The Journal of the Iowa Academy of Science: JIAS

Volume 107 | Number 3-4 Article 8

2000

A Relationship Between Trematode Metacercariae and Bullfrog Limb Abnormalities

James L. Christiansen
Drake University

Heather Feltman
Drake University

Jour. Iowa Acad. Sci. 107(3):79–85, 2000

A Relationship Between Trematode Metacercariae and Bullfrog Limb Abnormalities

JAMES L. CHRISTIANSEN[1] and HEATHER FELTMAN

Department of Biology, Drake University, Des Moines, Iowa 50311
[1] Corresponding author; Email: james.christiansen@drake.edu

This study is the first report of statistically significant relationships between limb abnormalities and trematode metacercariae in natural populations of frogs. We examined only parasite associations and did not investigate numerous potential pollutants that have been suggested by others to be associated with frog limb abnormalities. *Rana catesbeiana* of varying ages with a variety of limb abnormalities were found during the flood of 1993. By June 1994, the receding flood waters had left four pools, each with newly metamorphosing bullfrogs. Only one of the pools contained abnormal individuals and histological examination revealed significantly greater prevalence of trematode metacercarial infection in these frogs. Random tissue samples showed that within that pool, abnormal bullfrogs were significantly more likely to carry trematode metacercariae than were normal bullfrogs. Comparison of infected frogs from the study area showed that abnormal bullfrogs had significantly more severe infections than did infected normal bullfrogs. Samples of limbs abnormally lacking musculature, carried parasites while normal limbs did not and bullfrogs lacking back limbs always had subcutaneous metacercariae where the missing limb should have emerged. While this work indicates an association between parasites and limb abnormalities, it does not exclude the "easy target" hypothesis where by a chemically injured animal or chemically injured tissue might be more likely to be infected than a normal animal or normal tissue. Potential senerios are presented for both an "easy target" hypothesis and a "parasite cause" hypothesis. Our work suggests that trematode-abnormality relationships become evident only when samples are limited to metamorphosing frogs.

INDEX DESCRIPTORS: frog developmental abnormalities, parasites and limb abnormalities, frog trematodes.

Numerous reports of frogs with extranumerary (more or fewer than normal) legs suggest that the phenomenon may have multiple causes and that it has been occurring sporadically in a variety of environments in the last 250 years. Van Valen (1974) lists over 40 reports of this phenomenon. Ouellet et al. (1997) and many public media reports suggest that the condition has recently become more common.

Causes suggested from laboratory studies include temperature, physical rotation of the developing embryo, and chemical pollution (Muto 1969, Nace and Tremor 1981, Ouellet et al. 1997, Volpe 1985) for example. Because frogs can regenerate limbs following mechanical damage (Wessells 1977), one logical causative agent for some anomalies is some form of mechanical damage. Sessions and Ruth (1990) provided evidence for mechanical interference with development in their investigation of potential damage inflicted by trematode metacercariae. After finding metacercariae at the point of abnormal limb bifurcation and in the cloacal region in natural populations of pacific tree frogs (*Hyla regilla*) and long-toed salamanders (*Ambystoma macrodactylum*), they hypothesized that the supernumerary condition was caused by mechanical damage to the limb bud field by trematode metacercariae. They produced leg bifurcation by inserting resin beads the size of trematode metacercariae in early limb buds, supporting their hypothesis. However, because in their field studies, all their normal and abnormal frogs were infected, they could not show statistically significant association of metacercariae with the leg abnormalities in nature. The present study focuses only on this potential association in nature.

Our initial sampling in receding flood waters in eastern Iowa in 1993 yielded normal bullfrogs of varying size and age, some with extranumerary legs. The abnormal frogs appeared to have metamorphosed at different times that year and during the preceding year. Checks of a few of these indicated heavy trematode cyst infections but some normal frogs had large numbers of metacercariae and some abnormal ones had very few. This would be expected if tadpoles continued to accumulate metacercariae even after their limbs had developed. Specifically, if the problem were the result of trematode interference with a limb bud, an abnormal frog could have only a single cyst in the appropriate place, but a normal frog could have hundreds of cysts that were either acquired after the limbs developed or happened not to be in the appropriate place to cause an abnormality during limb development. The confusion caused by later accumulations of metacercariae could be reduced if large numbers of normal and abnormal frogs could be sampled from the same pools at nearly the same developmental stage.

By June 1994, the flood waters in our eastern Iowa study area had receded further and were now confined to four pools, all with a new cohort of metamorphosing bullfrogs, some of these with leg abnormalities. This provided a unique opportunity to study metamorphosing tadpoles that had shared a common body of water before limb bud development but were isolated into four populations at the time of limb bud development. It also allowed us to sample metamorphs of approximately the same age with exposure to similar environmental conditions. Specifically, if a parasite invasion must occur at the time of limb bud development, the abnormalities produced would be limited to the pools where the parasite was abundant. One might also expect that if the abnormality were the result of a lifetime of accumulation of a teratogenic pollutant, the abnormal frogs should be scattered throughout the four ponds that shared the same water the previous year and three of the ponds that shared common water until a few weeks before sampling. However, it would be possible that a concentrated chemical agent in one of the pools could reach critical levels not attained in the other pools.

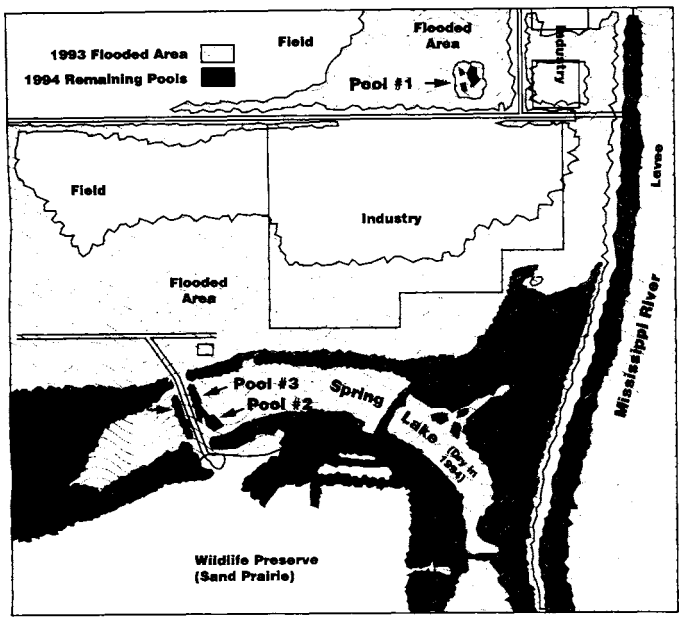

Fig. 1. The study area located on the south edge of Muscatine, Muscatine County, Iowa. Wavy lines indicate the area flooded in 1993. Most of the area was dry except for the numbered pools and the Mississippi River when sampling was conducted in 1994.

We have focused on the distribution of limb abnormalities and the distribution of trematode metacercariae in bullfrogs from the four pond study area. The study also compared presence or absence and abundance of parasites in tissue samples from normal and abnormal frogs of approximately the same age from the same pool. We further examined relationships between parasite presence and specific abnormalities such as dystrophic limbs in which the limb number was often normal but the musculature of one limb failed to develop. We also examined the relationship between parasite position and failure of limbs to develop. The study did not investigate any of the large number of chemical agents known to cause limb abnormalities.

METHODS

Frogs were sampled in September and October 1993 by walking edges of receding floodwaters and repeatedly driving roads in the study area located on the south edge of Muscatine, Muscatine County, Iowa. In 1994, sampling was limited to June, the time of bullfrog metamorphosis in four pools left in the same area by the receding flood. The four pools (Fig. 1) were an abandoned gravel pit (Pool 1), a man-made wildlife watering hole in the bottom of a dry lake (Pool 2), a ditch east of a road that crosses the dry lake (Pool 3), and a ditch west of the same road (Pool 4). Pools 3 and 4 were linked by a culvert below water until about 15 May, the approximate beginning of limb formation in most of the population. Pool 2 was linked to Pool 3 by a few inch deep channel until 1 June. This allowed a small amount of mixing of water and movement of tadpoles between these bodies until water levels dropped and the interconnecting water dried. At the time of sampling, concentrations of snails and dying fish were present in all pools but were most concentrated in Pool 4. Pools 3 and 4 dried completely a few days after sampling was concluded. However limb bud formation would have been around mid-May when the area and depth of these pools were much greater and fish death was not observed.

Sampling was conducted at night with flashlights and by hand and seine during daylight. All frogs were tagged, preserved in 10% formalin, and cataloged into the Drake University Research collection.

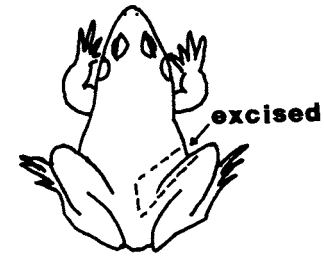

Fig. 2. Location of tissue sample removed for random sampling for parasite infections in 1994.

Gross abnormalities of frogs found in 1993 were recorded. These usually involved duplication, bifurcation, or absence of hind limbs. Tissues were removed and studied under a binocular dissecting microscope, placed in cassettes, fixed in 10% formalin, dehydrated, imbedded in paraffin, sectioned at 7 μm, and stained with hematoxylin and eosin. Because trematode metacercariae were often found in the kidneys and associated muscle and connective tissues of abnormal frogs, and because Sessions and Ruth (1990) had observed them to be concentrated in the cloacal region, tissue wedges (Fig. 2) containing the body wall, parts of internal organs, and an anterior portion of the right kidney were removed for random sampling of metamorphosing bullfrogs in 1994 to determine possible parasite-abnormal frog association. These tissues were prepared as those in 1993 and sectioning, staining and mounting was done equally for all locations and usually without knowledge of location. Metacercariae were counted in all sections and were recorded for statistical analysis from the single section where they were most numerous. Any remnant of a cyst was counted as a parasite. All counts were made by at least two people and when disagreement occurred, the slide was studied in presence of both until agreement on count was attained. Significance of difference between presence or absence in our slide sample for normal and abnormal frogs was established by Chi square analysis; significance of difference between means was determined by *t* test using pooled variance and two nonparametric tests, Wilcoxon two-sample test and Kruskal-Wallis test. Because identification of metacercariae to species from tissue sections is nearly impossible and because we suspect that it is the mechanical presence of an object or mechanical damage caused by an object that contributes to limb abnormalities, little effort was expended in trying to determine the identity of parasites to species.

For studies of the "withered leg" condition, all four limbs of the frog were removed along with adjacent tissue from the body wall. These were decalcified with Stephens Scientific™ decalcifying solution and serially sectioned through approximately 3/4 of the tissue as described previously. Miscellaneous other tissues were removed and prepared by the same methods. All slides are available for examination in the Drake University Research Collection.

RESULTS

Sampling of 139 amphibians from the study area in September and October of 1993 produced six species including 91 bullfrogs *(Rana catesbeiana)* ranging from 47–71 mm SVL and probably representing one to two years post metamorphosis. The only amphibians found to be grossly abnormal were bullfrogs and of these 15 (16.5% of the total bullfrogs) had limb abnormalities (Fig. 3a). This high percentage of abnormals reflects a sampling bias because the abnormal frogs were easier to catch and greater effort was spent in search for abnormals at this point in the study. Normal amphibians col-

Fig. 3. Abnormal frogs typical of those collected in (A) 1993 and (B) 1994.

Table 1. Incidence of appendage abnormalities and trematode metacercariae in random sections of kidney and nearby tissue in two slides of tissue wedges from metamorphosing bullfrogs collected in the study area in 1994. Locations are four pools that had been united during the 1993 flood. The numbers below are a representative sample and do not include all frogs seen. A sampling bias exists because frogs with severe abnormalities were easier to catch than were normal frogs. The % kidney infected is adjusted in Pool 4 to reflect four frogs that lacked kidney in the sections due to tissue orientation in the paraffin block.

Location	n	Abnormal (%)	Kidney Infected (%)	Kidney + Tissue Infected (%)
Pool 1	14	0	0	0
Pool 2	17	0	5.9	11.8
Pool 3	26	0	23.1	30.8
Pool 4	38	42.1	55.8	63.2
Totals	95	16.8	27.4	35.8

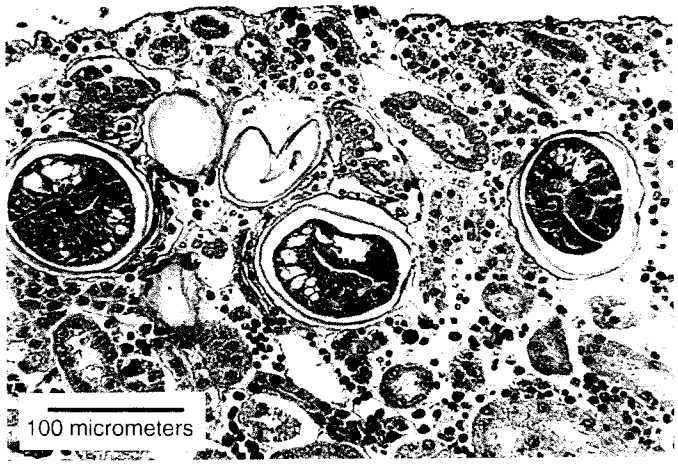

Fig. 4. (A) Metacercariae in the kidney of DU 2043, a bullfrog with an extra back leg originating from the pelvis. Maximum length of the largest sections through the renal metacercariae in this specimen ranged from 94–98 mm. Renal infections similar to this were found in most frogs with hind leg abnormalities.

lected in the same sample included 22 *Rana pipiens,* nine *R. blairi,* three *Bufo woodhousii fowleri,* 12 *Ambystoma tigrinum tigrinum,* and two *A. texanum.* A resampling of the area as bullfrogs were metamorphosing in 1994 yielded a new bullfrog cohort of which 95 (79 normal and 16 abnormal) were examined histologically (Table 1, Fig. 3b). As in 1993, the only species seen with abnormalities was *R. catesbeiana.* The abnormalities seen in 1993 and 1994 were similar and always involved asymmetrical changes. In all but one the changes were unilateral. Of the 16 abnormal frogs seen in 1994, 12 had back limb abnormalities including missing back legs (5), extra back legs (3), and underdeveloped (withered) back legs (4). Four had front limb abnormalities including missing front legs (2) and shortened or abnormally placed toes (2).

The 14 metamorphosing tadpoles examined from Pool 1 were all normal and all random kidney sections and associated tissues were free of parasites. Pools 2 and 3 also lacked abnormal frogs but had metacercaria prevalence of approximately 12% and 31% respectively. Bullfrogs from Pool 4 had an infection prevalence of 63% and yielded all 16 abnormal frogs seen that year (Table 1). While these prevalences were representative of the metacercaria population density in these frogs, actual prevalences were considerably higher. We know this because additional sectioning later demonstrated that our sample often missed cysts. A section of kidney (Fig. 4) shows typical renal metacercariae, always appearing in spherical cysts.

While the correlation between the parasite incidence in our sample and limb abnormality seems apparent from the forgoing comparison,

Table 2. Incidence of metacercariae in normal and abnormal bullfrogs collected in Pool 4 in 1994. One normal and three abnormal frogs are excluded where our random sections missed the kidney although all had metacercariae eventually located in other tissue.

	n	Infected (%)	X^2	Probability (P)
Kidney Only				
Normal Frogs	21	33.3		
Abnormal Frogs	13	92.3	11.29	0.001
Totals	34	55.9		
	Yates correction		9.03	0.003
Total Tissue on Slide				
Normal Frogs	22	40.9		
Abnormal Frogs	16	93.7	11.115	0.001
Totals	38	63.2		
	Yates correction		8.99	0.003

Table 3. Number of metacercariae in kidney sections of normal and abnormal frogs collected in Pool 4 and Pools 2, 3, and 4 in June 1994. The Pool 4 study included frogs with no visible infection in the sample slides and involved kidney only. Kidney + other tissue showed comparable levels of significance. Combined pool comparison included only frogs with metacercariae present in sections and included all tissue on slides (*eg.* the sample eliminated numerous specimens with no visible infection). *t*-tests used pooled variance and were one tailed. Wilcoxon 2-sample tests were made with normal approximation and continuity correction of 0.5.

	Normal		Abnormal		
Site	Range	Mean	Range	Mean	Probability
Pool 4					
t test (n = 38)	0–31	2.048	0–23	6.769	< 0.022
Wilcoxon 2-sample test		Z = 3.707			≤ 0.001
Kruskal-Wallis χ^2 Approximation		χ^2 = 13.854			≤0.001
Pools 2–4					
t test (n = 34)	1–31	5.53	1–27	7.667	≤ 0.191
Wilcoxon 2-sample test		Z = 1.993			≤0.046
Kruskal-Wallis χ^2 Approximation		χ^2 = 4.044			≤ 0.044

it would be possible that a chemical teratogen or some other factor in Pool 4 stimulated the abnormality and predisposed the tadpole to infection. It should be noted that the tadpoles were dependent on the tail for locomotion at this time and presence of a nonfunctional limb would not have significantly influenced locomotion, not making the tadpole from that perspective an easier than normal target for a parasite. To determine whether the parasites were significantly associated with presence of abnormalities where the tadpoles shared an exclusive body of water only through the time of limb bud formation, the incidence of detected parasitism and limb abnormalities was compared only within Pool 4. Random sections found trematode metacercariae in only 40.9% of 22 normal frogs and in all but one of 16 abnormals. χ^2 showed this association between abnormalities and presence of parasites to be highly statistically significant, both when the analyses of random sections were limited to kidney, and when renal and associated tissues were combined (Table 2). Statistically significant association of kidney infection alone with abnormalities is consistent with the hypothesis that early renal (mesonephric) *damage may* contribute to abnormal hind limb development in frogs.

If the parasites had to be in a critical location to cause an abnormality and if the distribution of parasites in the abdominal region of the frog were random, the larger the number of parasites, the greater the chance that one would be in a critical area and cause abnormality. While one would expect many exceptions, frogs with abnormalities should therefore average more parasites than normal frogs of the same age and from the same pool. This hypothesis was again tested within Pool 4 and the number of metacercariae in our sample was found to be significantly greater in frogs with abnormalities (Table 3) even though, in one abnormal frog, no parasite was found in our random sample and one normal frog had a very heavy infection with 31 metacercariae, all in a single kidney section.

It could be argued that some frogs with no parasites in our sample were entirely uninfected and therefore should be excluded from a study of extent of infection. It is the opinion of the authors that no parasites in this comparison is as important a number as is the presence of one parasite in demonstrating that large numbers of parasites are more likely to be associated with abnormality than are small numbers. However, we eliminated the frogs from Pool 4 with no metacercariae in our sample slides, and this greatly reduced the number of frogs for comparison. It was therefore necessary to repeat the comparison including all normal frogs bearing parasites in our ran-

dom sample (Pools 2, 3, and 4). This comparison included both renal and non-renal tissue and showed by two nonparametric statistical techniques that abnormal infected frogs had significantly more metacercariae than did normal infected frogs (Table 3). It is worthy of note that the single normal frog with 31 metacercariae increased the variance to the point that only the nonparametric tests indicated significance.

If the abnormalities observed were caused by parasites interfering with limb or organ bud development either by blocking the bud, dividing it, or forming a barrier between the bud and other tissue whose contact was necessary for bud cell determination or development, then parasites should be present in the general region of the abnormality, at least if examined near the time of limb development. We had observed that the hypoplastic (withered) hind limb of DU 2039 had a heavy infection, four metacercariae in the base of the withered leg, in addition to 13 in one section of the associated kidney. To determine if metacercariae were generally more associated with this limb condition than they were with normal limbs, all the normal as well as the abnormal limbs including the base of the limbs as they originated in the body, were examined for parasites for the two additional specimens with this condition, DU 2008 and DU 2011. These were serially sectioned, mounting one of every ten sections and retaining the block with approximately one-third of the leg uncut. This sample revealed two metacercariae associated with the base of the abnormal limb of DU 2008 and one multiple cyst (two metacercariae surrounded by a single connective tissue capsule) with at least three subcutaneous cysts at the base of DU 2011. No metacercariae were found associated with any of the three normal limbs of either frog. In addition, DU 2039 had a probably mobile, unencysted larval parasite between two underdeveloped muscle layers.

Recently metamorphosed frogs with multiple hind limbs always had metacercariae in the body at the base of the abnormal limbs, but we could not always determine a point of bifurcation. All but one of these frogs had renal infection with metacercariae in our tissue wedge sections.

Five bullfrogs with missing back legs (DU 2007, 2012, 2036, 2041, 2042) were observed to have metacercariae, some with double cysts in addition to individual cysts under the skin approximately where the leg should have emerged (Fig. 5). While inconclusive because we do not know the distribution of subepidermal parasites in the remainder of the body, this observation leaves open the possibility that a parasite, especially one blocking access of the mesonephros to epidermal tissue, may contribute to failure of the limb to develop.

No abnormal bullfrogs have been seen in the study area since 1994 but eight normal adult *R. catesbeiana* from the region of the Pool 4, presumable survivors of 1993–94, were collected for kidney examination in 1996. Six of these had renal metacercariae in four random sections with as many as 69 in a single kidney section. We were unable to identify the metacercariae found throughout this study because tissue sections passed through the cysts at various angles and depths. However, presence and absence of spines around the oral sucker suggested that one or more members of both a non-spine bearing family and the spine-bearing echinostomatids were present.

DISCUSSION

Our examination of normal adult frogs in 1996 and metamorphosing tadpoles in 1994 with large infections of metacercariae, suggests that tadpoles and adult frogs accumulate metacercariae throughout development and metamorphosis. For this reason, meaningful statistical comparisons for frogs with and without limb abnormalities must be made of frogs of about the same age and preferably from similar localities shortly after the time of limb formation. It is evident that a study of the location of metacercariae or mesocercariae during the earliest stages of limb development, earlier than in the present study, is necessary in establishing a cause-effect relationship. Such a cause-effect relationship has now been demonstrated by Johnson et al. (1999) who produced similar leg abnormalities with laboratory infestation of *Hyla regella* tadpoles. Considerable support for association of the type of leg abnormality we observed with trematode infestation has recently been provided by Sessions et al. (1999).

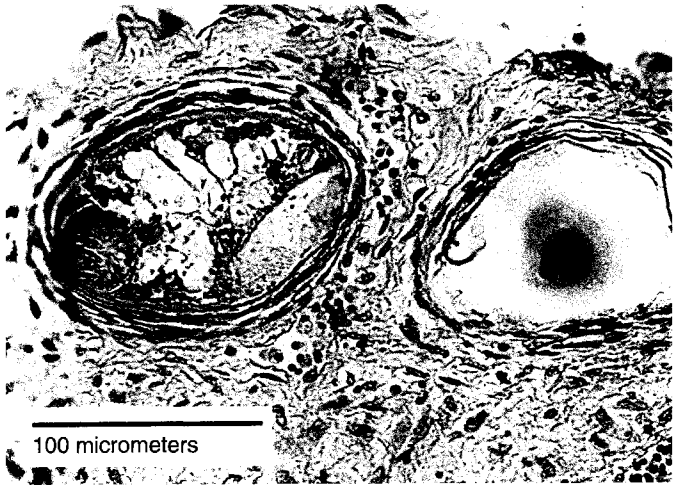

Fig. 5. A pair of cysts in the reticular dermis of DU 2036, a frog missing one back leg. The large metacercaria fully displayed in this section is 179 μ in greatest diameter and the pair of cysts shown with their associated connective tissue extend over 320 μ. Double cysts such as this could create a large barrier between the dermis and mesonephros.

In our study, pools producing frogs without or with low abundance of metacercariae, produced frogs that lacked abnormalities. It was also evident that in the pool where abnormalities existed, presence of metacercariae was highly significantly associated with presence of the abnormalities and further, frogs without abnormalities had significantly fewer metacercariae than those with abnormalities. These observations fit the hypothesis that if more metacercariae are present, the likelihood is greater that one will be in position to cause an abnormality but could also fit the hypothesis that animals could have been made susceptible to cercariae by a chemical agent. In addition, a site specific parasite species, (for example, a species that was so exclusively renal that it was never found in a limb bud) could distort conclusions if such a species were involved in the complexes seen in this study and if the presence of the parasite in the mesonephric kidney had no influence on limb bud development. We suspect that very early, very heavy, renal (mesonephric) infection may influence limb development.

We observed that a high percentage of our abnormal frogs including those with either missing legs or hind legs lacking musculature, had renal infection by metacercariae (Fig. 4). Leg reduction or loss similar to what we have seen in our frogs and typical of that caused by thalidomide syndrome, was induced by Smith et al. (1996) in *Ambystoma maculatum* solely by interfering with the developing mesonephros. He accomplished this both by removing the mesonephros and by placing a barrier between the mesonephros and the overlying ectoderm, this work confirming that a signal from the mesonephors is essential for limb development. This opens the possibility that the heavy kidney infections observed in this study may

have augmented the impact of metacercariae on the early limb bud in producing limb loss, reduction, or even bifurcation. One avenue of research might be to investigate the possibility that the parasite in combination with the mesonephros might influence embryonic production of retinoic acid.

Sessions (1990) explained well the mechanical mechanism by which metacercariae could cause limb bifurcation. Our examination of three frogs with hypoplastic limbs showed heavy infection in or at the base of the "withered" limbs of all three frogs. Histological examination indicated that only the affected limbs carried metacercariae even though an occasional cyst was expected in the normal limbs. This appears to support the "parasite cause" hypothesis but it can be argued that the tissue might have been chemically injured and be more susceptible to cercaria penetration. Similarly, we found parasites just under the skin of the body wall of bullfrogs and in underlying tissue where hind legs were missing. Interaction between the limb bud mesoderm and overlying ectoderm is required for normal limb bud development in vertebrates (Harrison 1918, Wolpert 1969, Zwilling, 1955). We suggest that the presence of parasites in this position, if they were in the same location at the time of limb bud formation, might have been sufficient to prevent normal limb bud development.

We have shown a significant association between presence of limb abnormalities in bullfrogs and trematode cysts, consistent with the hypothesis of Sessions and Ruth (1990) that supernumerary legs in at least some natural populations of amphibians may be caused by an object such as trematode metacercariae or another interstitial object at least as large. In addition to the disruption of the limb bud field demonstrated by Sessions and Ruth (1990), we have suggested two developmental mechanisms by which the parasites could have contributed to the abnormalities we have seen. Specifically, these are parasite-induced damage to the developing mesonephros and separation of early limb bud mesoderm from the overlying ectoderm by cysts. While we have no evidence, we recognize that, with industry present in the area, manmade agents may be involved in making the frogs more susceptible to parasite infection or that somehow the parasite could be attracted to tissue developing abnormally for other reasons. We present the following two hypothetical scenarios to suggest how some of the increased abundance of frog developmental abnormalities in the northern Midwest might have been caused and how it could be consistent with our observations of parasite associations. We urge consideration of the possibility that in some populations, abnormalities may result from parasite damage, in others from chemical damage, and possibly in others from a synergistic effect.

Chemical Injury, Easy Parasite Target Scenario

This hypothesis suggests that the association of the abnormalities with Pool 4 is the result of an unknown agent or combination of agents that were present in Pool 4 and that the agents produced the non-bilateral limb abnormalities seen there. It assumes that increased parasitism in Pool 4 was the result of increased susceptibility of chemically injured, abnormal frogs to the parasite. Further, it assumes that the abnormal frogs in Pool 4 had accumulated more of the agent than the normal frogs and because of increased susceptibility, accumulated more parasites. It reasonably follows that these or other such agents or combinations of agents may have been brought to the surface or otherwise in contact with frogs by the heavy rains of 1993 in the Midwest thereby bringing the concentrations over threshold levels and resulting in increased reports of abnormal frogs throughout the area.

Negative aspects of this scenario include the observation that most known teratogens have bilateral effects, large dosages of retinoids being an exception. No single teratogen (including retinoids) has yet been reported in concentrations high enough to produce the leg abnormalities seen here in bodies of water that yielded abnormal frogs. Laboratory FETAX studies of pond water and sediments from pools with a history of frog developmental abnormalities in Minnesota and Vermont have produced abnormalities in *Xenopus,* especially deletion abnormalities, but have not demonstrated the extensive supernumerary hind leg abnormalities we associate with parasites here (Fort et al. 1999a, 1999b). Because pools 2, 3, and 4 in the present study are in a controlled area, we are assured that no insecticides have been used in the area for at least 10 years. Finally, no source of teratogens, including mosquito insecticides, has been found that could logically have been expected in the wide variety or habitats that have produced abnormal frogs throughout the Midwest.

Parasite Cause Scenario

This hypothesis suggests that trematode eggs carried in the feces of snakes and other vertebrate predators of frogs, especially large birds, are often deposited on soil. The heavy rains and floods of 1993 introduced the newly hatched miracidia to snails introduced by the flood waters, completing the middle stage of the life cycle. Cercariae emerging from the snails found a tadpole population made more abundant by ideal breeding conditions and dilution of predators during the flood. This may have produced the first "outbreak" that we observed in eastern Iowa and that others observed in the Midwest in 1993. By 1994 the floodwaters were declining, concentrating abundant bullfrog tadpole populations produced the year before. Predators that had consumed the previous year's infected frogs, inoculated these pools containing large tadpole populations and further drying further concentrated tadpoles with infected snails creating a parasite paradise, resulting in an even larger population of abnormal frogs. We conjecture that by 1996 the spreading trematode epizootic from a few centers contributed to development of abnormal frogs in a variety of newly discovered locations in the Midwest.

Negative aspects of this scenario include the near certainty that a parasite cause is not consistent with all the types of abnormalities we have seen in the Midwest. Our unpublished studies of several Missouri frogs, each with a single missing eye, have shown no indication of parasite involvement. We have been unable to find metacercariae in critical areas of abnormal limbs in postmetamorphic frogs and had assumed that the parasites had been removed by the frogs immune system. However we have little evidence for this. Finally many species of trematodes may be involved and we are unable to identify them from our tissue sections. In addition, their life cycles may be incompletely known or unknown.

ACKNOWLEDGMENTS

We thank Dr. John Grzybowski for his useful suggestions, Mary Dunsmoor for her serial sectioning of many inadequately decalcified tissue blocks, and Iowa Lutheran Hospital for making work in their laboratory possible. We are grateful to Dr. Marie Klugman for help with nonparametric statistics, Dr. R. S. Wacha and Dr. R. A. Rogers, parasitologists, for verification of parasites, and Dr. Charisse Buising for her insightful analysis of the developmental biological aspects of this study. Special thanks are due Dr. Michael McKee for help throughout the study and to Monsanto Co. for funding much of the expensive histological work.

LITERATURE CITED

FORT, D.J., T.L. PROPST, E.L. STOVER, J.C. HELGEN, R.B. LEVEY, K. GALLAGHER, and J.G. BURKHART. 1999a. Effects of pond water, sediment, and sediment extracts from Minnesota and Vermont, USA, on

early development and metamorphosis of *Xenopus*. Environmental Toxicology and Chemistry 18:2305–2315.

FORT, D.J., R.L. ROGERS, H.F. COPLEY, L.A. BRUNING, E.L. STOVER, J.C. HELGEN, and J.G. BURKHART. 1999b. Progress toward identifying causes of maldevelopment induced in *Xenopus* by pond water and sediment extracts from Minnesota, USA. Environmental toxicology and Chemistry 18:2316–2324.

HARRISON, R.G. 1918. Experiments on the development of the forelimb of *Amblystoma*, a self-differentiating equipotential system. Journal of Experimental Zoology 25:413–461.

JOHNSON, P.T.J., K.B. LUNDE, E.G. RITCHIE, and A.E. LAUNER. 1999. The effect of trematode infection on amphibian limb development and survivorship. Science 284:802–804.

MUTO, Y. 1969. Hindlimb development and malformations of the toes in the larvae reared at high temperature in the toad, *Bufo vulgaris formosus*. Congenital Anomalies 9:1–12.

NACE, G.W., and J.W. TREMOR. 1981. Clinostat exposure and symmetrization of frog eggs. The Physiologist 24:S77–S78.

OUELLET, M., J. BONIN, J. RODRIGUE, J.-L. DESGRANGES, and S. LAIR. 1997. Hindlimb deformities (ectromelia, ectrodactyly) in free-living anurans from agricultural habitats. Journal of Wildlife Disease 33: 95–104.

SESSIONS, S.K., and S.B. RUTH. 1990. Explanation for naturally occurring supernumerary limbs in amphibians. Journal of Experimental Zoology 254:38–47.

SESSIONS, S.K., R.A. FRANSSEN, and V. HORNER. 1999. Morphological clues from multilegged frogs: are retinoids to blame? Science 284:800–802.

SMITH, D.M., R.D. TORRES, and T.D. STEPHENS. 1996. Mesonephros has a role in limb bud development and is related to thalidomide embryopathy. Teratology 54:126–134.

VAN VALEN, L. 1974. A natural model for the origin of some higher taxa. Journal of Herpetology 8:109–121.

VOLPE, E.P. 1981. Understanding Evolution. W.C. Brown Co., Dubuque, Iowa.

WESSELLS, N.K. 1977. Tissue Interactions and Development. W.A. Benjamin, Inc., New York.

WOLPERT, L. 1969. Positional information and spatial pattern of cellular formation. Journal of Theoretical Biology 25:1–47.

ZWILLING, E. 1955. Ectoderm-mesoderm relationship in the development of the chick embryo limb bud. Journal of Experimental Zoology 128: 423–441.