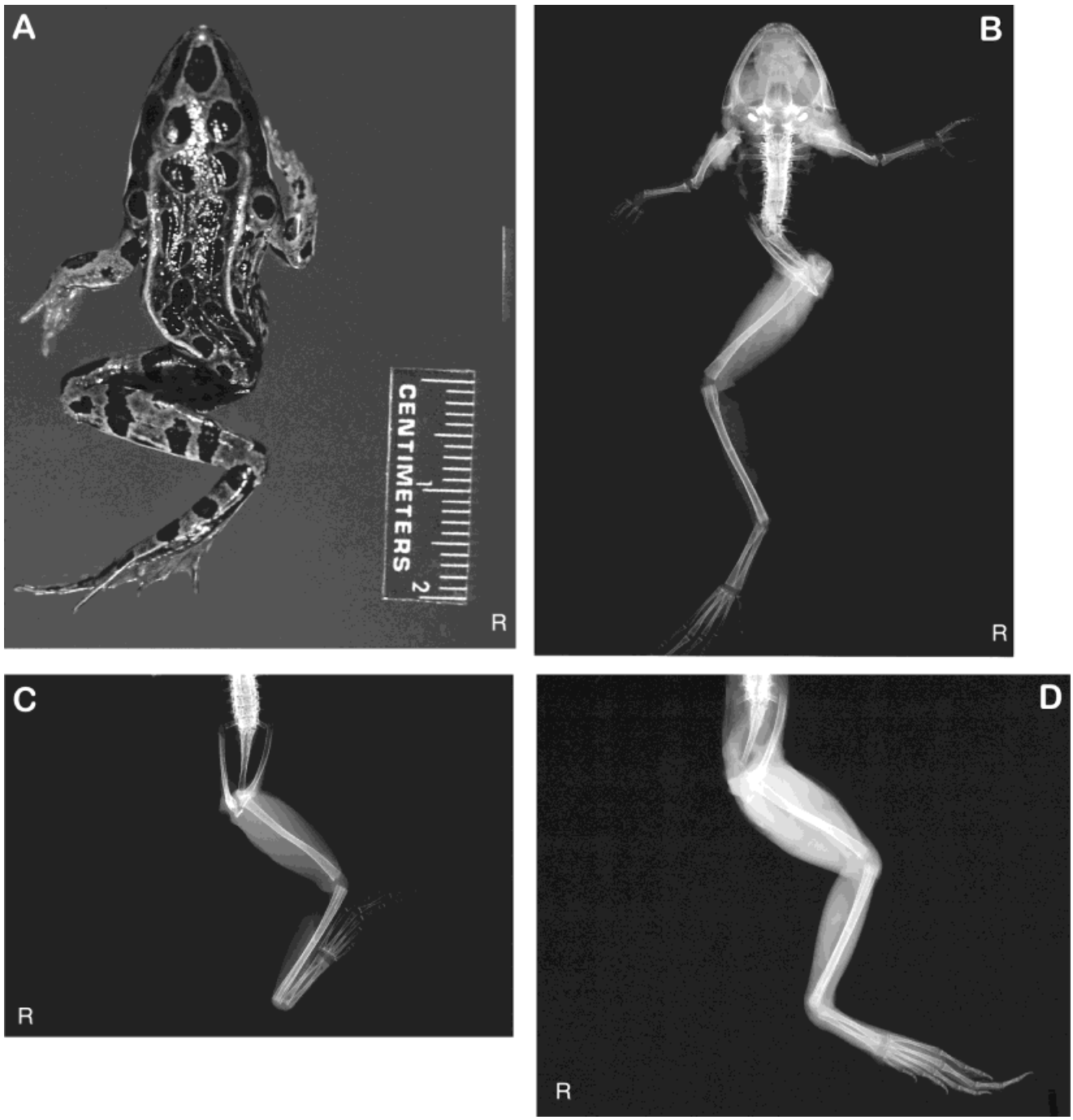

Fig. 2. Amelia in frogs from Vermont and Minnesota. **A,B:** In this frog, amelia is associated with agenesis of the ilium and dysgenesis of the ischium. Displacement of the coccyx gives the appearance of scoliosis although the vertebrae are properly aligned. **C:** Amelia with agenesis of the ipsilateral ischium and pubis. The distal right ilium has irregular cortices and prominent bone trabeculae. **D:** Amelia with agenesis of the ipsilateral ilium, ischium and pubis.

with a mid-shaft split of the metatarsus or phalanx is shown in Figures 4E, 5B, and 8B (Figs. 5B and 8B are two views of the same frog). Phalanges were also duplicated at the metatarsal-phalangeal joint (Fig. 5B,D) or at the distal phalangeal joint (Figs. 4A, 5C). Duplication at interphalangeal joints did not occur, with the exception of duplication of terminal phalanges. Each duplicated metatarsus or first phalanx completed its phalangeal pattern distally, with the exception of two digits shown in Figure 3B and one in Figure 5B, which lacked a terminal phalanx. The number of phalanges in a pair of duplicated digits usually equaled one another (except for the one shown in Fig. 5B) but did not necessarily correspond to the position of the metatarsus

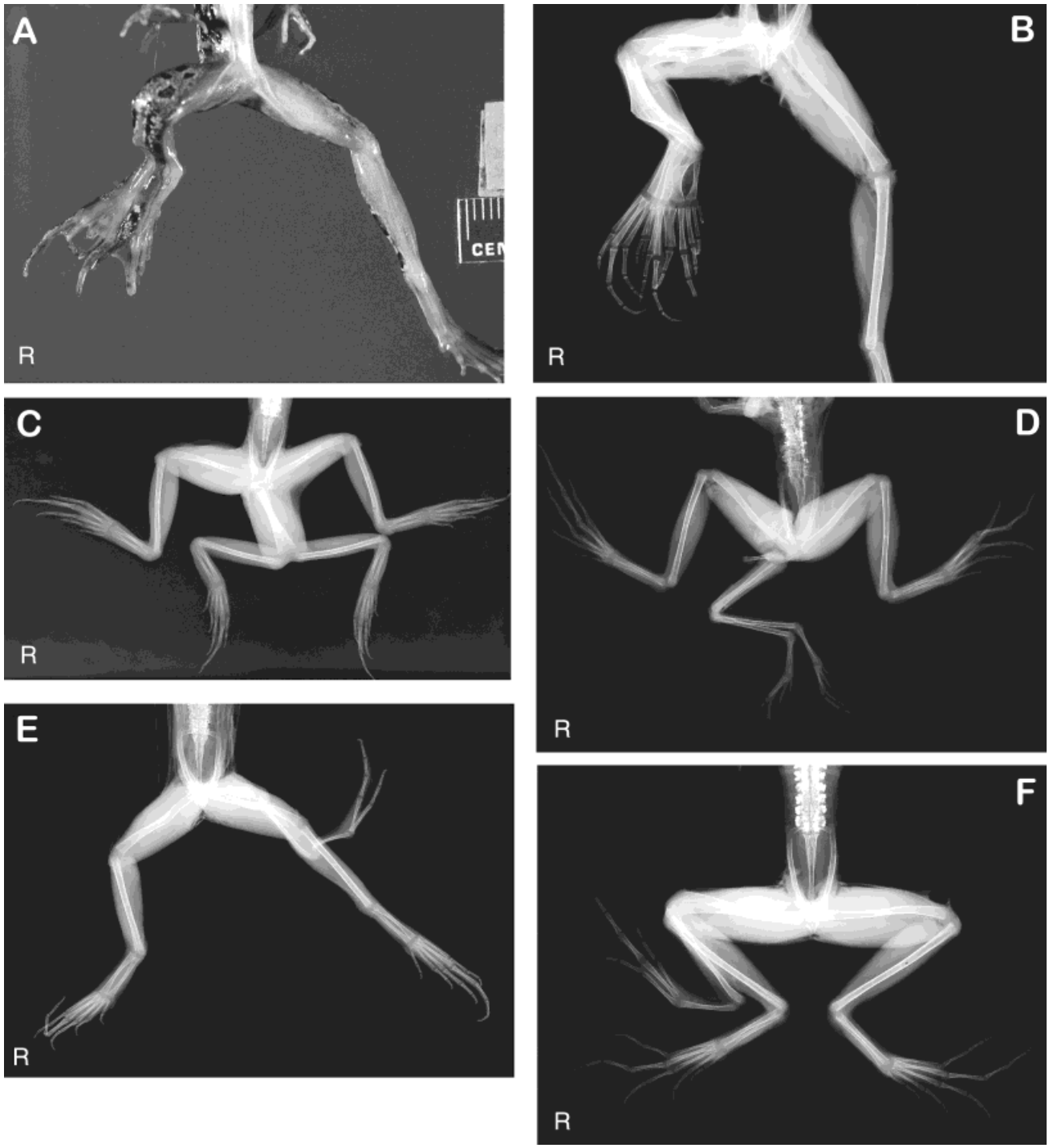

Fig. 3. Polymelia. **A,B:** Photograph and radiograph of a frog with a supernumerary limb originating at the pelvis. The femurs of the duplicated limbs fuse distally, and there are five zeugopodial elements (more distinct on alternate radiograph) and five tibiale/fibulare bones. The autopodium continues into 13 metatarsals. Two pairs of metatarsals are fused (5,6 and 9,10). The 5,6 pair give rise to what appears to be a D3 or D5, while the 9,10 pair continue into two D2. This foot has 12 digits in all, with the best-fitting pattern (medial to lateral) of: 1,2,3,4, [3 or 5], 4 [3 or 5], 2,2,3,4, [2 or 3 or 5]. The designation of the most lateral digit is difficult, as the anatomy of the metatarsus and first phalanx would suggest D3 or D5, but the number of phalanges present in the digit suggests that it is a D2. The most medial D3 and D4 have no terminal phalanx. **C,D:** In these frogs, supernumerary pelvic elements give rise to a complete extra pair of limbs. The supernumerary femurs in C are fused along their medial cortices. Rather than pair with each other, these limbs are oriented such that they pair with the adjacent primary limb, forming mirror-image duplications. From right to left, the limb polarity is: R, [L,R] L (the pair in brackets represents the supernumerary pair). In both frogs, the extra limbs functioned independently of the normal primary limbs. The primary pelvis of the frog in D has no ischium or pubis. **E:** The supernumerary limb in this frog arises from the level of the pelvis as an abbreviated femur. The zeugopodium contains only one element, as does the proximal autopodium. Only two digits, which resemble D4 and D5, are present. **F:** A supernumerary limb arose from the dorsolateral margin of the mid-shaft primary femur. This limb also has an abbreviated femur; a zeugopodium with only one element; and reduced elements in the autopodium with either the tibiale or fibulare, and only three lateral digits. A,B,C,E are from the same Minnesota site; D,F from Maine.

from which they extended. Duplication of phalanges at an interphalangeal joint was not observed.

Minnesota field survey findings (Table 4). Polydactyly and polyphalangy comprised 4.4% (25 of 570) of all malformations documented during field surveys at Minnesota sites during 1997 and 1998.

Mixed digit patterns. Nine frogs with ectrodactyly or brachydactyly as a primary malformation also exhibited polyphalangy (Tables 1–3), which presented as duplicated terminal phalanges (Fig. 4A,C,E,F; see Fig. 8F), a split phalanx (Fig. 4E) or a split metatarsus (Fig. 5E).

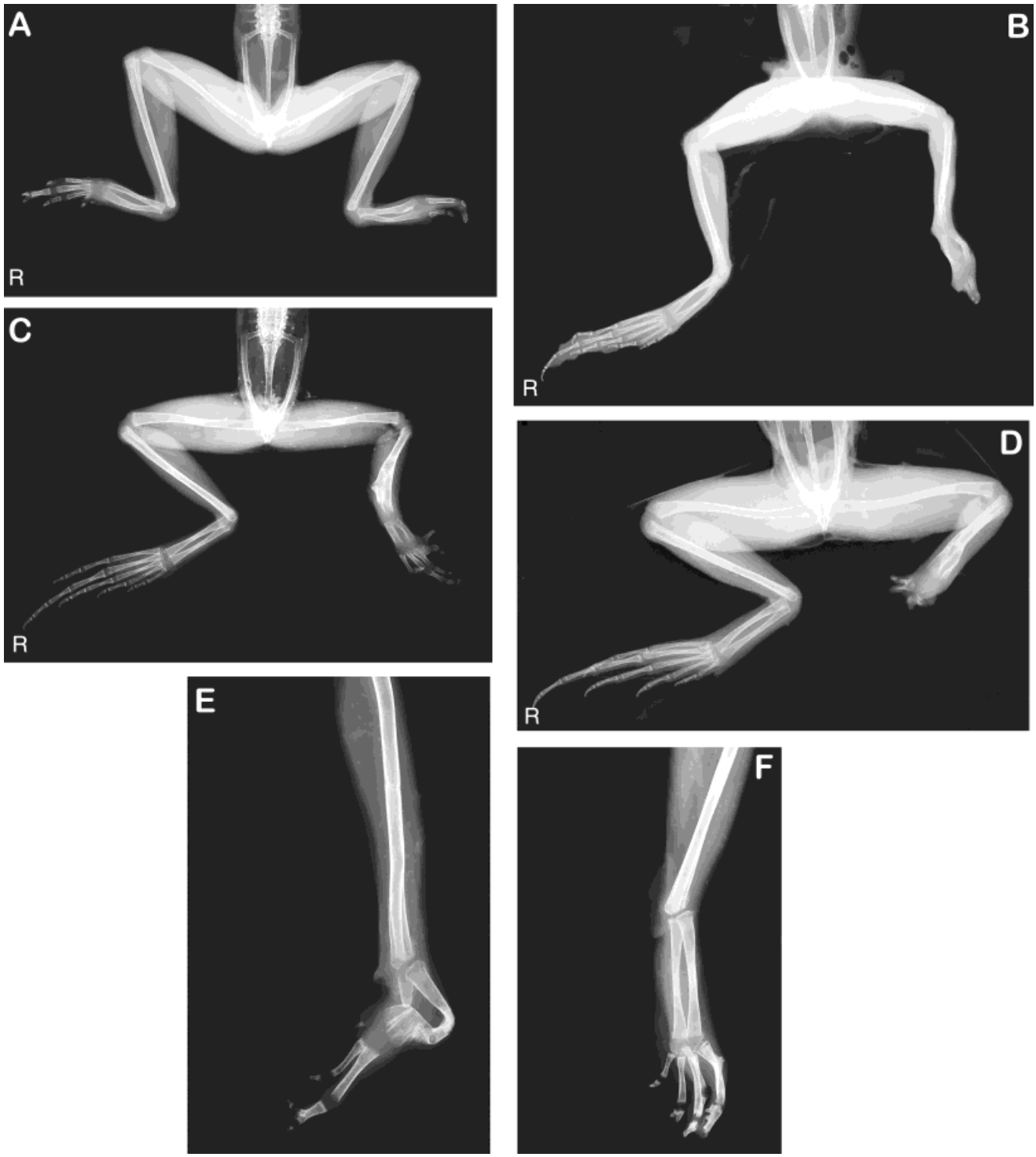

Figure 4.

Reduced limb segments

Phocomelia. Two frogs collected in Minnesota from one site on the same day had phocomelia of the right leg. These were the only two phocomelic specimens collected over the two study seasons in all three states. The limbs looked remarkably similar with an abnormal foot attached to a short limb composed of small, disorganized, and unrecognizable bones (Fig. 6). Two fused metatarsal bones and four terminal phalanges comprised the foot. The pelvis of these frogs was also abnormal.

Ectromelia of the stylopodium. Ectromelia, the premature termination of a limb segment, was the most common malformation seen in the frogs submitted to the NWHC (Tables 1, 2). Of these 55 frogs, 16 had femoral ectromelia, which was bilateral in one frog. Radiographs were taken of 11 frogs (12 ectromelic limbs) and the pubis was abnormal in four of these frogs.

The femoral terminations exhibited three types of morphology: (1) abrupt termination (five limbs; not shown); (2) bulbous termination (four limbs; Fig. 7A); or (3) termination with cortical separation (three limbs; Fig. 7B). Three of the five cases with abrupt termination lacked both pubis and a significant part of the femur. In the other two frogs, the femurs terminated further distally and, although there was no dysplasia at the point of termination, the bone was dysplastic at mid-shaft.

Four femurs were observed that terminated in a discrete bulbous expansile sphere (Fig. 7A). This type of malformation appeared to be unique to the stylopodium. All four frogs were collected in Minnesota; three were found at the same site. One of the frogs also lacked a pubis. Although radiographic studies of bone repair in frogs have not been done, the bulbous termination of the femur might be difficult to differentiate from a potential exuberant bone callus produced by weight-bearing forces.

Figure 7B represents one of three frogs in which a delicate radiodense ring formed around the distal cortex of the affected femur. The lesion was interpreted as cortical separation. Again, this malformation appeared to be unique to the stylopodium.

Ectromelia of the zeugopodium. Twenty-one of the 26 frogs with truncated tibiafibulae were radiographed. Both limbs were truncated in one frog, bringing the affected limb total to 27 (22 radiographed). One frog also lacked a pubis, although the femur appeared normal. Similar to the stylopodial truncations, there were three different morphologies: (1) abrupt termination (11 limbs); (2) distal dysplasia with broad, irregular cortices (7 limbs); and (3) mottled, thin cortices with little change in contour (4 limbs).

Figure 8C represents one of six limbs in the first group, which had a very short, abruptly terminating tibiafibula, of which two had distal femoral dysplasia and one of those had a femoral fracture (not shown). The zeugopodia of another four frogs in this group were abruptly truncated by one-half to one-third their length. Although these bones did not have dysplasia at their termi, the proximal bone was dysplastic and rotated. One frog had a complete tibiafibula but no development distal to the zeugopodium (Fig. 7E, right limb).

Very short dysplastic tibiafibulae were seen in two frogs collected from the same site (Fig. 7C). These bones were disorganized with indistinct distal cortical silhouettes and the femurs of both limbs had mid-shaft dysplasia. In five other frogs with dysplastic termini,

Fig. 4. Ectrodactyly and brachydactyly. **A:** The four digits (ectrodactyly) of the right foot have only one, two, two, and one phalanges, respectively (brachydactyly). The terminal phalanx of the longest of these digits is duplicated (polyphalangy). The bones of the metatarsus and the digits are mildly dysplastic in this foot. The left limb of this frog has only two digits, one of which has two phalanges and the other only a terminal phalanx. The bones of the metatarsus and the digits are mildly dysplastic in this foot. The joint spaces are abnormally wide in both feet. Additional limb malformations in this frog include bilateral rotation and mid-shaft dysplasia of the tibiale and fibulare, and an expanded, irregular cortex of the left fibulare. **B:** There are only a medial and a lateral digit in the left foot of this frog with one and two small proximal bones, respectively, and both have a terminal phalanx. Again, the joint spaces in this foot are abnormally wide. There is mid-shaft dysplasia of the left tibiafibula. Dysplasia of the tibiale and fibulare becomes more severe distally. **C:** The orientation of the left limb is rotated 180°, so that digit 1 points laterally. Digits 1–5 of this hind limb have brachydactyly; the number of phalanges are 0,2,2,3, and 1, respectively. D4 has a duplicate terminal phalanx. As in Figs. 4A–B, the joint spaces are wide. In addition, a skin web extends from hip to hock, and the elements of the zeugopodium and proximal autopodium are shortened and dysplastic. **D:** All three digits of the left limb have brachydactyly. D1 and D3 have a narrow proximal bone and a terminal phalanx. The more proximal bone of D3 curls medially. A single narrow bone comprises D2. The zeugopodium of this limb is dysplastic and rotated (without a skin web) and there is no evidence of either tibiale or fibulare. The joint spaces in the terminal aspects of this limb are wide. **E:** The most medial of the three digits of this left foot has only a terminal phalanx without evidence of a metatarsus. The metatarsus of the central digit is dysplastic and rotated, with only a terminal phalanx. The first phalanx of the longest (lateral) digit splits distally to articulate with two terminal phalanges. The tibiale and fibulare of this limb are very disproportionate in size and form a bone pattern that, on external exam, appeared similar to a bone bridge. The tibiale is short and stops abruptly as the fibulare curves laterally then medially with distal dysplasia. The fibulare is fused to a very wide bone with two medial cortical silhouettes that suggest fused distal tibiale and fibulare and the short tibiale abuts this bone. **F:** Though the proximal segments of this limb appear normal, the foot has a "mixed digit pattern" with both too many digits (polydactyly) and reduced number of phalanges (brachydactyly). The six metatarsal bones end abruptly, each with only a single phalanx; four of these are terminal phalanges. The terminal phalanx of the second metatarsus is duplicated. D3 and D4 are superimposed. The narrow third metatarsus is dysplastic proximally and has a central radiolucent region. A bone spur protrudes from the terminal phalanx of this digit. The thicker metatarsus is designated as belonging to D4, although it has only one irregular terminal phalanx. The metatarsals of digits 5 and 6 have separate silhouettes proximally but merge distally. The metatarsals are dysplastic with a proximal bone spur and two small bones projecting from the medial surface of the single (nonterminal) phalanx. The frogs in B and C are from the same site in Vermont; D–F are from one site in Minnesota; and A is from a separate Minnesota site.

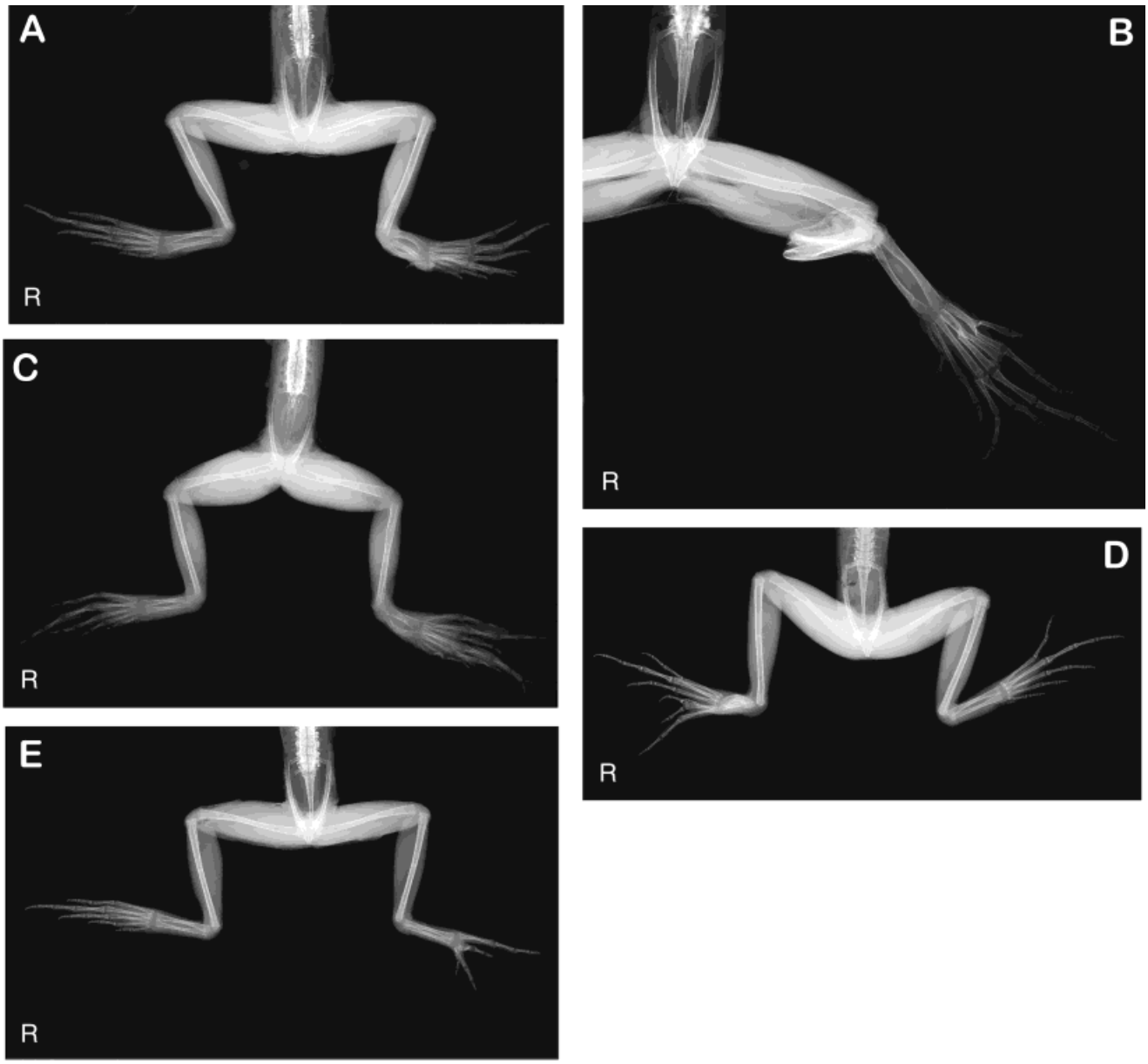

Fig. 5. Polydactyly and polyphalangy. **A:** The three distorted tibiale/fibulare in the left limb of this frog appear as separate radiographic silhouettes and terminate in an abnormally wide joint space. Duplicate D3s have fused metatarsi and a single, thick P2, but two separate P1 and P3. The eight digits are arranged in the order: 1,1,2,[3,3 fused],3,4,5 (medial to lateral). The abnormal D1 is rotated 180° such that it points cranially and has only a terminal phalanx. **B:** The stylopodium of the left limb is folded on itself and the folded bone creates a bone bridge (more distinct with the oblique orientation of this limb in Fig. 8B). The unremarkable tibiale and fibulare continue into five metatarsal bones and eight digits (polyphalangy). The metatarsus of D3 appears normal but articulates with two sets of phalanges such that it completes the phalangeal patterns of a D3 and D4. The split metatarsus of D4 gives rise to the phalanges of a D4 ventrally but none dorsally. The metatarsus of D5 is similarly split, continuing into the phalangeal pattern of a D5 (or D3) ventrally and a single terminal phalanx dorsally. **C:** The tibiale and fibulare of the left foot are curved and dysplastic in the mid-shaft. The wide joint space between them and the metatarsals contains a well but faintly defined synovial silhouette. Polydactyly includes the eight metatarsal bones with the best fit digital pattern: 1,2,3,4,[3 or 5],3,4,5 (medial to lateral). The digit in parentheses arises from the dorsal aspect of the fourth metatarsal-phalangeal joint The terminal phalanx of the medial D4 is duplicated. **D:** The tibiale and fibulare of the right limb are short and thick, curved, and show evidence of mid-shaft dysplasia. There are six metatarsals (polydactyly); the metatarsus of D2 is also thick, especially at its articulation with the duplicated phalanges (polyphalangy). The pattern of these seven digits falls into 3 planes: D1 ventral; D2 (with duplicated P1), and D3 dorsal; and the second D3, D4 and D5 in the medial plane. **E:** Polyphalangy and ectrodactyly in this foot give rise to a digital pattern of 1,(2,2),3. D2 (in parentheses) is split at the base of the metatarsus and forms two digits, each with a P1 and a terminal phalanx. A,C,D are from the same Minnesota site as the Gardiner and Hoppe study ('99); E is from a separate Minnesota site; B from Maine.

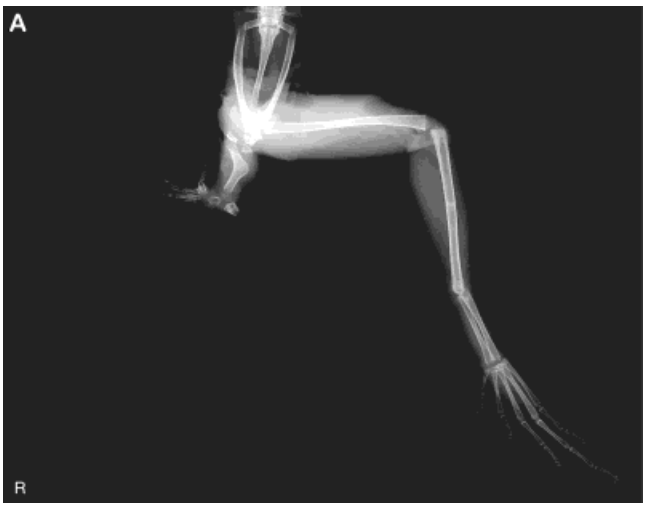

Fig. 6. Phocomelia. The proximal limb has small disorganized bones that are not individually identifiable. Four metatarsal bones are associated with four terminal phalanges. Metatarsal bones 1 and 2 are fused. The bony trabeculae of the caudal pelvic elements are prominent and disorganized. These two cases of phocomelia were the only ones found in this study, both at the same site in Minnesota.

the femur was unremarkable and the zeugopodium was somewhat longer (one-half to two-thirds its normal length; Fig. 7D) than the femur in Figure 7C. However, the tibiafibulae were twisted at the proximal-most site of dysplasia. As in the two frogs with the very short tibiafibulae, the cortices of these truncated bones were also mottled with poorly defined terminal borders. A broad pigmented soft tissue structure that ended in a point extended beyond the truncated bone.

The cortical silhouette along the full length of the tibiafibula was irregular and mottled in four other frogs (not shown). In three of four limbs, the cortex of the bone's terminus was very thin. One frog had a proximal hairline fracture of the tibiafibula surrounded by a delicate proliferative periosteum, suggesting periostitis. The femurs of these four frogs appeared normal.

Ectromelia of the autopodium. Of the 55 ectromelic frogs, we radiographed 12 of the 13 with truncated tibiale and fibulare. Ectromelia of the tibiale and fibulare was bilateral in one frog, bringing the total radiographed limbs with ectromelia of the autopodium to 13. The tibiale and fibulare were very short in five frogs (Fig. 7E) and bilaterally symmetrical in one frog (not shown). The tibiafibula of one of the 12 frogs was severely dysplastic and rotated. The cortices of the distal tibiale and fibulare were mottled, dysplastic, irregular, and dense in four other frogs, but the contours of the proximal tibiale and fibulare were unremarkable (Fig. 7F). In four frogs, the tibiale and fibulare were severely dysplastic with broad, irregular, thin and mottled cortices. One of the two bones were radiographically undetectable in two frogs from the same site. The diaphysis of the tibiafibula in one frog was mildly dysplastic. Two different frogs with ectromelia of the autopodium had dysplasia of the ipsilateral zeugopodium and in two others the contralateral zeugopodium was truncated.

Minnesota field survey findings (Table 4). Ectromelia was the most common malformation (35% (200 of 570), and at least one type of ectromelia was found at all of the Minnesota sites in each of the 2 years of the study.

Reduced limb elements

Missing tibiale and/or fibulare. Supernumerary limbs lacking either the tibiale or fibulare were observed in three frogs. A zeugopodial element was also missing in two of these limbs but the radiographs did not allow distinction between tibia and fibula (Fig. 3E,F). The primary limbs of seven frogs lacked one of the bones of the tibiale/fibulare pair. All these limbs had missing digits (ectrodactyly); six also lacked digital elements (brachydactyly). Both the tibiale and fibulare were missing from three frogs that had both ectrodactyly and brachydactyly (Fig. 4D).

Ectrodactyly. Twenty-seven of the 31 frogs with ectrodactyly lacked digital elements in existing toes (ectrodactyly with brachydactyly) (Fig. 4B, Table 3; see Fig. 8D). The tibiale and fibulare bones were dysplastic in three frogs that had ectrodactyly without brachydactyly (not shown). One of these feet lacked either the tibiale or fibulare; the zeugopodium of another limb was dysplastic; and the limb of a third frog had a skin web, and a bone bridge of the zeugopodium resulted in severe limb rotation.

Brachydactyly. Brachydactyly without ectrodactyly was seen in 11 frogs (Fig. 8F, Table 3). In three frogs, the tibiale and fibulare were dysplastic, as were the metatarsals associated with affected digits in two other frogs. Long bone rotation, skin webbing, and dysplasia of the distal tibiafibula occurred in two frogs with brachydactyly (Fig. 4C). Bone bridges without a skin web were seen in the tibiafibula of two frogs and in the femur of another; in the latter, the tibiale and fibulare were dysplastic as well (not shown). All ten hind digits of one frog and five on one foot of another lacked a single proximal phalanx (Fig. 8F, right foot), although the digits were otherwise complete. The joint spaces in the feet of both of these frogs were wide. The frog in which all 10 digits were affected in this way also had dysplasia of the left tibiafibula and a bone bridge of either the right tibiale or fibulare.

Ectrodactyly with brachydactyly. When ectrodactyly and brachydactyly occurred in the same foot, the digits lost their positional orientation and, without the normal number of phalanges, the digits could not be assigned identity. Of the 27 autopodia in this category, nine lacked a tibiale or fibulare (Fig. 8D); three lacked both (Fig. 4D); and in another 13, these bones were dysplastic (Fig. 4A,B,E; see Fig. 8F). Eight frogs had rotated long bones, four of which also had a skin web. The tibiafibula was severely bent and bridged in two frogs (Fig. 8D). Abnormally wide joint spaces were observed frequently between phalanges, between the

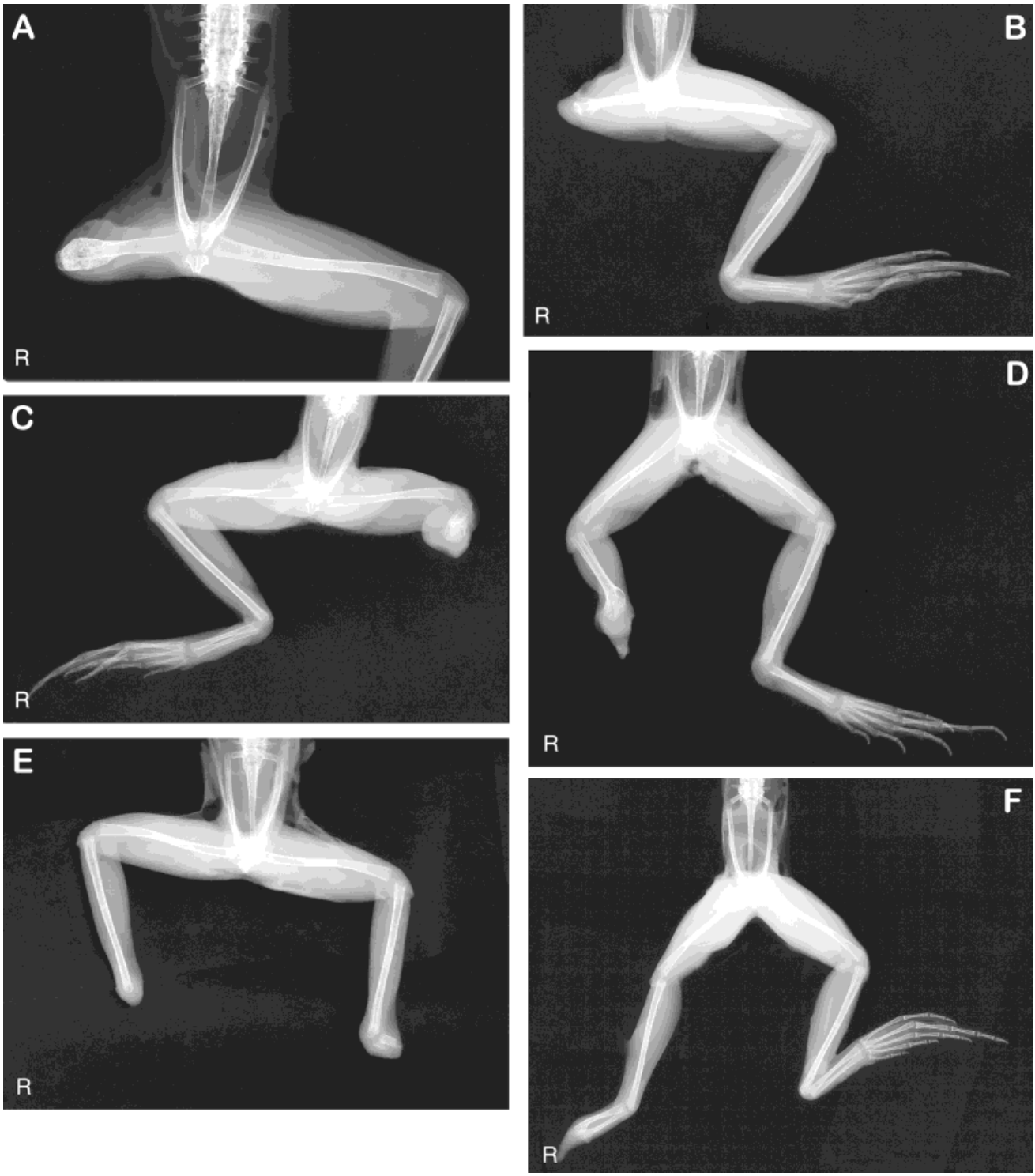

Fig. 7. Ectromelia. **A,B:** Frogs with two of the three types of femoral ectromelia. In A, the distal end of the truncated bone is characterized by terminal trabecular hypertrophy, hyperplasia, and marked expansion of the thin cortical bone. In B, the cortex of the distal femur is separated and an expansile, radiodense ring has formed around the inner cortex. A prominent, narrow band of disorganized trabeculae appears at the very terminus of the bone. **C,D:** Frogs with ectromelia of the tibiafibula. The segment is C is very short and broad, and has a disorganized distal cortical silhouette, coarse trabeculae, and an indistinct terminal border. The truncated tibiafibula in D is rotated proximally and dysplastic in the mid-shaft region. Mottled, expansile bone with a coarse trabecular pattern obscures the terminal borders of the segment. The segment terminates in a broad soft tissue silhouette that tapers into a point that contains a small round structure of bone density. **E:** Bilateral ectromelia. The elements of the zeugopodium in the right limb appear normal and fully developed, but the limb lacks any distal structures. The left tibiale and fibulare are truncated, but with minimal cortical disruption at the bone termini. **F:** Ectromelia of the tibiale and fibulare. In the right limb of this frog, the cortices of the distal tibiale and fibulare are broad and thin. The coarse, disorganized trabeculae traverse the medullary space. The terminus of the limb is covered with a pointed soft tissue mass similar to that seen in the frog in D. Frogs in B–F are from Minnesota, B–D, from the same site; in A, from Vermont.

metatarsals and phalanges, and between the tibiale/fibulare and metatarsal bones (Fig. 4A–C,E; see Fig. 8D,F). Because polyphalangy occurred in four of the 27 frogs (Fig. 5E, Table 3) with both brachydactyly and ectrodactyly, they are classified in Table 1 and 2 with "mixed digit patterns."

When ectrodactyly and brachydactyly (with or without polyphalangy) were present in the same foot, the phalangeal patterns were unique for each foot. Two frogs had only two digits on the malformed foot. The number of phalanges on each of these two digits were as follows, with each foot in brackets: [0,1]; [1,2]. Six frogs had only three digits on the malformed autopodium. The number of phalanges on each of these three digits was as follows, with each foot in brackets: [1,2,1]; [2,3,2]; [1,1,2; no central metatarsus]; [2,(2,2),3; split central metatarsus]; [2,(1,1)1; split central metatarsus each with single nonterminal phalanx]; [1,1,(2,2); split P1 on third digit each with a terminal phalanx]. The malformed foot on seven other frogs had four digits; the number of phalanges on each of the four digits were as follows with each foot in brackets: [1,1,1,1]; [1,2,2,2]; [1,2,3,1; first and fourth are single small bones]; [1,(3,3),2; second and third metatarsals are fused]; [1,(0,2),4,3; split second metatarsus]; [1,(3,3),2; central metatarsals fused]; and [1,2,2,1; duplicated terminal phalanx on third digit].

Ten additional frogs with ectrodactyly and brachydactyly were very similar in appearance; all were from Vermont. The tibiale and fibulare of these frogs were dysplastic, or unilaterally or bilaterally absent. The digits presented as a single, small, unidentifiable bone. Four frogs had one such digit, two frogs had two single-bone digits (Fig. 4B), and four frogs had three (Fig. 4D). Two additional frogs from Vermont had slightly different autopodial malformations with no detectable tibiale or fibulare and only a terminal phalanx on each of five metatarsals.

Minnesota field survey findings (Table 4). Ectrodactyly and brachydactyly comprised 32.5% (185 of 570) of all malformations documented during field surveys and these malformations were found at all of the Minnesota sites. The frequency of this type of malformation stayed fairly consistent for 1997 and 1998 at each site.

Distally complete but malformed limbs

Long bone rotation. The limbs of 53 frogs were abnormally formed, although all bone elements were present. Bones that appeared twisted without bone bridging or skin webbing were categorized as rotational malformations. The hind limb rotation dramatically affected the orientation of the foot. Primary rotational malformations were always bilateral and severe. Progressive bone dysplasia was present in the femur, tibiafibula, and, to a lesser extent, in the tibiale and fibulare. Dysplasia appeared radiographically as a prominent, proliferative, trabecular pattern in bone cortices, and occurred at the points of directional change in bone growth. Rotational changes appeared very similar in both right and left limb (Fig. 9A), and among the four frogs that had these changes. The four frogs with rotational malformations were collected in 1997 on the same day from one site in Minnesota.

Bone bridges. Bent long bones (femur, tibiafibula, tibiale or fibulare) with sharp, mid-shaft angles (Fig. 8) displayed two types of cortical change. One appeared as though the cortical bone split to form the base of a triangle whose apex was the bend in the diaphysis (Fig. 8C). In the second type, which characterized 10 of the 13 bridges, bony trabeculae flared from the diaphyseal angle to form a thin plane between the proximate surfaces of the long bone (Fig. 8A,D,F). The morphology of these bone fans was consistent among affected limbs. The trabeculae appeared to radiate from the angle in the long bone to the base of the imaginary triangle formed by the flexure. The trabeculae replaced the cortical margins at the apex of the bone angle, originating as thick, widely spaced, poorly organized bone spicules. The resulting appearance was that of a single triangular plate of bone (Fig. 8A,B,E), although the bridge was in fact two thin triangular veneers of bone between which was bone marrow.

Similar lesions have been described by Gardiner and Hoppe ('99) as "bony triangles" in polymelic mink frogs from Minnesota. Their description suggests that their "bony triangles" may be similar to our bone bridges, although the trabecular and cortical patterns of those triangles were not described.

A unique type of bone structure was seen in one frog represented in Figure 4E. This structure resembled a bone bridge grossly but radiographically it was formed by a very short tibiale and a longer fibulare, which curved laterally then medially, where it fused to a bone mass that was distal to the tibiale.

Additional malformations were found in 11 limbs with bone bridges. Eight frogs with bone bridges also had skin webs (described below) crossing a joint of the same limb. Two of the eight also had ectrodactyly, and another had supernumerary phalanges. Ectromelia of the tibiafibula; polyphalangy and brachydactyly; and polyphalangy, ectrodactyly, and brachydactyly were combinations of malformations found in three other frogs with bone bridges. Bone bridges occurred between bent margins of long bone and did not involve joints.

Skin webs. Broad bands of skin that joined the femur with the tibiafibula (Fig. 9B) were found in frogs from all states but were particularly common in Maine and at one Minnesota site (Tables 2, 4). The skin webs compromised extension of the stifle joint and in some cases held the hock so close to the rump as to immobilize the limb entirely. Limbs with skin webs were rotated even if the bones were radiographically unremarkable (Fig. 9B). Although 16 limbs with skin webs contained all bone segments, five of these frogs had pelvic abnormalities, and five of the long bones that were spanned by skin webs were dysplastic. Distal malformations were also seen in frogs with skin webs: 10 limbs with ectrodactyly, brachydactyly, or both; five with polydactyly or polyphalangy; and one each with

ectromelia of the tibiafibula and femur. Of the 69 Minnesota frogs with skin webs, 62 of them were collected at the same site as the Gardiner and Hoppe study ('99) (Table 4).

Micromelia. Two frogs had a hind limb that was structurally normal but short relative to the contralateral limb. The muscle mass in these shortened limbs appeared to be severely reduced.

Other malformations. A large central femoral bone mass was present in an otherwise unremarkable limb in one frog. This frog was not radiographed. In another frog, the toes of a structurally normal limb had syndactyly.

Minnesota field survey findings (Table 4). Complete but malformed limbs comprised 18.4% (105 of 570) of all malformations documented during field surveys. At least one was seen at each Minnesota site in

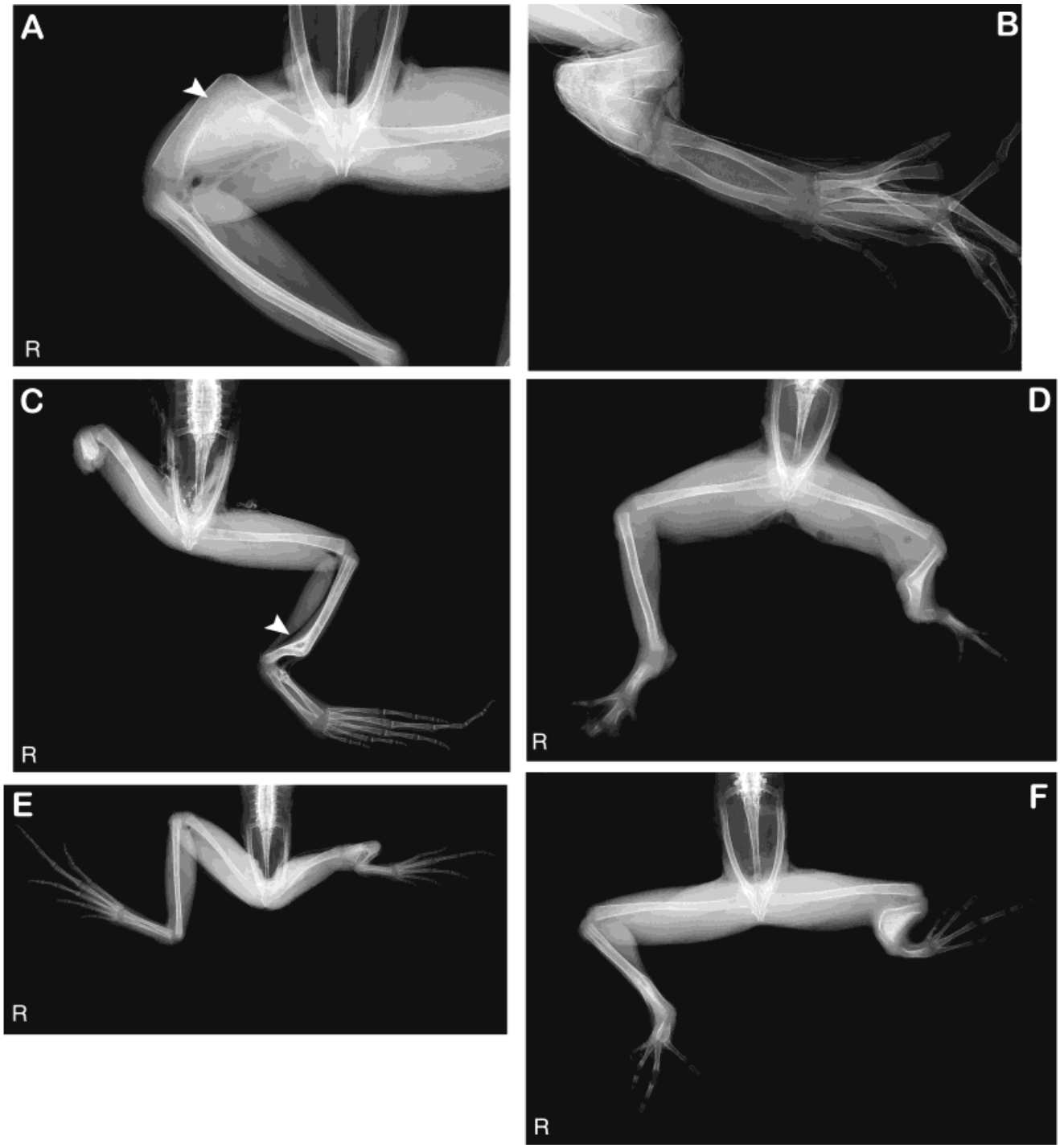

Figure 8.

1997 and 1998. Of the four types of malformations in this category, skin webbing was the most frequent (67.7% of this category) and of the 69 frogs with skin webbing, 67 (97.1%) were from one site in Minnesota.

Bilateral malformations

Lesions were symmetrical in only eight of the 22 frogs with bilateral malformations, and included the four frogs with bilateral rotational malformations; ectromelia of both femurs and no pubis (one Vermont frog; not shown); ectromelia of both tibiafibula with coincident luxation of the pelvic symphysis (one frog from Minnesota; not shown); ectromelia of left and right tibiale and fibulare bones (one Vermont frog; not shown); and bilateral, symmetrical brachydactyly with all ten hind digits missing a single proximal phalanx. This frog from Minnesota (not shown) also had a bone bridge on one limb and severe dysplasia of the tibiafibula of the other limb.

Bilateral asymmetrical malformations included one Vermont frog with amelia and ectrodactyly with dysplasia of the opposite tibiafibula. One frog from Vermont with ectromelia of the femur had brachydactyly, skin webbing, and long bone rotation in the contralateral limb (not shown). One frog from Vermont with tibiafibula ectromelia had a bone bridge of the tibiafibula in the contralateral limb, and two other frogs (one from Minnesota and one from Vermont) with tibiafibula ectromelia had ectromelia of the contralateral tibiale and fibulare (Fig. 7E). In frogs with bilateral brachydactyly and/or ectrodactyly, the proximal bones of the limbs were also abnormal. Bone bridges, dysplastic tibiafibula, dysplastic or missing tibiale and/or fibulare, rotated bones, and skin webs were found in various combinations in these limbs.

Skin webs were a common lesion, but only four frogs with bilateral skin webs were submitted (Fig. 9B). Three Minnesota frogs with unilateral skin webs had bone bridges in either the femur or the tibiale/fibulare of the opposite limb. Contralateral pelvic abnormalities were found in two frogs with skin webs of the left limb. Finally, it is notable that unilateral hind limb malformations exhibited no left/right preferences (Table 1).

DISCUSSION

This study presents the range and complexity of hind limb malformation exhibited by frogs that have survived to metamorphosis at several different locations in the United States. The malformations are the result of environmental factors impacting anuran (frogs and toads) limb development and a thorough study of the skeletal phenotypes can contribute to our understanding of causes. Toward this aim, we have classified the malformations according to their anatomy in order to identify recurring patterns. A detailed radiographic analysis has allowed a more precise description of the skeletal malformations than would have been possible if the specimens had simply been cleared and photographed. The findings emphasize the great variation of phenotypes and patterns of occurrence within and between collection sites and times. The results contribute to multidisciplinary analyses of the abnormalities and further define the complexity of the amphibian malformation problem.

Fig. 8. Bone bridges. **A:** A fine trabecular array fans caudally from the 90° angle in the right femur in this limb. The trabeculae replace adjacent cortices (arrowhead). A similar bone "fan" is seen in the lesions depicted in B, D, E, and F. The femur in A is bent and rotated to point the hock dorsally and the stifle ventrally. The skin follows the contour of the bone, making it impossible to tell whether the skin web preceded the bone rotation, or vice versa. The contralateral limb, although otherwise normal, has a skin web across the stifle. The coxofemoral joint of the malformed right limb appears incomplete. **B:** The same limb depicted in Figure 5B. Here we focus on fusion of the stifle joint at approximately 135°, and the left proximal tibiafibula has a severe bend directed caudally at the mid-shaft. Rays of linear bone trabeculae radiate perpendicular to, and filling the space between bent bone, replacing adjacent cortices. **C:** The lesion in the left zeugopodium of this frog shows a variation of the bone bridge. In place of the bone fan seen in the other images of this figure, a delicate band of cortical bone traverses the base of the triangular space that is created by the bend (approximately 45°) in the original cortex of the tibia-fibula (arrowhead). The proximal fibulare of this limb has a horizontal fracture and coarse bony callus with an expanded cortex. The right tibia-fibula is truncated proximally with little cortical disruption. **D:** The bone bridge in the shortened left tibiafibula of this frog combines the cortical band across the diaphyseal bend as in C with the trabecular fan seen in A and B. The tibiafibula is short, and the angle of the bend is approximately 45°. The autopodium of this limb is also abnormal. A single small, round bone represents either the tibiale or fibulare. Ectrodactyly and brachydactyly characterize the foot: the number of phalanges in the three digits are 2, 3, and 2, respectively. The joint spaces in the foot are wide, as was often seen with these autopodial malformations. The distal aspects of this frog's right limb are also malformed. The distal tibiale and fibulare are curved and mildly dysplastic. Similar to the left foot, this one also has only three digits with wide joint spaces, although their structures differ. The medial-most digit is composed of a single, unidentifiable bone. Fused metatarsals extending into a single proximal phalanx and a small, displaced bone that may be a terminal phalanx characterize the central digit. The lateral digit has a single metatarsus, as well as a proximal and terminal phalanx. **E:** The left tibiafibula is very wide proximally, appears unfused and bends sharply (~160°) mid-shaft. The angle of the mid-shaft bend is traversed by parallel trabeculae that obscure the cortical borders of the long bones, as described in A. The tibiale and fibulare are short, and the cortex of the medially curved tibiale is replaced by delicate short trabeculae projecting from the periosteum. Further distally, however, the foot appears normal. At the proximal end of this limb, a slightly rotated femur with mid-shaft dysplasia extends from a malformed pelvis. The cranial aspect of the left ilium is not fused with the caudal (acetabular) portion. The misshapen acetabular portion of the ilium with coarse trabecular pattern may be due to folded bone. Medial to the left distal ilium is a rudimentary bone that is suggestive of a duplicated wing of the ilium. The pubis is missing. **F:** Both limbs are abnormal. On the left, a bone fan fills the concavity of the bent tibiafibula. The trabeculae radiate from the lateral aspect of the bone and replace the cortex. The tibiale is a small, oval bone and the fibulare is short, curved, and dysplastic at mid-shaft. All but the second digit are missing a proximal phalanx, and the terminal phalanx of D4 is duplicated (as in Figs. 5A,C,E,F). The right tibiafibula is broad, unfused, and rotated. The cortices of this bone are thin and the distal margin is indistinct. Both the tibiale and fibulare of this foot are short, oval bones, and the joint spaces in the foot are wide. All five of the digits are present but each is missing a proximal phalanx.

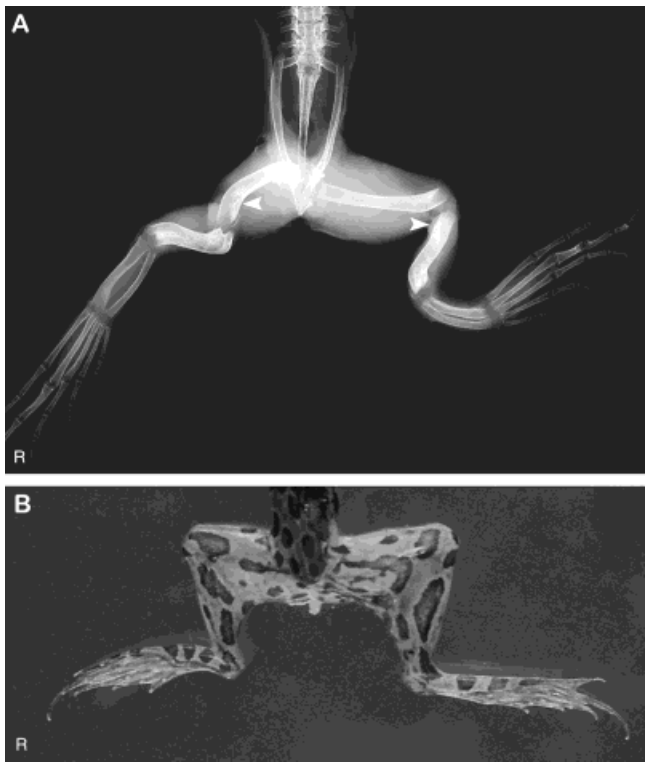

Fig. 9. A: This Minnesota frog had multifocal bilateral rotational malformations of the long bones in the rear limbs. The metaphyseal dysplasia seen at the points of curvature (arrowheads) was characteristic of this type of malformation in four frogs from the same Minnesota site. The proximal phalanx of D4 is dysplastic bilaterally, characterized by disruption and thickening of the cortex. The proximal aspect of P2 and the distal aspect of P3 of D4 of the left foot are also dysplastic. The left ilium and pubis are luxated. **B:** Bilateral skin webbing and a retained remnant of tail on a frog from Maine. The skin webs connect the hip and hock medially, flexing the stifle joints and distorting the orientation of the limbs. The range of motion in these limbs was restricted.

Although the malformations described in this report may greatly underrepresent not only the incidence, but also the variety of malformations that occur in free-living frogs, it does appear that particular types of malformations tend to occur at high frequencies in specific geographic locations, and that these phenotypes demonstrate a temporal influence. This temporospatial clustering suggests that tadpoles in a given location and in the same stage of development may have been exposed to common events (single, successive, or multiple) and that different phenotypes may be produced depending on the timing, sequence, and severity of the insult. For instance, both phocomelias collected in 1997 were similar, from the same site in Minnesota, and collected on the same day. Polydactyly/polyphalangy was found in more than 30% of the frogs collected from the site in Maine. All 65 frogs collected from four sites in Vermont exhibited some degree of limb reduction (amelia, ectromelia, ectro/brachydactyly). At one of our study sites in Minnesota (CWB, Table 4), frogs with polymelia were found in August of one year, but not until October of the second year. This was the same site at which Gardiner and Hoppe ('99) reported that 18 of the 23 mink frogs (*R. septentrionalis*) had supernumerary limbs (our collection periods at this site overlapped by 1 year: Gardiner and Hoppe 1996–1997; ours 1997–1998). Of the 570 malformations documented during the course of our study in Minnesota during 1997 and 1998 (Table 4), only 31 were classified as polymelia (5.4%). Moreover, all 31 were found at two of the eight study sites, and 17 of them at the same site as Gardiner and Hoppe's mink frog study. At that same study site 62 (91%) of the 69 frogs had skin web lesions that we observed in Minnesota frogs; all 23 of Gardiner and Hoppe's mink frogs had skin webs. Overall, however, skin webs constituted only 12% of the malformations found in the eight Minnesota sites. A similar cluster of skin webs occurred in the Maine site, affecting 58% of the frogs collected there. Taken together, our results, like those of Ouellet et al. ('97), clearly indicate that multiple limbs are not the distinguishing feature of the recent increase in malformation. In addition, the diversity of the malformations presents an intriguing challenge, and, based on our current level of knowledge, suggest that it is unlikely that a single environmental factor can be defined as causative.

Several factors, including exposure to ultraviolet (UV) radiation, parasites, retinoids, multiple chemical exposures, and predation, have been identified as contributing to the etiology of anuran limb malformation (Sessions, '90; Ankely et al., '98; Burkhart et al., '98; Fort et al., '99a,b; Gardiner and Hoppe, '99; Johnson et al., '99; Sessions et al., '99). Continuous experimental exposure of *R. pipiens* to UV radiation for 24 days was sufficient to produce rear limb malformations with or without exposure to teratogens (Ankley et al., '98). This continuous exposure to UV radiation may have prevented repair of the AER and/or mesoderm. The necessity of AER integrity for normal anuran limb development and the potential for the anuran AER to repair itself under normal circumstances is outlined in Tschumi ('57). However, the lesions produced by experimental laboratory UV exposure are primarily bilateral symmetrical truncations, an especially rare finding in our study.

Recent reference has been made to the potential role of trematodes in anuran polymelias (Johnson et al., '99; Sessions et al., '99). More than 250 trematode species are known to infect tadpoles; immature trematodes (cercariae) can burrow into developing tadpoles to form metacercarial cysts. The observation that the cercariae of the trematode genus *Ribeiroia* can induce polymelia in the Pacific tree frog (*Hyla regilla*) is very interesting and warrants further investigation into the mechanisms of this relationship (Johnson et al., '99). Sessions et al. ('90, '99) describe the supernumerary limbs among free-living Pacific tree frogs from the California site reported by Johnson et al. ('99) as mirror-image duplications. They suggest the etiology of the limb duplications is likely to involve perturbation of the limb field by the parasites because the affected frogs are

heavily infected with trematode cysts in and around the hind limb region. Johnson et al. ('99) do not describe the nature of the duplications that they induced in their experimental tree frogs. It might be useful to compare mirror-image duplications among the studies, as we also had mirror-image duplication in the two cases of paired polymelia in our study (Fig 3C, D). It should be emphasized again, however, that although polymelia was a predominant malformation in some limited circumscribed studies (Gardiner and Hoppe, '99; Johnson et al., '99; Sessions et al., '99), they were infrequent in the wide geographic area of our study and Canada (Ouellet et al., '97) and metacercariae were not found in the connective tissue of some of the malformed frogs (Meteyer et al., '00; M. Ouellet, personal communication).

Disruption of retinoic acid (RA) metabolism and signaling pathways can produce a wide variety of malformations in most species (Lammer et al., '85; Chambon, '93). Abnormalities can be induced by altering the endogenous pathways or by exposures to exogenous retinoic acid with variation associated with time, dose or other treatment variables. Experiments using transient exposure to retinoids during particular stages of development produces proximalization of distal limb elements (Sessions et al., '99, and references therein). This is one of the few limb malformations that we did not observe in the present study despite the wide geographic range. The lack of this finding in the free-living frogs suggests that simple exposure to exogenous retinoic acid is not the definitive cause of anuran malformations (Sessions et al., '99). However, chemical signaling during development is highly complex and involves interaction among a variety of cascades and nuclear receptors. Therefore, local disruption in cellular communication involving RA and related pathways cannot be excluded from possible factors that might play a role in amphibian malformation.

Studies based on field specimens provide insight into the impact of environmental factors in their natural context. However, many uncontrolled variables, both known and unknown, are inherent in the field, such as time of egg laying and rate of tadpole development at each study site; stage of the tadpole during potential exposure to teratogens; variation in cofactors between study sites, such as water chemistry and constituents; and the intensity of UV radiation. In order to control some of these variables, laboratory experiments exposing *X. laevis* to field samples are underway (Burkhart et al., '98; Fort et al., '99a,b). In these studies, sediment extracts from different sites produced different types and percentages of malformations and extracts of sediment combined with site water had greater teratogenic affect than extracts mixed with laboratory water, suggesting that multiple factors are likely contributing to malformations. In some cases, when these investigators added thyroid hormone to the field extracts, malformations in the test frogs were ameliorated, suggesting that alteration of the thyroid hormone signal may be contributing to these malformations. Still, interpretation of results need to be uniform between experimental and field studies, and our understanding of amphibian physiology and biochemistry remains limited. The outcome of teratogenic activity on limb development may, for instance, depend on unique characteristics at the study sites that have not yet been identified.

Limb truncations at various levels comprised nearly one-half the malformations seen in the present study, and approximately 75% of the reductions occurred in the stylopodium and zeugopodium, rather than in the autopodium. Two-thirds of the supernumerary limbs in Gardiner and Hoppe's 1999 study were also classified as "truncated distally," but truncation was not defined in terms of which bones were missing. Assuming that it referred to the complete loss of segments, this observation differed from findings in our study where all segments were represented in the supernumerary limbs although some lacked specific elements. The closest similarity was one limb that lacked all digits but had the autopodium represented by the tibiale or fibulare.

There has been some speculation that truncated limbs in these newly metamorphosed frogs could be due to amputation by predators. Malformed limbs are likely to increase the vulnerability of newly metamorphosed frogs to predation. However, several lines of evidence from this study do not support predation of metamorphosed frogs as a major contributor to current deformation among free-living frogs. The truncated limbs examined in these newly metamorphosed frogs did not present with obvious evidence of soft tissue reactions such as scarring and pigment changes in the skin, hemorrhage, or inflammation normally associated with recent trauma. Frogs with amelia did not have a coxofemoral joint and 73% also had reduced pelvic elements. All pelvic elements were absent on the side of amelia in two frogs. Both frogs with phocomelia had an abnormal pelvis, and the pelvis was abnormal in 25% of frogs with femoral ectromelia. Four frogs with ectromelia were also missing the pubis on the side of the malformation. The association of abnormal pelvic structures with truncation supports an early developmental error rather than amputation.

The hind limb of a tadpole lies close to the body and tail, and it is difficult to visualize how a developing limb could be amputated without concomitant damage to adjacent tissue or to the whole tadpole. However, if traumatic amputation did occur during the tadpole stage it is possible that healing could have occurred before metamorphosis. Limb buds of *X. laevis* tadpoles can regenerate in the pre-foot paddle stages of development (Dent, '62; Overton, '63; Muneoka et al., '96). The toad, *Bufo andersoni*, will produce a normal hind limb even after amputation of the limb at the foot paddle stage when the 4th and 5th digital ridges are present (Niazi, '78). Some regeneration may take place thereafter, but regeneration becomes increasingly incomplete until metamorphosis when it fails completely. The corresponding stage specificity for regeneration is not known for *R. pipiens*, but we assume it to be simi-

lar. There remains the possibility that injury to the limb bud by trauma, parasites, predators, or infection, together with an additional teratogenic insult, may result in abnormal limb bud regeneration (Niazi and Saxena, '78; Scadding and Maden, '86). Although amputated limb buds could regenerate completely on their own at the stages noted above, when limb buds are exposed to chemicals such as retinoic acid, malformations occur whether the limb bud is undamaged (Scadding and Maden, '86), or previously amputated (Niazi and Saroj, '78; Scadding and Maden, '86). Variations in type of malformations produced by retinoic acid were influenced by dose, duration of exposure, and by whether or not limb bud trauma was part of the experimental regime. In these studies, the presence of retinoic acid was required for malformations to result from amputation. The implication for a natural environment is that limb bud damage during early repair-competent stages would likely require exposure to a teratogen to produce a malformation.

Bone bridges may represent an error in pattern completion. Alternatively, bone bridges may represent bone repair. Radiographic documentation of changes that occur during normal fracture healing in frogs has not been performed. However, a microscopic study of bone repair in frogs describes changes that may have similarities to our radiographic findings associated with bone bridges (Pritchard and Ruzicka, '50). If bone bridges are a consequence of bone repair influenced by weight-bearing forces, they would have to occur after metamorphosis and could be a consequence of bending or fracture of poorly ossified bone. Alternatively, if skin webs are restricting the normal development of long bones they might mechanically restrict normal bone formation in tadpoles, resulting in bone bridges. Bone bridges in the primary limbs of frogs in the present study occurred most frequently at the site in Minnesota that was identical to the Gardiner and Hoppe study and an additional Minnesota site (Table 4). Gardiner and Hoppe report similar lesions, which they term "bony triangles," in 41 of 56 of the supernumerary limbs in their study, and all their frogs had some degree of skin webbing.

This report provides a baseline for classifying the range of hind limb malformations in leopard frogs that survive to metamorphosis. However, the malformations presented here do not represent the full variety of malformations in the free-living frogs. Limb malformations likely increase the vulnerability of newly metamorphosed frogs to predation and malformed frogs do not appear to successfully overwinter, as they are rarely found in Spring. There may also be phenotypes that are fatal to the tadpole. Malformations submitted to the National Wildlife Health Center that did not involve hind limbs were also not included in this report and will be described in subsequent papers. Recognizing the complexity and potential interactions among factors that might influence patterns of development, it is critical that interpretation of future experiments and field investigations be done with great care using methods, terminology, and interpretation that will allow comparison among studies. Taken together, the observations that similar phenotypes occur in frogs collected at the same time from a particular site suggest that tadpoles in a given location and in the same stage of limb bud development may have experienced a common combination of insults. The convergent environmental and developmental events then materialized as malformations that share common characteristics. Tadpoles at other stages of development may have received a similar insult, but interpretation in a different ontogenic context suggests that they may have developed different malformations, or may even have developed normally.

From the broad range of malformations in *R. pipiens*, it is clear that there is no simple and effective way to utilize the current laboratory-based molecular insights about limb development to explain the complexity of environmentally induced malformations in wild anurans. Although it may be tempting to focus on one type of malformation or one potential cause, it is our opinion that the data do not lend themselves to simplification. In fact, these data are a call to widen our knowledge about the environmental causes and conditions that bring about these malformations.

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