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Carol U. Meteyer
National Wildlife Health Center

Rebecca A. Cole
National Wildlife Health Center

Kathryn A. Converse
National Wildlife Health Center

Douglas E. Docherty
National Wildlife Health Center

Mark Wolcott
National Wildlife Health Center

See next page for additional authors

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Defining Anuran Malformations in the Context of a Developmental Problem

CAROL U. METEYER[1], REBECCA A. COLE[1], KATHRYN A. CONVERSE[1], DOUGLAS E. DOCHERTY[1], MARK WOLCOTT[1], JUDY C. HELGEN[2], RICHARD LEVEY[3], LAURA EATON-POOLE[4], and JAMES G. BURKHART[5]

[1]U.S. Geological Survey, National Wildlife Health Center, 6006 Schroeder Road, Madison, Wisconsin, 53711 Phone: 608-270-2462 Fax: 608-270-2415
[2]Minnesota Pollution Control Agency, 520 Lafayette Road, St. Paul, Minnesota, 55155 Phone: 612-296-7240 Fax: 612-297-8683
[3]Vermont Agency of Natural Resources, Department of Applied Ecology and Aquatic Studies Section, 103 Main Street, 10 South Waterbury, Vermont 06571-0601 Phone: 802-241-1368 Fax: 802-241-3008
[4]Ecological Service—Region 5 United States Fish and Wildlife Service, 22 Bridge Street Unit - 1 Concord, New Hampshire Phone: 603-225-1411 Fax: 603-225-1467
[5]National Institute of Health—National Institute of Environmental Health Science, Environmental Toxicology Program, MDC4-07: NIEHS, 111 Alexandra Drive, Research Triangle Park, North Carolina 27709, Phone: 919-541-3280 Fax: 919-541-3137

This paper summarizes terminology and general concepts involved in animal development for the purpose of providing background for the study and understanding of frog malformations. The results of our radiographic investigation of rear limb malformations in *Rana pipiens* provide evidence that frog malformations are the product of early developmental errors. Although bacteria, parasites and viruses were identified in these metamorphosed frogs, the relevant window to look for the teratogenic affect of these agents is in the early tadpole stage during limb development. As a result, our microbiological findings must be regarded as inconclusive relative to determining their contribution to malformations because we conducted our examinations on metamorphosed frogs not tadpoles. Future studies need to look at teratogenic agents (chemical, microbial, physical or mechanical) that are present in the embryo, tadpole, and their environments at stages of development that are relevant for the malformation type. The impact of these teratogenic agents then needs to be assessed in appropriate animal models using studies that are designed to mimic field conditions. The results of these laboratory tests should then be analyzed in such a way that will allow comparison with the findings in the wild-caught tadpoles and frogs.

INDEX DESCRIPTORS: amphibians, environmentally-induced malformations, malformations, teratogenesis, *Rana pipiens,* microbiology.

Malformations represent the end point of processes that have incorporated errors early in development; the causes are temporally distant from the effects we see in the fully developed animal. An understanding of normal developmental principles is necessary to identify and define the errors occurring in abnormal development.

Development begins at fertilization with initiation of intricate interrelationships between cells and cell layers resulting in very organized and highly specialized tissues. The development of organs and other specialized structures is called morphogenesis. Morphogenesis occurs through cell division and proliferation, cell migration, cell differentiation and programmed cell death (apoptosis). As cells divide they follow well defined, genetically predetermined maps. As cell layers interrelate to each other, they profoundly influence the growth and character of cells around them. The timing of this intercellular communication is critical and is carried out by chemical signals that affect gene expression.

Limb bud formation is initiated by the committed mesoderm which carries all of the information needed to induce a limb as it interacts with the overlying ectoderm. If the lateral mesodermal plate (Fig. 1) is surgically transplanted onto the back of a tadpole, an entire limb will form in response to that committed mesoderm (Gilbertson 1997). During later morphogenesis of the limb, the apical ectodermal ridge (Tarin and Sturdee 1971 and 1974) is responsible for providing the cellular environment, called the progress zone (Fig. 1), that allows the limb to continue growing, and the mesoderm becomes responsible for directing bone formation. In limb development as in other organs, morphogenesis is achieved through cell division, migration, differentiation and apoptosis. If something goes wrong with one of these processes and produces pathology, it is called a malformation or a deformation.

Malformations are primary errors in development. Deformations are usually considered to arise later in development as the result of mechanical factors (such as amputation) that alter shape or anatomy of a structure that has otherwise developed normally (Doige 1988). The occurrence and the type of malformation produced by an error in the developmental process is influenced by the type of abnormal event and the developmental stage at which the error occurred. Therefore, the phenotype of a malformation can be used to indicate the stage at which the developmental error occurred. If the malformation is an incomplete organ, such as an incomplete limb, then the factor or insult acted during a susceptible period prior to organ completion (Robbins et al. 1984).

Teratogenesis is the name given to the abnormal development, at any level of morphogenesis, which results in malformations. Development is complex, therefore the manifestations of teratogens are also complex. Teratogenesis can be caused by genetic or by environmental factors which are intra-uterine factors in mammals. The same teratogen presented at different developmental stages can initiate

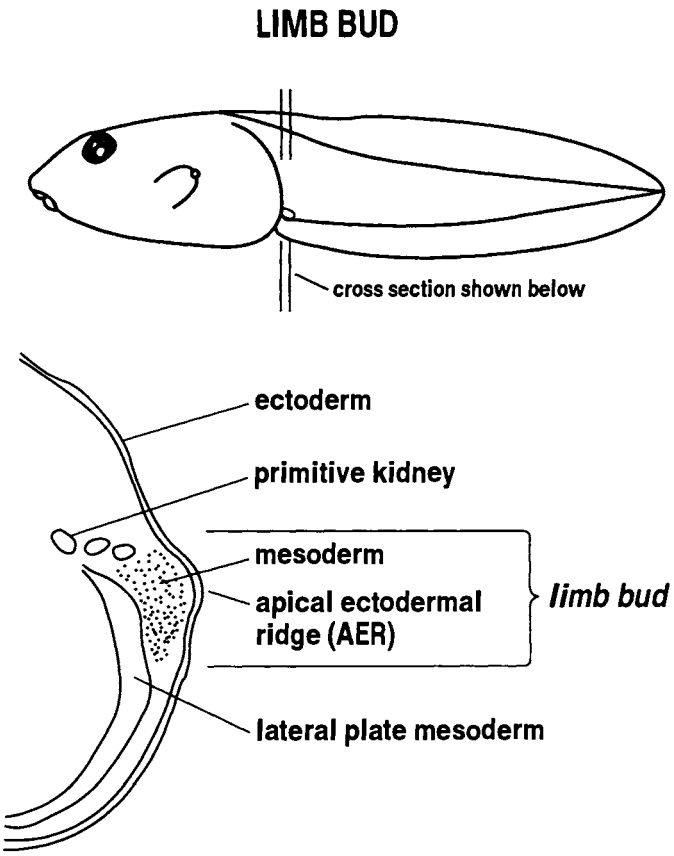

Fig. 1. Anatomy of a limb bud adapted from Gilbert, S.F. (ed). 1997. Development of the tetrapod limb. In: *Developmental Biology,* pp. 704.

different malformations, whereas different teratogens presented at the same developmental stage can result in similar errors leading to the same malformation. Examples of environmental factors that can cause malformations include radiation, hyperthermia, poor nutrition, low oxygen, high carbon dioxide, and chemicals. A few examples of natural and man-made chemicals that can cause teratogenesis are hormones, retinoic acid and its analogues, mercury, cadmium, and chemotherapeutic agents (Robbins et al. 1984, Rutledge et al. 1994). Chemicals can influence development by altering intracellular function and intercellular communication. Anything that causes trauma or death to populations of cells at critical stages of organogenesis can also cause malformations. Chemotherapeutic chemicals work through this mechanism as well as some infectious agents. To increase complexity, teratogenic agents may work singly, or synergistically and may be influenced by site-specific variables.

Teratogenic viruses may not be pathogenic in adults, but fetal infection can cause tissue injury and cell death during early stages of organ development (Jubb et al. 1993). Apoptosis is the developmental process that allows for tissue remodeling during normal organ development. Current work suggests that some viruses might cause malformations through abnormal induction of apoptosis as well as inhibition of apoptosis (Kosugi et al. 1998). Again, the pattern of viral induced malformations corresponds to the developmental stage of the embryo or fetus at the time of infection.

The anatomy of a malformed metamorphosed frog can give us an idea of the approximate window during which the developmental insult was initiated and might even suggest the type of event (mechanism) that may have occurred, however, morphology does not dictate the cause. To define causal mechanisms of frog malformations we need to use well designed investigations that are different from traditional tests used in acute toxicity or disease pathogenicity studies. Toxicity assessment studies typically establish the dose of a chemical that will kill 50% of the animals exposed (LD 50 dose). Pathogenicity tests define the clinical signs, pathology and immune response that is characteristic of animals experimentally infected with an organism. Both of these diagnostic approaches evaluate causes and effects that are closely related in time using results that are relatively easy to measure. When investigating malformations in metamorphosed frogs, however, we are looking at the effect of exposure to an agent that occurred early in tadpole development. Studies to determine causes of malformations need to look at agents that are present in the field and in the tadpoles at these early developmental times. Laboratory experiments need to expose embryos and tadpoles to suspect agents at appropriate developmental stages and look at acute results as well as the entire developmental process to determine the actual teratogenic impact of an agent. This means holding animals past metamorphic climax to assure that the anatomy and physiology of the adult have developed to completion. As we look at field collections of abnormal frogs, we need to keep in mind that these collections reflect survivors only. We are looking at malformations that were not fatal to tadpoles. We cannot assume that because we do not collect other malformations, they did not exist. More work needs to be done on the developing tadpole, in the field and in the laboratory, to better elucidate the range, frequency, character and causes of anuran malformations.

MATERIALS AND METHODS

Specimen Collection

This 1997 study of frog malformations was conducted on northern leopard frogs (*Rana pipiens*) from three sites in Minnesota (50 frogs) and four sites in Vermont (70 frogs). These sites were chosen as study sites because they had 7% malformed newly metamorphosed frogs examined in 1996. Five frogs without malformations were submitted from each Minnesota study site. Normal frogs were submitted from a fifth Vermont site which had less than 2% malformations detected in newly metamorphosed frogs in 1996. Frogs were shipped alive in hard sided coolers via overnight delivery to the National Wildlife Health Center (NWHC).

Specimen Examination

Frogs were given brief clinical examinations and anesthetized by partial submersion in a 1:2000 dilution of MS 222 in saline within 36 hours of arrival. Once the frogs were nonresponsive to external stimuli, they were photographed and received external and internal examinations. Internal organs were removed for histopathology and microbiology. Frogs were 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 future radiography.

Radiography

All radiographs were taken with the frogs on their backs, creating a ventral-dorsal (VD) view. Frogs were radiographed without magnification using a *Faxitron* Specimen Radiography System Model MX-20. Radiographs were exposed at 18 kilovolts for 120 seconds with 0.3 milliamps of continuous current. After initial VD radiographs, frogs with malformations of the pelvis and digits were radiographed again, at 4× magnification to clearly define pelvic and

Table 1. Tissue location of metacercariae or mesocercariae in 1997 metamorphosed frogs classified by malformation.

	Pancreas	Head	Eyes	Intestinal contents	Lung	Liver	Kidney	Muscle	Subcutis
Vermont									
Amelia									
Poultney	−	−	−	−	+	+	+	+	−
Alberg	−	−	+	−	−	+	+	−	−
Hemimelia									
Poultney	−	−	−	+	+	+	+	−	+
Alberg	−	+	+	−	+	+	+	+	+
Lapans	−	+	+	−	−	+	+	+	−
Ectrodactyly/Brachydactyly									
Poultney	−	−	+	−	+	+	+	−	+
Missisquoi	+	+	+	−	+	+	+	−	−
Lapans		+	+	−	+	+	+	−	−
Normal									
Poultney	−	+	+	−	+	+	+	−	−
Alberg	+	−	+	−	+	+	+	−	−
Missisquoi	−	−	−	−	−	−	+	−	−
Lapans	+	−	+	−	−	+	+	−	−
Mud Creek	+	−	+	−	−	+	+	−	−
Mud Creek	+	−	+	−	−	−	+	−	−
Minnesota									
Amelia									
NEY							+	−	−
NEY							+	−	−
Hemimelia									
CLE							−	+	+
SUN							+	−	−
Ectrodactyly/Brachydactyly									
SUN							+	−	−
Rotation									
CLE							+	−	+
Craniofacial									
SUN							+		
Normal									
CLE							−	+	+
SUN							+	−	−
SUN							+	−	−
NEY							+	−	+
NEY							+	−	−

digital structures. Oblique positioning was used to radiograph frogs with malformations that were obscured by overlying bone.

Virology

Virus isolation was attempted on five frogs from each site as well as frogs that had tissue pathology. Liver, spleen, and kidney from each frog were pooled as a single inoculum for fathead minnow epithelial tissue culture, rainbow trout gonadal tissue culture, and bullfrog tongue fibroblast tissue culture (American Type Culture Collection, Rockville, Maryland). Viral isolates from cell cultures were identified and characterized using electron microscopy. Direct electron microscopy was also used on homogenized pooled tissues in an attempt to detect any viruses that were not isolated in cell culture.

Bacteriology

Liver, heart, and kidney, as well as any tissue with visible pathology, were placed directly into tryptic soy broth (TSB) at necropsy. Tubes of TSB broth with tissue were vortexed for 5–10 seconds and subcultured immediately onto blood agar, eosine-methylene blue agar or MacConkey agar, sabouraud-dextrose agar, thioglycollate broth, and cooked-meat broth (CM). The media was examined at 18–24 hour intervals for up to 72 hours before being discarded as negative. Positive cultures were subcultured and identified at least to genus level. Tryptic soy broth that was negative (no turbiditiy of TSB) on initial culture after 24 hours was subcultured for an additional 24 hours. Sabouraud-dextrose agar was held until sufficient growth developed or there was no growth at the end of 7 days.

Table 1. Extended.

Skin	Tail bud	Rear leg muscle	Back muscle	Abd muscle	Coelom	Gastro-intesti-nal serosa	Urinary bladder	Esoph-agus	Heart	Hard palate	Fat body
+	−	+	−	+	−	−	+	−	−	−	−
−	+	−	+	−	−	−	−	−	−	−	+
−	+	−	+	+	+	−	−	−	−	−	−
+	+	−	−	−	+	−	−	−	−	−	+
+	−	−	+	+	−	−	−	−	−	−	−
−	+	+	+	+	+	+	−	−	+	−	−
+	+	−	+	−	+	−	−	−	+	−	+
+	+	−	+	+	−	+	−	+	+	−	−
+	+	−	−	−	+	−	−	−	−	−	−
+	+	+	+	+	+	+	+	−	−	−	−
−	−	−	−	−	+	−	−	−	−	−	−
+	+	−	+	−	−	−	+	+	−	−	+
+	−	−	+	−	−	−	+	−	−	−	+
+	+	−	+	+	+	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−
+	+	−	−	−	−	−	−	−	−	−	−
+	+	+	+	+	+	+	+	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−
+	−	−	−	−	−	−	+	−	−	−	−
−	+	−	−	+	−	−	−	+	+	−	−
+	+										
−	+	−	−	−	−	−	−	−	−	+	−
+	−	−	−	−	−	−	−	−	−	−	−
−	−	−	+	−	−	−	−	−	−	−	−
−	+	+	−	−	−	−	−	−	−	−	−
−	+	−	−	−	−	−	−	−	−	−	−

Bacterial isolates were identified using commercial identification systems, primarily the API system (bioMerieux Vitex, St. Louis, Missouri).

Parasitology

All examinations of frogs and respective tissues were done with the aid of a dissection scope and/or microscope. Frogs collected from Vermont sites were skinned and muscles and fascia examined. Hind limb muscles were teased apart and examined for metacercariae and mesocercariae (Table 1). Internal organs from Minnesota and Vermont frogs were examined grossly, parasites removed, prepared for fixation and fixed in an alcohol formalin acetic acid fixative or 70% ETOH depending on the class of parasite (Laboratory Procedures in Parasitology, 1961). Eviscerated carcasses from Minnesota and Vermont were placed in 10% buffered formalin and later cleared according to Hanken and Wassersug (1981). Cleared frogs were examined for the presence of metacercariae and mesocercariae. Relative number and location of larval trematodes were recorded. This procedure allowed for identification to class.

RESULTS

Radiology

Radiographs successfully identified bone patterns in malformed frogs and demonstrated the broad range of variation in malformations (Meteyer et al. 2000). Radiographs of frogs that were missing a limb (amelia) usually had malformations of the pelvis (8/9), and in extreme cases half of the pelvis was completely missing.

Both Minnesota frogs with multiple limbs (polymelia) also had malformations of the pelvis (2/2). One frog had two complete femurs, two tibiafibula, 3 sets of tibiale and fibulare bones and 3 sets of toes; 12 total. This frog also had very thickened pelvic bones, suggesting a duplicated but fused pelvis, on the side with the multiple limb. One Vermont frog, found just after metamorphosis, had extreme bilateral femoral ectromelia with abrupt termination of both limbs close to the hip. There were essentially no functional rear legs in this frog. Pelvic changes were also seen in 3/9 frogs radiographed with ectromelia of the femur (limb ending mid femur), and 1/19 frogs with ectromelia of the tibiafibula (limb ending mid tibiafibula).

Two frogs from the same Minnesota site had a very short limb with a small, poorly formed foot near the body. The radiographs of these limbs showed short, unidentifiable bones associated with an abnormal foot. The term given to this type of malformation is phocomelia (Wise et al. 1997). The pelvis of each of these frogs also had malformations.

Multiple elements of the foot were seen in 2 frogs from one Minnesota site and were not seen at any of the Vermont sites. Vermont had 23 frogs that had feet with either less than 5 toes (ectrodactyly), or reduced number of bones in their toes (brachydactyly). Minnesota had only 4 frogs with either ectrodactyly or brachydactyly.

A high proportion of frogs from one particular site in Minnesota had webs of skin that extended from their hip to the back of the ankle (hock). These skin webs traversed the angle of the knee preventing extension of the leg and securing the feet close to the body.

Malformed frogs from another Minnesota site had bilateral rotation of the long bones. This rotation caused the propelling surface of the feet to be misdirected, hampering mobility. Radiographs of these bones showed abnormal bone patterns at intervals along the cortex.

Craniofacial malformations such as small heads (microcephaly) or mandibular malformations (brachygnathia) were seen in 3 frogs from the same Minnesosta site. When the mouth was closed the mandibular malformations could be seen as a large gap between the closing margin of the upper and lower jaw and the margin of the lower jaw was irregular. Radiographs show that instead of a smooth mandibular arch, both sides of the abnormal jaw had a dramatic angle which drew the closing margin of the lower jaw away from the upper jaw. The absolute length of the mandibular bones appeared to be normal.

There was suggestion of site specificity for the frog malformations. Frogs with complete but severely rotated limbs were from the same Minnesota site. Frogs submitted with lower jaw abnormalities were all from another Minnesota site; two of three Minnesota frogs submitted with complete absence of one limb (amelia), as well as the frogs with multiple limbs (polymelia) were also from this site. Frogs from one Minnesota site were similar to frogs seen in Vermont with truncations of limbs and/or digits without multiple limb structures. All malformed frogs from Vermont had maformations representing reduced limb structures regardless of specific site.

Virology

Iridovirus was isolated from the kidney, liver and skin of dying frogs at one Minnesota site during the summer of 1997. Frogs from this site also had skin webbing, and multiple limb elements.

Bacteriology

Cultures of livers, spleens and kidneys of frogs resulted in 265 bacterial isolates. Most bacteria were in the order Enterobacteriaceae (*Enterobacter* spp., *Citrobacter* spp., *Escherichia* spp., *Klebsiella* spp.) and were considered incidental isolates and not primary pathogens. These bacteria were isolated from controls as well as abnormal frogs. Although *Aeromonas hydrophila,* a known pathogen in amphibians (Emmerson et al. 1905), was occasionally isolated from healthy frogs, it was commonly isolated from frogs that had iridovirus infections as well as from 2 frogs with traumatic lesions.

Parasitology

Infection with metacercariae (immature flukes) was light in Minnesota frogs and metacercariae were found in both malformed and control frogs. Vermont frogs had moderate loads of metacercariae in both malformed and control frogs. Metacercariae were most commonly found in the kidney. However, these renal metacercariae, most likely Echinostomatidae (Fried et al. 1997, Martin et al. 1990), were morphologically distinct from the metacercariae seen in other locations. Skin, tail bud, liver, soft tissue adjacent to eyes, epaxial muscles were also common sites where metacercariae, most likely Diplostomatidae (Cook 1978), were found. Both normal frogs and malformed frogs had metacercariae. Malformed frogs were examined that had metacercariae only in the kidney (Table 1). Because of these results, even though the number of frogs submitted for parasitologic examination was small precluding statistical analysis, we considered the presence of metacercariae in metamorphosed frogs to have poor specificity for malformations. Nematodes were uncommon in the newly metamorphosed frogs and were not associated with pathology.

DISCUSSION

Examinations of wild caught northern leopard frogs suggest that the majority of skeletal abnormalities were malformations rather than deformities from recent trauma, bacterial or viral infections. Completely missing limbs in frogs with abnormal or missing pelvic structures indicate early primary errors in development which may involve the lateral mesodermal plate, or communication between the apical ectodermal ridge and the mesoderm (Fig. 1). The teratogenic event leading to the development of duplicate or triplicate sets of bones would have also occurred very early in the morphogenesis of that limb; possibly a primary error in the mesoderm which controls differentiation of bone. Phocomelia (short limbs with unidentifiable segments) indicates a potential error in both the mesoderm resulting in abnormal differentiation of bone, as well as an error in the signals from the ectoderm resulting in abnormal growth. Skin webbing suggests an error in apoptosis and remodeling of ectoderm. Severe and progressive rotation of long bones might reflect errors in the mesoderm that occurred intermittently as the limb was developing. The appearance of the severely truncated limbs might suggest that chemical communication had been interrupted between the apical ectodermal ridge and the mesoderm of the limb bud, halting growth early in development.

If, after analysis of the larger set of field data, the distribution of malformation types continues to indicate site specificity, this could suggest that tadpoles at different sites are being exposed to teratogens during different stages of development, different teratogens or combinations of teratogens, different doses of teratogens or duration of exposure to teratogens, or a combination of the above. However, morphology of a malformation alone does not define the cause. For example, numerous chemicals can cause phocomelia including thalidomide, retinoic acid and its analogues, as well as cadmium (Plowman 1994, Sanders et al 1991). While we can say with confidence that frogs in the wild are not being exposed to thalidomide, rigorous laboratory and field testing need to be completed to determine the true cause or causes. The mandibular malformations seen in the leopard frogs collected from Minnesota are similar to those described by researchers inducing osteolathyrism in experimental frogs. Osteolathyrism is an abnormality of elastin and collagen formation that can be induced through exposure to semicarbazides during the later stages of tadpole development (Dawson, et al. 1991). The morphol-

ogy of experimentally-induced osteolatherism and malformations of some of the wild leopard frogs are similar, but a direct association cannot be made without further testing.

Water and sediment extracts from the Minnesota field sites investigated in this study cause malformations in *Xenopus* embryos in laboratory experiments (Burkhart et al. 1998, Fort et al. 1999, Fort et al. 1999). Sediment extracts from different sites produced different types and percentages of malformations in these experiments and extracts of sediment combined with site water had greater teratogenic effect than extracts mixed with laboratory water suggesting 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.

Cercariae (immature flukes) migrate through the skin and body openings forming metacercarial cysts in tissues. Field investigations (Sessions et al. 1990) in California documented an association of metacercariae with malformations in Pacific tree frogs (*Hyla regilla*). Experimental studies exposed the tadpoles of *Hyla regilla* to different numbers of the specific cercaria (*Ribeiroia* sp.) found at this California site (Johnson et al. 1999). These tadpoles metamorphosed into frogs with malformations (primarily multiple limbs) that were more frequent and severe as the dose of cercariae increased (Johnson et al. 1999). The geographic distribution of *Ribeiroia* sp. in the United States and its influence on other species of frogs is unknown at this time. However, NWHC records show reports of the adult stage of this fluke in osprey from several locations the United States and Puerto Rico. Further investigations to determine the significance of this parasite as a cause of malformations in frogs, particularly ranids from the upper midwest and the northeast are warranted. However, these investigations should look for *Ribeiroia* sp. in the tadpoles during relevant stages of development and determine the significance of the metacercarial infection to the developmental process. Presence of metacercariae in metamorphosed frogs provides limited information regarding their potential contribution to the teratogenic process in the tadpole.

Results of bacterial cultures in our study were similar to other investigations. *Aeromonas hydrophila* has been isolated in normal and dying frogs (Hird et al. 1981, Nyman 1986). Combined infection of *Aeromonas hydrophila* and iridovirus in frogs has also been previously reported in Great Britain with the suggestion that iridovirus may be the primary pathogens in these coinfections (Cunningham et al. 1996). Iridovirus and *Aeromonas hydrophila* were isolated from frogs at one Minnesota site in 1997 and 1998. Whether the iridovirus or bacteria isolated from the skin of frogs were somehow involved in the skin webbing and multiple limb elements in the frogs at this site is unknown. This is the same collection site and mortality event referred to in a subsequent paper published by Gardiner and Hoppe (1999). Gardiner and Hoppe describe malformations seen in dying mink frogs (*Rana septentrionalis*). Isolation of this virus or identification of a bacteria or parasite in the fully developed frog or in tadpoles that are late in development indicates that the organisms are present in the environment of this population but does not provide relevant information regarding its significance at the time the malformation was occurring early in limb development.

Microbiological studies in the 1997 frogs from Minnesota and Vermont could not be used to support the hypotheses that parasites, bacterial infections or viruses were the primary cause of malformations in those frogs. Although, with the exception of the iridovirus, there did not appear to be a correlation between microbes and malformed frogs, the number of frogs evaluated in 1997 was relatively low and, most importantly, the samples examined included only metamorphosed frogs. The insults that may have occurred during the larval stages, when the limb is developing, are still unknown and need to be determined.

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