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Environmentally Induced Limb Malformations in Mink Frogs (*Rana septentrionalis*)

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ABSTRACT In recent years, there has been an increase in the incidence of frog deformities throughout many of the northern states of North America. The most readily noticed malformations involve the hindlimbs of peri-metamorphic animals. We have analyzed skeletal preparations of metamorphosing mink frogs (*Rana septentrionalis*) collected from a site in Minnesota, in order to develop a better understanding of the possible causes. In this paper we describe the categories of abnormalities found at this site. The spectrum of deformities includes missing limbs, truncated limbs, extra limbs (including extra pelvic girdles), and skin webbings. We also describe a newly recognized malformation of the proximal-distal limb axis, a bony triangle. In this abnormality, the proximal and distal ends of the bone are adjacent to one another forming the base of a triangle. The shaft of the bone is bent double and protrudes laterally, the midpoint of the bone forming the apex of the triangle. In this paper we consider several recently proposed explanations for the recent outbreak of amphibian deformities. Based on our analysis, we conclude that the spectrum of abnormalities seen in these frogs is remarkably similar to the range of abnormalities that has been reported as a result of exposure of developing vertebrates to exogenous retinoids. Given the potential implications of this possibility for the welfare of humans as well as wildlife, further studies are needed to determine whether environmental retinoids are responsible for the frog deformities at the site we have examined. *J. Exp. Zool. 284:207–216, 1999.*

In recent years there has been an unusual increase in the occurrence of deformed amphibians in North America. Although deformed amphibians have been periodically observed in the wild (Rostand, '58; Van Valen, '74), occurrences historically have been episodic, restricted to scattered locations, and usually affecting a single species at a given site (Hoppe, '99). In contrast, the recent occurrence of amphibian malformations is persistent, widespread, and affects multiple species, often at the same site.

It is unclear when the recent epidemic of amphibian malformations first started. The field surveys by the Canadian Wildlife Service in the summers of 1992 and 1993 clearly established that significant numbers of animals at specific sites were deformed. Each summer since that time, large numbers of animals have been observed, particularly in northern (mid-west and eastern) states of the United States and in southeastern Canada (Narcam, '97). The issue of amphibian malformations has been the subject of several workshops sponsored by agencies of the United States Government (USEPA, NIEHS, NSF). The consensus developed at these workshops is that the background rate of amphibian malformation is likely less than 1%. Hoppe ('99), for example, found a frequency of 0.2% malformed frogs among 2,433 preserved *Rana pipiens* collected in Minnesota from 1958 to 1963. Recent reports (e.g., Bonin et al., '97; Ouellet et al., '97) put the current abnormality rate much higher and place the incidence of deformities well above background. This raises the possibility that a recent environmental change is inducing congenital malformations in the amphibians of North America.

Although multiple organ systems have been reported to be abnormal in deformed frogs, limb deformities are the most apparent and universal abnormalities. Fortunately, the developing and regenerating tetrapod limb is the most extensively studied of all organ systems. Progress in recent

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years has led to a considerable understanding of the developmental and molecular mechanisms controlling limb growth and pattern formation. These discoveries have been facilitated by experimental approaches that induce limb malformations either genetically or chemically as a way of identifying the important steps in the regulatory cascade controlling limb development. For that reason, there is a wealth of information about the types of malformations that are induced in response to disruption of the various developmental signaling pathways involved in the control of limb development.

In this paper we report the results of an analysis of the anatomy of environmentally induced limb malformations in a severely affected species of anuran amphibian. We draw upon the wealth of information about developmental and molecular mechanisms controlling limb growth and pattern formation to generate inferences as to what agent or agents in the environment are likely to be responsible for inducing these congenital malformations.

MATERIALS AND METHODS

Specimens of newly metamorphosed mink frogs (*Rana septentrionalis*) were collected at one lake in central Minnesota in late June and early July of 1996 and 1997. These frogs were dead or dying during episodes of high mortality that apparently are associated with high frequencies of malformation at this site. Animals were preserved in 10% neutral buffered formalin and stored in 70% ethanol. For skeletal analysis, specimens were eviscerated, postfixed with Bouin's fixative and processed for Victoria Blue staining (Bryant and Iten, '74). Stained specimens were cleared and stored in methyl salicylate for subsequent analysis and photography.

RESULTS

A total of 27 deformed frogs were examined. All had abnormal hindlimbs, whereas none had abnormal forelimbs. For skeletal analysis, we prepared 23 of these animals, as well as two normal animals collected from the same site, as controls. The 23 deformed animals had a total of 101 hindlimbs (Table 1). There were only seven hindlimbs on seven animals that were anatomically normal, and all of these were primary limbs (see below). In the abnormal limbs, defects were observed in the skeleton, the skin and the soft tissues. Skeletal abnormalities were present in 78% of the limbs, and skin abnormalities (webbing) were present in 79% of the limbs analyzed (Table 1). Many limbs with a normal skeletal pattern were abnormally thin. Such limbs are apparently deficient in muscle mass to varying degrees, including entirely missing muscles (not illustrated). It is likely that the associated neural and vascular tissues were also abnormal, and a detailed analysis of muscle, nerve and vascular patterns is a priority for future studies.

TABLE 1. Limb abnormalities in 101 limbs from 23 specimens of Rana septentrionalis

Limb morphology		No. of limbs
Normal		7
Abnormal		94
Skeletal dysplasia only		15
Skin webbing only		16
Skeletal dysplasia/skin webbing		63
Characterization of abnormalities		
Skeletal dysplasia		78
Triangles		41
Hypomorphism (ectromelia, ectrodactyly)		39
Tapering truncations	31	
Blunt truncations	8	
Split limbs		28
Supernumerary distal elements		20
Skin webbing		79
Inter-limb webbing	31	
Inter-segmental webbing	37	
Intra-segmental webbing	41	

Skeletal abnormalities

The most obvious skeletal abnormality was the presence of more than the normal number of hindlimbs (supernumerary or polymelia) (Fig. 1A, B, C, E). Among the many hindlimbs, a pair of limbs that may themselves be abnormal—but which are typically larger (close to normal size) and more mature than the other hindimbs—can be identified. The degree of maturity is apparent in Victoria Blue stained specimens, which stains cartilage, but not the more mature calcified cartilage or bone (Fig. 1). In addition, these larger, more mature pairs of hindlimbs are associated proximally with pelvic bones that have an appropriate anatomical association with the axial skeleton. We refer to these limbs as the "primary" limbs. By these criteria, all but one of the 23 deformed frog skeletons examined has a pair of primary limbs. The one exception lacks all hindlimb elements, including the pelvic girdle, on one side (Fig. 1B). Another specimen appears to lack a primary limb on one side based on examination of the external morphology. In actuality, a limb had

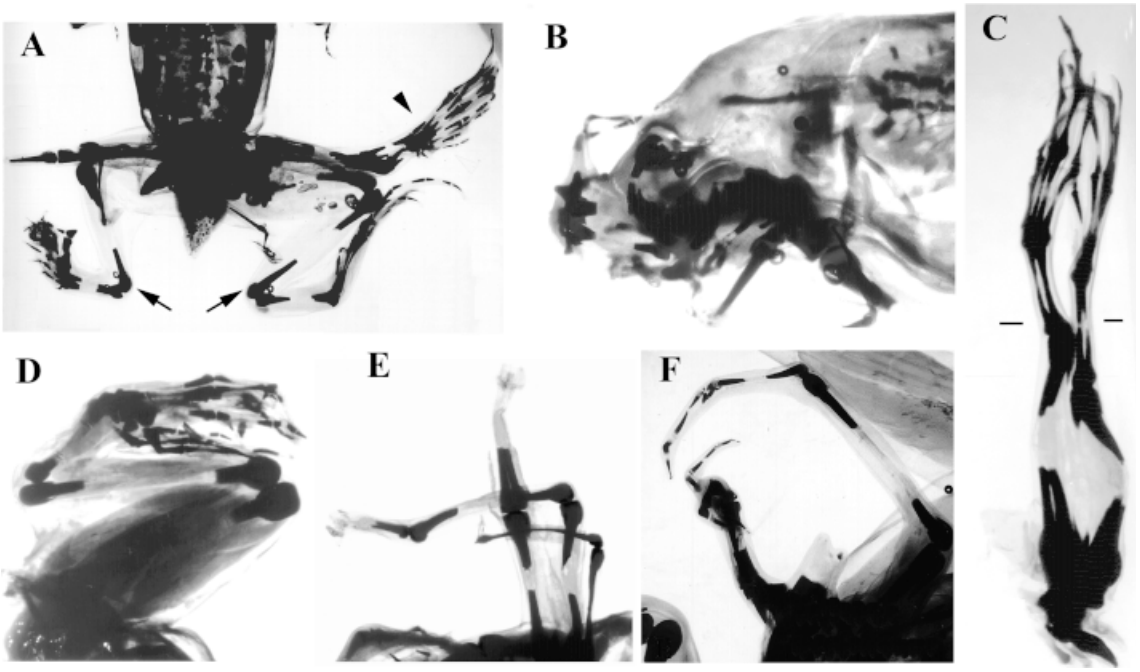

Fig. 1. Environmentally induced limb malformations in mink frogs (*Rana septentrionalis*). Cartilagenous skeletal elements have been stained with Victoria Blue and the soft tissues rendered transparent in methyl salicylate. **A:** Dorsal view of the hindlimb region of an animal with a pair of "primary" limbs (arrows) and several supernumerary limbs, including a complete pair of normal limbs projecting laterally from the right side of the body (arrowhead). **B:** Dorsal view of the hindlimb region of an animal oriented with its head toward the right of the image. This animal is missing all limb structures on the left side of the body (top), and has a complex tangle of nine limbs on the right side (bottom). **C:** An example of a "split limb" in which a complete pair of extra hindlimbs have developed within a single mass of soft tissues up to the level of the distal zeugopod (hash marks). The limbs are separated at more distal levels, giving rise to two normal feet. **D:** A hindlimb with extra distal elements. The femur, tibia-fibula, and tibiale-fibulare are normal; the foot has nine digits, rather than the normal five. **E:** Three supernumerary limbs that are incomplete distally (truncated). The two larger limbs are classified as "blunt truncations." **F:** Two supernumerary limbs that taper distally to single spikes and are examples of "tapering truncations."

formed, but because it is folded back on itself several times (Fig. 2A, D; a phenotype described in detail below), it had become embedded in the body wall (not illustrated) and thus is not observable externally.

The majority of the frogs (18 of 23) have supernumerary limbs, and these are typically smaller and less calcified than the primary limbs. The five remaining specimens each have just the two primary hindlimbs, one or both of which is abnormal. The 18 frogs have a total of 56 supernumerary limbs, with an average of three extra limbs each (ranging from 1 to 9 each). In the case of the frog with no limbs on one side (Fig. 1B), the contralateral side has a complex array of nine limbs, within which there are no obvious primary limbs. Since pairs of supernumerary limbs often arise lateral to the primary body axis (Fig. 1A), it is possible that the same could occur with the primary limbs. Thus this specimen might have a pair of laterally displaced primary limbs embedded in the tangle of nine limbs. Alternatively, the primary set of limbs may have failed to form in this specimen.

With one exception, the supernumerary limbs of all frogs are complete proximally, whether or not they are normal, incomplete (hypomorphic) or have supernumerary elements distally. The one exception to this observation is a supernumerary limb consisting of a complete set of ankle and foot skeletal elements but no other more proximal structures. This supernumerary limb originated within the connective tissue of another limb that itself is complete in the proximal-distal axis. Of the 56 supernumerary limbs, half (28) are present as paired limbs that are proximally complete and associated with supernumerary pelvic elements (Fig. 1C). Such a pair of limbs thus resulted from the induction of a supernumerary pelvis and associated limbs. The paired limbs share a common mass of soft tissues and skin proximally and then separate distally to give rise to discrete limbs. Ex-

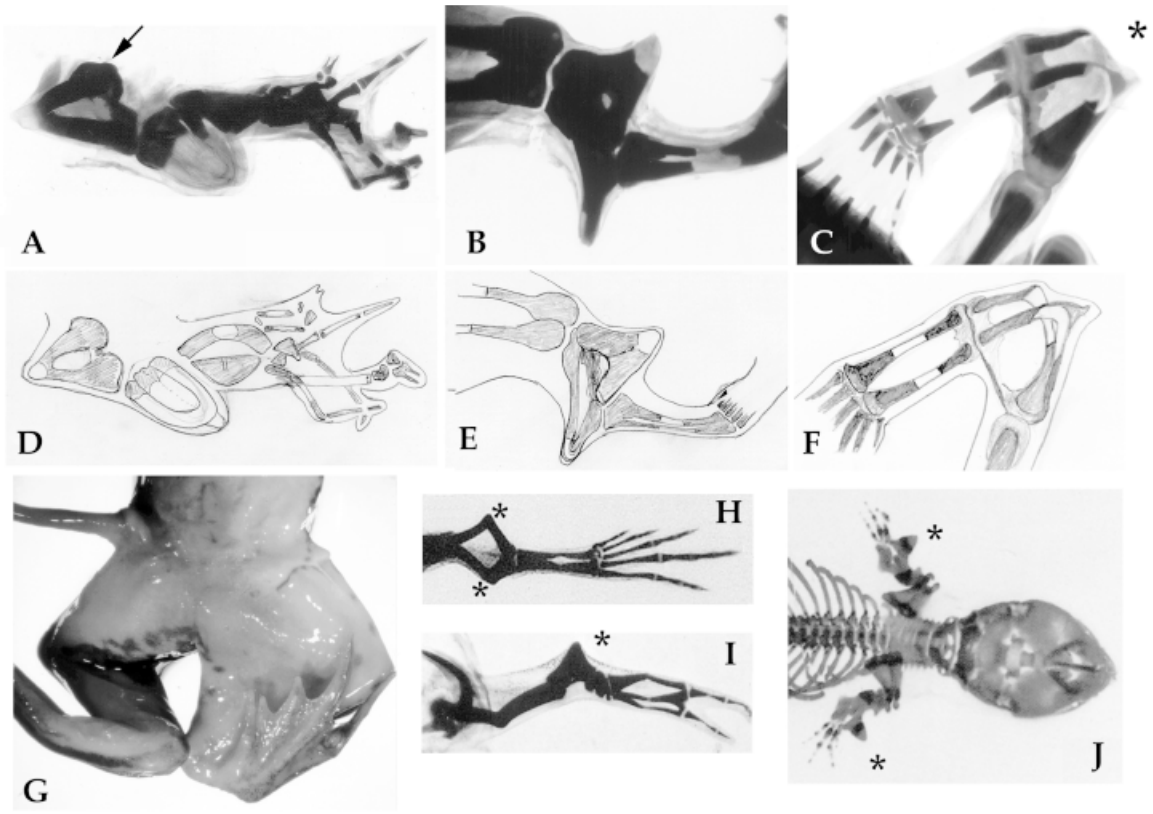

Fig. 2. Environmentally induced bony triangles and skin-webbing in comparison to retinoid-induced bony triangles. **A:** A limb from a mink frog with bony triangles in all limb segments along the proximal-distal axis. The anatomy is depicted in the corresponding cartoon (**D**) below. Proximal is to the left, distal is to the right. The proximal head of the femur (arrow) has been dissected free of the pelvic girdle. **B:** A "split limb" in which bony triangles have been induced in the zeugopodial segments of both limbs. The anatomy is depicted in the corresponding cartoon (**E**). Proximal is to the left, distal is to the right. All the skeletal elements are contained with a single mass of soft tissues, with two femurs and two tibia-fibulae connected distally to a single tibiale-fibulare and foot. **C:** A limb with a bony triangle in the zeugopodial segment of an otherwise normal limb. The anatomy is depicted in the corresponding cartoon in (**F**). The distal part of this limb bends back on itself (anteversion) and would have an immobile "knee" joint which is actually a pseudo-knee corresponding to the apex of the bony triangle (asterisk). **G:** A ventral view of the hindlimb region of an animal in which the left limb is enclosed within one continuous skin webbing. Such a limb is completely immobilized and cannot be extended. **H–J:** Bony triangles (asterisks) induced by retinoic acid treatment of developing limbs of *Xenopus laevis* (I) and the mouse (J), and regenerating limb buds of *Rana temporaria* (H). Illustrations reprinted with permission of the authors and the Company of Biologists Ltd. (I: Scadding and Maden, '86), and Academic Press (H: Maden, '83; J: Scott et al., '94).

ternally, such limbs would be classified as distally duplicated or 'split limbs' (Table 1; Narcam, '97).

Several limbs (20%, Table 1) had supernumerary skeletal elements distally (Fig. 1D). Extra distal elements occurred more frequently in primary limbs (16 out of 45, or 35%) than in supernumerary limbs (4 out of 56, or 7%). Extra distal structures included extra phalanges, metatarsals, and tarsal elements. When extra tarsals are present, they show normal anatomical relationships with extra metatarsals, which in turn are articulated with extra phalanges. The largest number of total digits associated with a single limb was 12.

Entirely missing limbs are rare, but many limbs are missing some, but not all, skeletal elements. With the exception of the one limb described above that was complete distally but incomplete proximally, all hypomorphic limbs are missing distal elements (ectromelia, ectrodactyly). A significant number of hypomorphic limbs were observed (50% of the limbs with skeletal abnormalities, Table 1), as has been reported for other species and from other locations (Ouellet et al., '97; Burkhart et al., '98). Because these limbs are incomplete distally, we refer to them as truncated and have categorized them as either "blunt" or "tapering" based on their anatomical appearance. With one exception (a tapering truncation of a

primary limb), all truncations occurred only in supernumerary limbs, and 38 of the 56 supernumerary limbs (nearly 70%) were hypomorphic.

Blunt truncations represent a minority of the truncated limbs (20%, 8 of 39). These limbs end abruptly, either at the end of a limb segment (not illustrated) or in the middle of the segment (Fig. 1E). The anatomy of the limb proximal to the level of a blunt truncation is normal. Given the jagged appearance of the end of blunt truncations ending in the middle of a limb segment (Fig. 1E), these may represent traumatic amputations.

Most hypomorphic limbs are tapering truncations (80%, 31 of 39). Tapering truncations are characterized by a progressive loss of skeletal structures at progressively more distal levels in the limb. Loss of structures occurs either as a failure of a skeletal element to develop (Fig. 1F) or as a fusion of two elements to form a single element (not shown). As a result, the anatomy of the limb proximal to the terminal end of the limb is not normal, in contrast to the situation with blunt truncations.

A large number of limbs (40% of all limbs examined and 50% of all limbs with skeletal dysplasias; Table 1) appear to have either shortened or missing skeletal elements when examined externally. When we examined these limbs in cleared and stained preparations, we discovered a novel skeletal dysplasia. The bone within a given limb segment is bent back on itself, such that the proximal and distal ends of the bone are adjacent to one another and the midpoint of the shaft forms the apex of a triangle (Fig. 2A–F). The degree of angulation varies from bowing (Fig. 2A, D) to complete folding to form a triangle (Fig. 2C, F). In what appears to be the extreme condition, the element fuses to form a solid triangular skeletal element (Fig. 2B, E). We refer to these dysplasias as "bony triangles," although they are variably ossified (bony) and chondrified. Nearly 80% of the animals we analyzed had at least one limb with at least one bony triangle.

We have observed bony triangles at all levels along the proximal-distal axis of both primary and secondary limbs at equal frequency in each (40%). In some limbs, bony triangles occurred in more than one limb segment (Fig. 2A, D); however, in most limbs, only one segment was induced to form a bony triangle (63% of the limbs with bony triangles). Because the bending occurs in the middle of a limb segment, a bony triangle results in the formation of a pseudo-joint (e.g., Fig. 2C, F). From external examination, such a pseudo-joint can appear as an immobile joint, flanked on the proximal side by a segment that appears to be too long and on the distal side by a segment that appears to be too short. Because the proximal-distal orientation of the segment is all but reversed, the animal appears to be holding its limb in an abnormal position, which would be characterized as an anteversion from external morphology. Overall, the limbs appear externally to be grossly distorted and to have partially missing skeletal elements and bony excrescences. Limbs with these characteristics have been reported previously, but the internal anatomy has not yet been described (Ouellet et al., '97).

Skin abnormalities

All 23 of the animals with limb deformities exhibited abnormal development of the skin on at least one hindlimb. Of the total number of limbs, approximately 80% had abnormal skin development (Table 1). The abnormality is characterized externally as skin webbing between limbs or limb segments that normally are separated. At an extreme, one of the limb segments is separated from the others, and the entire array of skeletal elements is enclosed within a continuous envelope of skin (Fig. 2G). At this point we do not know how skin webbings develop, and thus do not understand the developmental mechanisms involved. We also do not know if there is a functional relationship between abnormal skin development and abnormal skeletal development, a possibility we currently are investigating.

We have classified the limbs into three categories based on the type of skin webbing. About 30% of the limbs analyzed had webbing between adjacent limbs (interlimb webbing; Table 1). These included the split limbs that were enclosed proximally in a single mass of soft tissue and associated skin. Nearly 40% of the limbs had webbing between adjacent segments of a single limb (intersegmental webbing; Table 1). Intersegmental webs most commonly occurred between the femur and the tibiofibula (86%). The third category of skin webbing is intrasegmental and occurs in association with bony triangles, which by their nature result in an abnormal juxtaposition of proximal and distal within a limb segment (Table 1). As a result the two ends of the segment are interconnected by a bridge of skin that would not normally be present.

DISCUSSION

Although the malformations observed in the population of mink frogs we studied appear gro-

tesque and extreme, they are exemplary of the types of malformations being observed throughout North America (Narcam, '97). Reports to date have often been scattered and anecdotal but include one systematic field survey initiated in 1992 that has continued to this day (Ouellet et al., '97). At this time, the data on malformations from the various reports have not been systematically analyzed and compared; however, it appears that there are geographical as well as species differences in the frequencies and types of malformations observed, as reported at a recent NSF workshop. In general, the most abundant category of malformation at most sites is missing or hypomorphic limbs (Ouellet et al., '97; Burkhart et al., '98; Helgen et al., '98), whereas the occurrence of extra limbs is significantly less common. However, the predominant type of malformation can vary between sites. For example, in the site sampled here, 27% of mink frogs examined in 1997 had extra limbs, while only 7% had hypomorphic or missing limbs. In the extreme condition, none of the limb segments are separated from the others, and the entire array of skeleal elements in enclosed within a continuous envelope of skin (Fig. 2G). The frequency of malformation also varies from a background rate of less than 1%, to 10% at many sites (Ouellet et al., '97; Burkhart et al., '98), to 30% among northern leopard frogs (Helgen et al., '98), to as high as 60–80% for species such as the mink frog at the site reported in this study (D.M.H., unpublished data).

At the present time, it is difficult to directly compare morphological data between different studies. The characterization of types of malformations has been based mainly on observation of external morphology, which can be misleading, as evidenced by several of the results in this study. For example, limbs that appear missing externally can be tightly folded and held close to the body by the skin. Further, it is not possible to identify one of the most frequent of the skeletal dysplasias, "bony triangles," with certainty by external morphology. Such structures could be variously recorded as "phocomelia" (Scadding and Maden, '86), "anteversions" or "bony excrescences" (Ouellet et al., '97). Similarly, the paired limbs contained within a single mass of soft tissues observed in the present study would be reported as "split limbs" (Narcam, '97).

In spite of variability in the identification and categorization of the types of malformations, there is general agreement that they represent environmentally induced congenital malformations. Since the same types of malformations occur in multiple species over a wide geographical range, it is extremely unlikely that a specific genetic alteration has arisen independently on multiple occasions and in multiple species. Given the conservation of the mechanisms controlling limb development in vertebrates, it is likely that there are one or more environmental agents disrupting the normal functioning of these developmental processes. Although there may be multiple agents responsible for inducing these malformations, they share the common property of targeting and disrupting the developmental signaling pathways controlling growth and pattern formation in the developing limb.

As in the mink frogs we analyzed, nearly all the limb malformations reported for other species and at other locations are restricted to the hindlimbs. There are reports of forelimb malformations occurring at low frequencies (Ouellet et al., '97), and there is one report from Japan where all the limb deformities within one population occurred in the forelimbs (Takeishi, '96). The striking bias towards hindlimb defects likely reflects a fundamental property of the mechanism of teratogenesis; however, at this time, it is unclear what that might be. One possibility is that the agent(s) causing the deformities specifically causes disruption via an aspect of hindlimb development that is unique and distinct from that of forelimbs. Though the mechanisms specifying the differences between forelimb and hindlimb development are not well understood, candidate genes have been identified. These include members of the HoxC complex and Tbx genes that are differentially expressed in forelimbs (Hoxc-6 and Tbx-5) and hindlimbs (Hoxc-10 and Tbx-4) (Oliver et al., '88; Burke et al., '95; Burke and Tabin, '96; Chapman et al., '96; Gibson-Brown et al., '96, '98; Isaac et al., '98; Logan et al., '98). It is also possible that there is differential mortality associated with the induction of forelimb malformations. In such a case, an agent that disrupts development in the anterior region of the embryo, in the region of the forelimbs, might also induce corresponding malformations in other anterior organs such as the heart and brain. These latter malformations would likely be more lethal than malformations at a more posterior level, and thus animals with forelimb malformations would be unlikely to survive. Although the bias towards hindlimb defects likely provides a clue concerning the nature of the environmental agent(s), its interpretation will require additional information.

Although there may be multiple agents capable

of inducing congenital malformations in frogs, there are a limited number of mechanisms by which such agents can act to disrupt normal limb development. To date, several possible agents have been suggested to cause frog malformations, for which the proposed mechanisms fall into three categories: mechanical disruption, ultraviolet (UV) irradiation, and chemicals. There is experimental evidence indicating the feasibility of these mechanisms in the induction of at least some types of malformations.

A large number of experiments involving surgical rearrangement or removal of limbs tissues have demonstrated that malformations can be induced by mechanical means. Although early-stage anuran limb buds undergo regeneration in response to amputation, there is a progressive loss of regenerative abilities at later stages of development. Thus a late-stage amputation caused by a predator that removed the limb without killing the tadpole could result in a truncated limb. However, analyses to date have not found evidence of traumatic limb amputation in frogs with hypomorphic limbs. Further, amputation could not account for animals that lack all limb structures, including pelvic girdles, and it is difficult to imagine that an animal with hypomorphic limbs on both sides of the body was attacked by predators twice and survived both attacks with only the loss of the limbs.

Both hypomorphic limbs and limbs with supernumerary structures can be created by microsurgical rearrangement of limb tissues to create chimeric limbs with altered interactions between limb cells (French et al., '76; Bryant et al., '81). Supernumerary structures can include duplications of distal limb elements; however, such experiments do not result in the induction of duplicated structures at extreme proximal levels (pelvic girdles) or the induction of bony triangles. In such experiments, the induction of supernumerary limb structures requires the juxtaposition of tissues from opposite sides of the limb, and it is difficult to imagine how such cellular rearrangements could occur in nature with the precision required to induce the observed malformations. It has been proposed that trematode parasites could cause cellular rearrangements within limb buds during parasite infection and in so doing, mimic microsurgery. Consistent with this idea is the observation that the implantation of multiple, very small resin beads as mimics of parasite cysts can induce the formation of extra distal structures in both *Xenopus* and *Ambystoma* (Sessions and Ruth, '90). In the mink frogs we examined, there were no massive parasite infections such as those previously suggested as causes of limb malformations (Sessions and Ruth, '90), and no trematode cysts were observed in association with either extra distal structures, hypomorphic limbs, or bony triangles.

In recent laboratory experiments (Ankley et al., '98), it has been demonstrated that ultraviolet irradiation can induce hypomorphic limbs. The hypomorphic limbs are "blunt" and appear comparable to what is observed when the apical epidermis (AER) is removed from developing limb buds (Saunders, '48; Tschumi, '57). During development, the AER is required for continued outgrowth, and treatments that interfere with its function result in truncation of limb outgrowth. It seems reasonable that the mechanism of action of UV exposure is to interfere with the normal functioning of the apical limb bud epidermis. This interpretation is supported by earlier studies in newts that showed that UV irradiation kills the epidermis of regenerating limbs (Thornton, '58). The relationship between the UV dosage under the experimental conditions and the dosage in the field has yet to be determined.

Although UV can induce hypomorphic limbs, the relationship between these experimentally induced malformations and those observed in animals from the field is unclear. First, the UV-induced truncations are characteristically "blunt" rather than "tapering" as is the case in the majority of the mink frogs in the field. In addition, the UV effects are bilateral and roughly symmetrical on both sides of the affected animals, with both limbs exhibiting blunt truncations at the same proximal-distal level. This is in contrast to what is observed in mink frogs in the present study, as well as what has been reported for other species at other locations, in which the malformations tend to be unilateral or bilaterally asymmetrical. Although both left and right limbs may exhibit malformations, the types of malformations are usually not the same, and often one limb is normal whereas the other is malformed. Asymmetrical teratogenic responses have been observed previously and have been hypothesized to occur as a result of asymmetries in the blood circulatory system of the organism (Wise and Scott, '86). By inference, the asymmetrical occurrence of the various frog malformations could occur as a result of exposure to chemicals via the circulation rather than directly through the epidermis of the skin. In contrast, the symmetrical malformations induced by UV light would be indicative of a relatively uniform exposure externally.

There are many experimental studies demonstrating that chemical agents can disrupt normal embryonic development and induce congenital malformations. Collectively these studies form the basis for the scientific disciplines of developmental toxicology and teratology. In fact, much of what is known about normal development is derived from controlled, experimentally induced teratogenesis by both chemical and genetic means. Recent advances in techniques in molecular genetics have enabled direct tests of the function of molecules in controlling limb growth and pattern formation. As a result, several endogenous chemicals have been identified whose normal patterns of expression are necessary for normal limb formation and whose absence or misexpression leads to limb malformations. These include molecules such as shh, wnt, fgf, rfng and bmp that are involved at various points in several signaling pathways (Johnson et al., '94; Johnson and Tabin, '97).

We are struck by the fact that there exists one class of chemicals, the retinoids, that have been shown in experiments to be able to induce all of the separate limb malformations observed in frogs in the wild. A large number of studies have demonstrated that retinoids, in particular retinoic acid, are teratogenic for many stages of vertebrate development, including the stages of limb development (Kochhar, '77; Kochhar et al., '84; Lammer et al., '85). The mode of action of retinoids is well characterized, and the effects, both normal and teratogenic, are mediated through activation of members of the nuclear hormone receptor family (RARs and RXRs) by endogenous as well as synthetic retinoids. Retinoids have important functions in normal development, and any experimental treatment that results in either abnormally high or low levels of retinoic acid results in abnormal development (Bryant and Gardiner, '92; Maden et al., '96; Stratford et al., '96; Tanaka et al., '96; Lu et al., '97; Crawford and Vincenti, '98).

In the case of the frog malformations, a parsimonious explanation is that an environmental agent is disrupting a retinoid-sensitive signaling pathway. Candidates would include retinoid mimics, retinoid antagonists or inhibitors, as well as agents that interfere with the functioning of molecules that are either upstream or downstream of retinoids. Upstream targets could include genes involved in the synthesis or removal of retinoids (Båvik et al., '97; Hoffmann et al., '98) or relevant transcription factors. Downstream targets include Hox genes such as Hoxb-8, which in turn control the expression of other transcription factors and of signaling molecules such as sonic hedgehog (Lu et al., '97; Stratford et al., '97).

Among the spectrum of teratogenic effects of retinoids that correspond to the observed frog malformations, the induction of extra hindlimbs and the induction of bony triangles (Fig. 2H, I, J) are particularly significant since we know of no other chemical that can induce these malformations. Extra hindlimbs can be induced by retinoid exposure during normal development of mouse embryos (Rutledge et al., '94; Niederreither et al., '96) and during tail regeneration in several species of frogs (Mohanty-Hejmadi et al., '92; Maden, '93; Maden and Corcoran, '96). Images in published papers show that bony triangles can be induced by retinoid exposure during normal limb development in many vertebrates, including frogs, chick, mouse and humans (Tickle et al., '85; Scadding and Maden, '86; Rizzo et al., '91; Maden, '93; Scott et al., '94; Die-Smulders et al., '95). The specific mechanisms whereby these malformations are induced are unknown. However, in the case of the induction of extra hindlimbs in frogs, there appears to be an interaction between retinoid signaling and signaling by another member of the nuclear hormone receptor family, thyroid hormone (Maden and Corcoran, '96).

The characteristics of the bony triangle dysplasia provide information about the nature of the exposure to the teratogenic agent. In affected limbs, triangles are most often found in a single segment (63% of the limbs with bony triangles; e.g., Fig. 2B, C, E, F), but that segment can be at any level along the proximal-distal axis. Given that the proximal-distal limb axis is specified and differentiates in a proximal to distal sequence (Saunders, '48), segments will be sensitive to teratogens in a sequence from proximal first to distal last. From this we can infer that limbs that form a bony triangle in a single segment (e.g., the zeugopod [Fig. 2B, C, E, F]) were exposed to the teratogenic agent during a developmental window in which only the zeugopod was sensitive. In addition, because in this example the earlier formed (more proximal) and the later formed (more distal) segments are unaffected, we infer that the teratogenic signal was not present continuously during the period of limb development. Although the length of the sensitive period for individual segments is unknown, the entire proximal-distal limb axis of *Xenopus laevis* forms over a period of about two weeks (Nieuwkoop and Faber, '56). Hence, it is likely that the maximal length of the sensitive window for an individual segment is 5–

6 days, and it is likely that an exposure of just a few days could induce a bony triangle in a single limb segment. Our analysis of the bony triangle dysplasia leads us to the strong inference that the frog malformations arise as a consequence of acute exposures—either a single exposure, or more likely, a series of exposures to a teratogenic agent—rather than a continuous, chronic exposure. Sampling protocols that attempt to identify the agent(s) responsible for these malformations need to accommodate this likelihood.

Although the environmental agent(s) responsible for inducing the frog malformations have yet to be identified, it is evident that the mechanism of teratogenesis either directly or indirectly involves disruption of a retinoid-sensitive developmental pathway. Therefore, it is possible that at least one such agent is an environmental retinoid, which is of concern given the fundamental role of retinoids in the regulation of normal development of most, if not all, organ systems. Given that the molecules involved in developmental signaling pathways are conserved structurally and functionally among all vertebrates, a retinoid that disrupts the development of mink frogs in Minnesota will likely have adverse effects on the development of other vertebrates, including humans.

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