Diagnostic Findings Associated with Malformed Frogs from Vermont Summer 1997

Final Report

In Fulfillment of Milestones for:

Investigation of Deformities in the Northern Leopard Frog (*Rana Pipiens*) at Selected Sites in Vermont - FY 97

Radiographic Characterization of Abnormal Frogs at Selected Sites in Vermont-FY 97

Biological Resources Division
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APPENDICES

I. SUMMARY

Ninety three frogs were submitted for evaluation. Frog malformations were classified into 6 major categories and 12 subcategories based on necropsy observations of 72 frogs (Appendix J) and the findings of radiographs performed on 69 of these 72 frogs (Appendix D). All of the primary malformations in frogs from Vermont were relatively uniform, involved missing or incomplete limbs, which is quite different from frog malformations found in other states where our lab has conducted studies. Unlike malformations in 3 other states (1997 and 1998 National Wildlife Health Center data), Vermont frogs collected in 1997 were unique in having no multiple limbs, no phocomelia or complete but extremely abnormal limbs, no pure rotational/skin web malformations, and only one frog with a possible craniofacial malformation. It was interesting that, on the average, all malformed frogs from all Vermont sites were in poor to emaciated body condition without discrimination by site or malformation type (Appendix F, Tables #1 and #2). Again, this was not our finding in other states. Radiographs were extremely helpful in determining the degree of malformation in the limbs as well as the type of abnormal termination in both long bones and digits. Malformations of the hip were also seen in some of the Vermont frogs and these would not have been detected without the use of high detail radiography. Radiographs were also helpful in differentiating trauma from malformation, although necropsy observations and histopathology were also important in ruling out trauma. Histopathology was performed on 19 frogs (Appendix G). Histopathology on malformed bones showed two general types of bone changes. One of the characteristic types of microscopic change seen at the termination of the malformed limb was very disorganized and hyperplastic cartilage that was not ossifying normally and interfered with the formation of bone cortex. The second type of bone change seen at the point of termination was the formation of small nests of endosteal type cartilage matrix with some organization but no obvious maturation or ossification. None of the malformed limbs had inflammation and they often had very poor skeletal muscle development at the terminal end. Traumatic amputations did have microscopic evidence of skeletal muscle necrosis and inflammation. There were no microscopic changes in internal organs that could be related to malformations. The brain and spinal cord of the frogs submitted often had dilated ventricles and we are in the process of consulting with a neuropathologist to determine the significance of this finding. Virology cultures were conducted on 51 tissues on 2 different cell lines adapted for amphibians and electron microscopy was performed on selected homogenates of frog tissues to determine if viruses were present. No viruses were isolated or identified in any Vermont frogs. Bacterial cultures were performed on 44 tissues and 98 bacteria were isolated and identified. There did not appear to be any direct association between bacterial isolates and the observed malformations. Parasitologic examinations were conducted on 14 frogs. Although the parasitology sample size was small, no association could be found between the presence of metacercaria and the occurrence of malformations.

II. BACKGROUND

During the summer of 1995, a group of school children conducting a wetland nature study observed a high rate of abnormal leopard frogs in Henderson County, Minnesota. Since 1995, occurrence of deformed frogs have been reported in 42 states and 3 Canadian provinces (Gilbertson). The species reported have included northern leopard frog (*Rana pipiens*), spring peeper (*Hyla crucifer*), American toad (*Bufo americanus*), gray treefrog (*Hyla versicolor*), bullfrog (*Rana catesbeiana*), wood frog (Rana *sylvatica*), green frog (*Rana clamitans*), pickerel frog (*Rana palustris*), and mink frog (*Rana septentrionalis*).

Studies, funded by the USGS Biological Resource Division's Eastern Region in coordination with the Vermont Agency of Natural Resources, and the U.S. Fish and Wildlife Service, were initiated through the National Wildlife Health Center in the summer of 1997. The objectives of the study were to characterize frog abnormalities, document the distribution of malformed frogs, determine the frequency of malformations, collect baseline morphometric measurement of frogs, and collect water and sediment samples for analysis. The National Wildlife Health Center (NWHC) was requested to assist in defining the developmental problems in Vermont frogs. The role of the NWHC was to perform diagnostic assessments of abnormal and normal frogs from Vermont sites that were known to have malformed frogs. The diagnostic evaluation included gross necropsy examination, histopathology, virology, bacteriology, parasitology. In the spring of 1998 funding became available to perform radiology on a subset of the Vermont frogs.

A report of preliminary findings was presented at the NIH/NIEHS Workshop on Strategies for Assessing the Implications of Malformed Frogs for Environmental Health held December 4-5, 1997 at the National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina. The data we presented at the conference made it clear that teratogenesis was occurring in tadpoles of the northern leopard frog. Factors that have been shown in the lab to cause teratogenesis are numerous falling into the general categories of genetic or environmental. Some environmental factors that can influence development are; chemical, thermal, infectious, or trauma-related factors such as UV radiation which can cause cell damage.

Tadpoles are free-living postembryonic organisms that achieve all of their intricate developmental milestones without the protection of a shell or uterus. The environment in which these tadpoles develop is complex and dynamic. Sorting out the significant physical and chemical (molecular) factors that are contributing to teratogenesis in the frogs from those factors that are coincidentally present in the environment without direct effect is important.

Malformations can indicate a general time frame during which factors are influencing the teratogenic process. Knowledge of the possible developmental stages that have incorporated an error can provide guidance in choosing appropriate times for collecting field samples for chemical analysis and guide researches in developing bioassays that mimic the timing of factors present in the field. Careful characterization and categorization of malformations occurring in the frogs can provide clues to potential causes through comparison with similar malformations that have been produced in lab experiments. Some of these laboratory experiments involving development date back as far as 1929 (Vogt). However, associations based on morphologic evidence are circumstantial and without further field and lab analyses, conclusions cannot be drawn regarding the causes of malformations.

To determine the causes of malformations, agents will need to be identified in the field at appropriate developmental times, and in the appropriate geographic locations in relation to occurrence of malformations. The agents must be capable of consistently producing comparable lesions in appropriate species of frogs. Lab experiments will need to take into consideration the role played by such variables as timing of exposure, dose and duration of exposure, and interactions between other environmental or host factors in the potential for producing the wide range of malformations seen in the field. Laboratory results will need to be reproducible under field conditions.

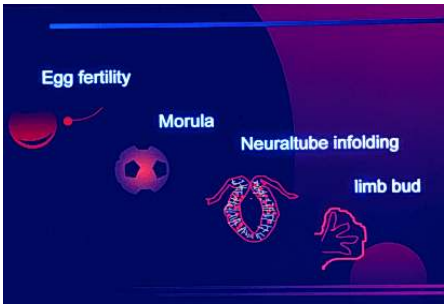
III. DEFINING THE PROBLEM

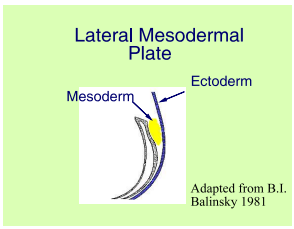
14897-011

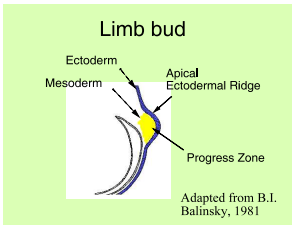
The pathology of many of malformed frogs is obvious, the cause is not. Syndromes like these have not been clearly defined in frogs and even the direction to look for answers is under debate. These changes are not produced by a sudden and catastrophic insult. Malformations represent the end point in a developmental process that incorporated an error long before the external manifestation of pathology. What are the errors in development and what questions can we ask to clarify the possible causes of these errors?

A. Morphogenesis

From the time an egg is fertilized, an intricate relationship exists between cells and cell layers which gives rise to very organized and highly specialized tissues. This process is called morphogenesis. Morphogenesis occurs through cell division and proliferation, cell migration, cell differentiation, and programmed cell death. Cells divide and proliferate following well defined maps as they move and fold, becoming more differentiated and specialized. 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 thought to be carried out primarily by chemical signals that affect genetic expression.







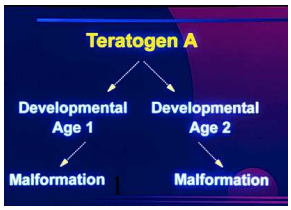
In limb bud formation, the mesoderm carries all of the information needed to induce a limb as it interacts with the overlying ectoderm. If the lateral mesodermal plate, represented in this figure, is surgically transplanted onto the back of a tadpole, an entire limb will form in response to that committed mesoderm. During later morphogenesis of the limb the mesoderm is responsible for directing bone formation and the ectoderm is responsible for providing the cellular environment that allows the limb to continue to grow.

B. Malformation vs. Deformation

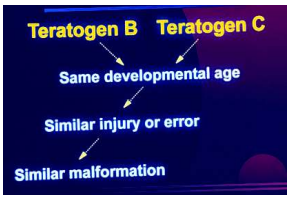
If something goes wrong during morphogenesis and an error occurs in the sequence of cell division, cell migration, cell differentiation or programmed cell death, pathology is likely. This pathology is called a malformation or a deformation. Malformations represent primary errors in development; deformations arise later in development and usually result from mechanical factors. Timing of the error or insult influences both the occurrence and the type of malformation produced. And the appearance of the malformation can indicate timing. For example, if the malformation is an incomplete organ, the factor or insult must have occurred during a susceptible period prior to complete formation of that organ to halting development.

C. Teratogenesis

Teratogenesis is simply the name given to the abnormal development that results in malformations. These errors can involve any level of organ formation; cell division and proliferation, cell migration, cell differentiation, and programmed cell death. Teratogenesis can be caused by either genetic or environmental factors (intra-uterine factors in mammals). Examples of environmental factors include radiation, hyperthermia, low oxygen, high carbon dioxide, poor nutrition and chemicals. Factors or compounds causing malformations are called teratogens.



The same teratogen presented at different developmental ages can initiate different malformations. However, different teratogens presented at the same developmental age can result in similar errors in development causing the same malformation.



IV. FINDINGS

A. *PHYSICAL CHARACTERISTICS OF FROGS WITH SIMILAR MALFORMATIONS*

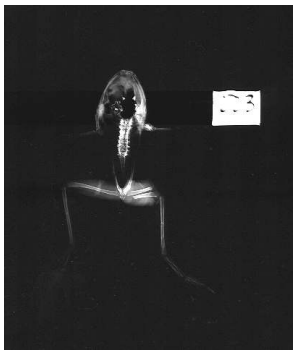
During July and September 1997, 93 recently metamorphosed leopard frogs were submitted to the National Wildlife Health Center (NWHC) for diagnostic evaluation. Frogs were submitted from Alburg Dune (12 abnormal and 6 normal), Lapans Bay (14 abnormal and 6 normal), Missisquoi NWR (15 abnormal and 6 normal), Mud Creek (6 normals), Poultney River (21 abnormal and 7 normal) and shipped, alive, to the NWHC. Diagnostic methods and procedures are detailed in the study plan (Appendix B).

Necropsy evaluations did not reveal developmental abnormalities in the internal organs of the frogs, although there were renal flukes (most likely Echinostomatae) in various stages of development and inflammation of the cloaca was occasionally seen. The most common primary abnormalities in these frogs involved the rear legs and examples of these limbs with radiographic detail of the bone changes provide information that assists in classification of the pathology as malformations or deformations.

1. Deformations

a. Trauma

Very few frogs submitted were convincing deformities or trauma. Again, deformities are not primary errors in development but structural abnormalities that can be caused by mechanical forces, trauma, infectious agents or inflammation.


14895-003

Gross observations of northern leopard frog 14895-003 from Poultney River indicated a short femur on one side suggesting a malformation. Radiographs, however, show us that the femur actually has a overriding fracture which is a deformity caused by trauma.

Although frog 14897-003 from Missisquoi is missing a limb, the changes in this limb are consistent with trauma and not a primary error in development. Characteristic changes associated with trauma include an abrupt end to the leg associated with an irregular depressed center and red, inflamed, raised margins. Microscopic examination of leg of the frog shown at right (14897-003) showed hemorrhage, inflammation and acute necrosis of the skeletal muscle surrounding the end of the femur. No radiographs were available from this frog as the limb was sectioned for microscopic evaluation.


14897-003

Two of the frogs (14896-008, 14897-003) were in fair body condition suggesting recent trauma. Frogs 14897-013 and 14895-003 were in poor body condition suggesting a more chronic condition.

Trauma n=4; (left=3 right=1)

Suspect trauma from VT: Alburg Dune 14896-008; Missisquoi 15897-003, 013; Poultney River 14895-003 (femur fracture).

AVERAGES FOR FROGS WITH TRAUMA
Snout to vent length: 3.35 cm
Weight: 2.83 gm
Body condition: 2

2. Malformations

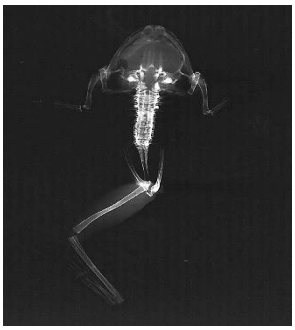
The limb abnormalities seen in the Vermont study that represent primary errors in development follow and include; complete or partially missing limbs, rotation of long bones, and skin webbing.

a. *Amelia*

14895-002

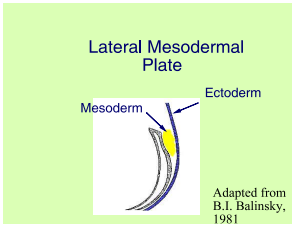
This frog is missing a limb. This is a very early primary error in development.

If we look at the radiograph of one of these frogs with amelia (below 14895-015) we see that not only is the right leg missing, but the right acetabulum (joint for the femur) of the hip also never developed and the two bones (ischium and pubis) are absent. The hip and hip joint on the opposite side with a normal limb are unremarkable. There is no evidence of inflammation, fractures or vestigial bones in the regions where the right leg should be. The limb never developed.



14895-015

Where are the errors in development? The lateral mesodermal plate is responsible for the pelvic development as well as initiating the development of the rear limb. The lateral mesodermal plate must have been present, as evidenced by the presence of at least a portion of the pelvis on the affected side. However, there must have been a very early error because 2 of the 3 pelvic bones never developed. Apparent lack of initiation of a limb also suggests an early primary error in the formation of the limb with either an error in the development of the apical ectodermal ridge or in communication between the mesoderm and ectoderm.



Radiographs of three frogs with completely missing limbs showed two types of changes. Two of the frogs radiographed were missing the ischium and the pubis of the pelvis only on the side without the limb, as in the radiograph shown above. The other radiographed frog with amelia had all of the pelvic elements present.

Frogs with amelia were emaciated.

Amelia n= 6 (right limb=4, left limb=2)
Alburg dune 14896-007, 009; Lapans Bay 14898-003; Poultney River 14895-002, 006, 015

AVERAGES OF FROGS WITH AMELIA
Snout to vent length (n=6): 2.93 cm
Weight (n=5): 1.76 gm
Body condition (n=3): 1

b. Polymelia

Multiple limbs (n=0)

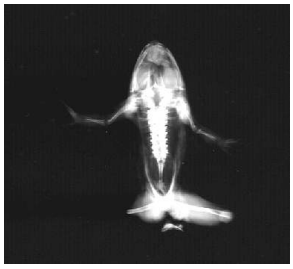
None of the Vermont frogs examined by the NWHC in 1997 had multiple limbs or multiple elements associated with the limb.

c. Hemimelia

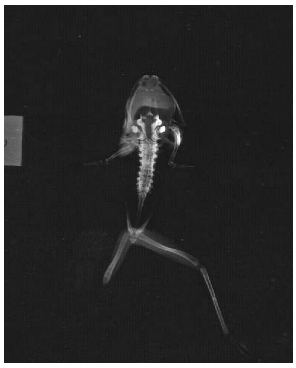
Truncation of the limb suggests an error prior to the completion of limb development but an error that probably occurred later in development than those errors occurring in frogs with amelia.

14896-003

Frog 14896-003 from Alburg Dune does not have the characteristic changes expected in a traumatized limb. There is no swelling, redness, inflammation, or irregularity at the termination of these limbs. There is no scarring and the pigmentation over the end of the limb is normal. Radiographs of these limbs show no fractures in the segment of femur that remains and the termination is blunt. Frogs with hemimelia have truncated limbs but the proximal segment of limb that is present is relatively normal. This is one of 2 types of bone changes seen radiographically in association with truncation of bones (hemimelia).

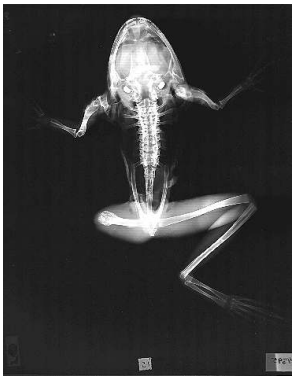


14896-003



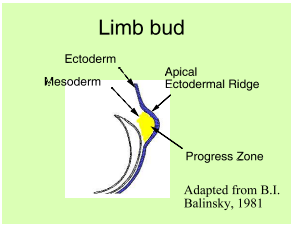
14896-014

If we look at these two radiographs, we see 14896-014 had an abrupt termination of the femur without bone reaction similar to that seen in 14896-003. In the radiograph of 14895-013, however, the distal femur has very abnormal bone development at the end of the bone with irregular widening of the bone silhouette and increased lacy bone trabeculae traversing the marrow. There is no attempt at bone development beyond this abnormal bone.



14896-013

The bone changes in 14895-013 suggest that there was an error at the progress zone; with mesodermal signals; or with communication between the ectoderm and mesoderm which eventually stopped growth but may not have stopped as abruptly as in 14896-003. The bone development apparently lost its control and became very irregular abnormal fashion in 14895-013 but it did continue to produce bone. In 14896-003 and 014, we see no irregular bone growth at the termination, the bone just ends suggesting a more abrupt halt to development.



Microscopically, the proliferative bone changes as seen in 14895-013 confirmed thick, partially ossified trabeculae traversing the marrow with irregular and thickened cortices which were sometimes bent at the point of abnormal development.

Frogs with a portion of one limb missing were generally in poor to emaciated body condition.

Hemimelia:
Unilateral n =29 (right limb=18, left limb=11)
Bilateral n= 4;

Lapans Bay 14898-002, 005, 007, 008, 009, 011, 012, 014.; Missisquoi 14897-006, 008, 009, 010, 011, 012, 014, 017, 022; Alburg dune 14896-003, 005, 006, 010, 014; Poultney River 14895-004, 005, 007, 010, 011, 012, 013, 019, 020, 021

Hemimelia of femur n=11
Hemimelia of tibiafibula n=16
Hemimelia of tibiale and or fibulare n=3
Adactyly n=3

Bilateral hemimelia received counts for each leg for the occurrence of hemimelia, hence the 3 extra malformation designations above. In the bilateral hemimelia frogs, one had bilateral femoral hemimelia, one had a tibiafibula hemimelia and a tibiale/fibulare hemimelia, and the other frog had bilateral adactyly.

AVERAGES OF FROGS WITH HEMIMELIA

Hemimelia of femur
Snout to vent length n=11: 3.25 cm
Weight n=11: 2.66 gm
Body condition n=7: 1.71

Hemimelia of tibiafibula = 16
Snout to vent length n=15: 3.27 cm
Weight: 2.45 gm
Body condition n=11: 1.82

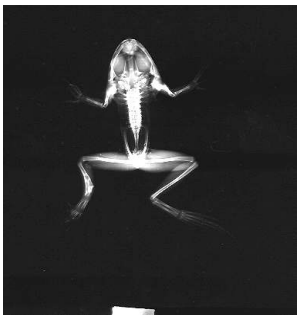
Hemimelia of tibiale, fibulare (n=3)
Snout to vent length: 3.53 cm
Weight n=: 2.73 gm
Body condition (n=2): 1.0

Adactyly (n=3)
Snout to vent length n=2: 3.35 cm
Weight n=2: 2.50 gm
Body condition n=1: 1.0

d. Abnormal feet or missing digits (Ectrodactyly or Brachydactyly)


14895-008

Frog 14895-008 from Poultney River has a short limb and, although difficult to recognize, this disorganized limb ends with a structure resembling a foot.


14895-008

The radiograph of this frog shows that not only is the growth abnormal but differentiation of the bones is abnormal as well. There appears to be abnormal development of the distal tibiafibula and there was 180˚ rotation of the tibiafibula that resulted in the tibiale bone in a lateral position and the fibulare bone in medial location. The tibiale and fibulare are short. The digit positions are also reversed 180˚ (they are aligned in the appropriate order relative to the tibiale and fibulare bones) and are missing phalanges. These malformations suggests an error in the mesoderm that may have occurred later in the developmental sequence resulting in abnormal skeletal elements in the distal limb only.

Another example of abnormal development of the feet can be seen in the radiograph of frog 14895-18. There are only two digits and these were within the same web of skin. The tibiale and fibulare bones are again short and curved and there was no functional hock joint. It is interesting that the mid-shaft of the tibiafibula is also abnormal suggesting that the insult to the bone that caused the error began earlier in bone formation with a more protracted effect than would have been appreciated without radiographs.


14895-018

Frogs in this group with malformations of the feet and digits were also generally in poor to emaciated body condition.

Abnormal development of feet or digits; n= 24 (right limb=15, left limb=9)

Lapans Bay 14898-001, 006, 010; Missisquoi 14897-001, 004, 005, 007, 015(adult), 016, 018, 019, 020, 021, 023; Alburg Dune 14896-001 (adult), 004, 009; Poultney River 14895-001, 005, 008, 009, 014, 016, 018.

AVERAGES OF FROGS WITH ECTRODACTYLY: (Not including adults)

Ectrodactyly (n=17)
Snout to vent length (n=12): 3.25 cm
Weight (n=12): 2.77 gm
Body condition (n=7 recorded): 1.57

Brachydactyly (n=4)
Snout to vent length: 2.88 cm
Weight: 1.88 gm
Body condition: 1

e. Rotation of long bones and skin webbing

The radiograph of the Vermont frog shown (14895-010), although missing the distal limb on one side has rotation of the lower limb of the more normal leg with a bend in the bone that is traversed by a "bone bridge". Rotational abnormalities in the Minnesota frogs were very common. These rotational abnormalities involved the limb proximal to the hock causing the flexed hock to have either a dorsal or ventral orientation rather than pointing in the more normal medial direction. Although there were no Vermont frogs with rotation as its primarily malformation rotations did occur in relation with other malformations and did resemble some of the Minnesota rotations. Rotations were most likely to occur in frogs with ectrodactyly or brachydactyly (missing or shortened digits) suggesting that errors in the bone formation was occurring proximal to the affected digits (4 frogs). Limb rotation also occurred in two frogs with femoral hemimelia of the opposite limb and in the same limb as tibiafibular hemimelia in another frog. It appeared that the more proximal the source of rotational abnormality (ie femur vs tibiafibula), the more severely the propelling surface deviated from the norm.

14895-010

Limb Rotation n=7; (right limb=2, left limb=5)
Missisquoi 14897- 004, 006, 011; Poultney River 14895-001, 005, 008, 010.

AVERAGES OF FROGS WITH ROTATION

Snout to vent length n=7: 3.32 cm
Weight n=7: 2.26 gm
Body condition n=6: 1.17

f. Craniofacial abnormalities / Body conformation

Although jaw and skull malformations have been common in other states, none were seen in the submissions from Vermont.

One of the frogs from Alburg Dune (14896-011) was runted with possible compression of the vertebrae.

Short compressed (runted) body type (n=1; only with full assessments)
Snout to vent length: 2.6 cm
Weight: 1.3 gm
Body condition: 1

g. Normal

Normal frogs varied in size between sites. The average weight and length of normal frogs from Mud Creek, which was the control site, were 4.35 gm and 3.97 cm respectively, average measures for weight and length of normal frogs from sites with malformations were Alburg Dune: weight 2.0 gm and 3.97 cm and average weight and length of normal frogs submitted from Lapans Bay were 1.4 gm and 2.6 cm respectively.

AVERAGES FOR NORMAL FROGS FROM CONTROL SITE
Mud Creek 14899-001, 002, 003, 004, 005, 006
Snout to vent length 3.97 cm
Weight 4.35 gm
Body condition 3.7

AVERAGES FOR NORMAL FROGS FROM SITES WITH MALFORMATIONS

Alburg Dune: 14896- 012, 013, 015
Snout to vent length: 3.13 cm
Weight 2.0 gm
Body condition 1 (recorded in 012 only)

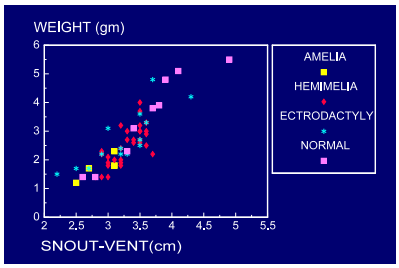
Lapans Bay: 14898-015
Snout to vent length: 2.6 cm
Weight: 1.4 gm
Body condition: (not recorded)

Missisquoi NWR and Poultney River
No Normal frogs submitted

3. INTERPRETATION OF DATA

Although the number of frogs submitted from each site was low, it seems important to look at the data to see if there was an association between malformation type and body condition. The potential impact a particular abnormalities might have on frog success, as represented by general body condition or nutritional status, might also be accessible through data analysis. Data from the field studies will be needed to determine if the malformations submitted from each site for this study were truly representative of the population in both malformation type and frequency of malformations present.

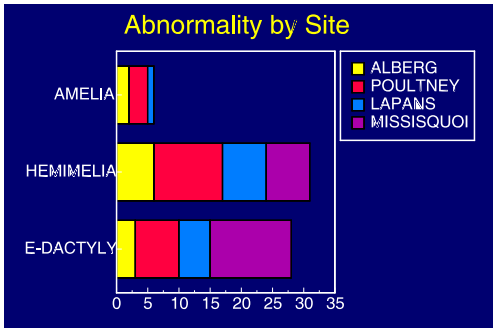
Although data should be corrected for population variation due to site variables as well as for weight bias secondary to absence of body parts in particular abnormalities, I feel looking at the frog morphology data, as in this scatter diagram, could provide insight into the association between the type of abnormality in the frog and body condition of those frogs, represented by the frog's weight in relation to length. The average body condition for all of the types of malformations was poor to emaciated with the exception of frogs with amelia that were all very emaciated.



B. MORPHOLOGIC FINDINGS IN FROGS FROM EACH STUDY SITE:

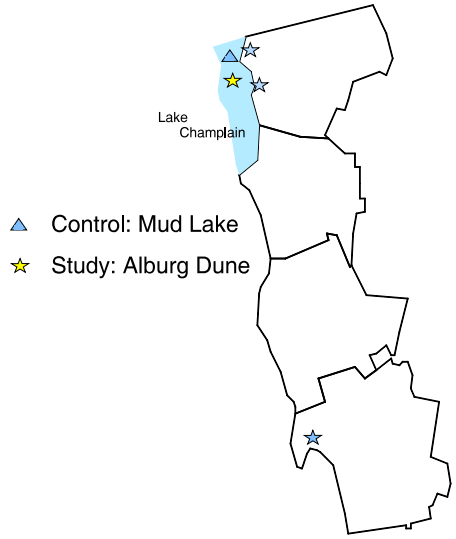
Malformations from Vermont were 93% reduction malformations (amelia, hemimelia or ectrodactyly). Only 3 frogs submitted with malformations evaluated had an abnormality that did not involve truncation of a limb. Of the 61 malformations, (4 were bilateral) 10% had no limb on one side, 40% involved abrupt termination of either the femur, tibiafibula or tibiale and fibulare and 43% had loss or truncation of the digits.

Frogs from Missisquoi had the most complete limb development with truncations involving the lower limb distal to the femur. Frogs from Poultney River and Alburg Dune (although fewer number of total frogs were submitted from Alburg Dune) had the most severe truncations with the primary abnormalities involving apparent lack of any initiation of limb (amelia) or truncation of the limb at the femur (femoral hemimelia). Average body condition in frogs from all of the study sites was poor to emaciated. Frogs with no apparent malformations (controls) collected at the study sites also were in poor to fair body condition but the sample size was too small to make an extrapolation to the overall population of leopard frogs at these sites. Lapans Bay had rear limb malformations primarily involving the tibiafibula and the foot.



This chart of **association of severity of malformation with site of collection** might suggest errors occurring either at different developmental stages in frog development, or different dose or duration of exposure to a teratogen.

1. Alburg Dune



ALBURG DUNE (14896)

Total Frogs with malformed rear limbs: 11

Amelia: 2 = 18% of all malformations from Alburg Dune

Hemimelia (one frog was bilateral): 6 = 55% of all malformations from Alburg Dune and 67% involved the femur

Abnormal feet or digits: 3 = 27% of all malformations from Alburg Dune

Trauma: 1

Primary Site Malformation: Rear leg **hemimelia/ ectromelia**

4 femur, 2 tibiafibula, 3 digits or phalanges

This represented 27% of all long bone hemimelia submitted from Vermont (femur or tibiafibula) and 13% of malformations submitted that had missing digits or phalanges. Although only 2 frogs from Alburg Dune had completely missing limbs, this represented 33% of all of the amelia submitted from Vermont. One frog (14896-011) had stunted body composition.

Average measurements for malformed frogs from Alburg Dune (n=9)

Snout to vent length: 2.99 cm

Weight: 1.93 gm

Body condition (n=7): 1.86

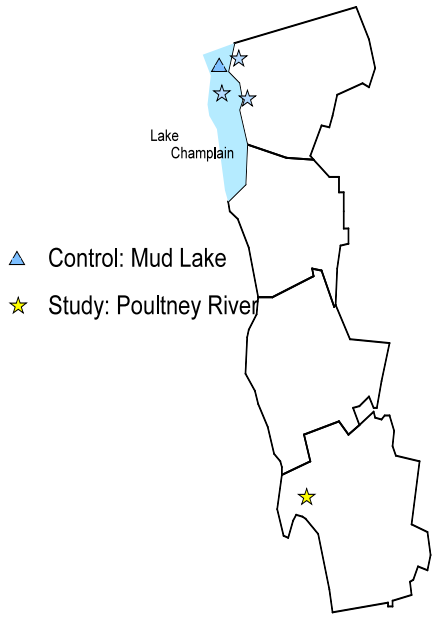
Average measurements for normal control frogs from Alburg Dune: (n=3)

Snout to vent length for normal frogs: 3.13 cm.

Weight of normal frogs: 2.03 gm

Body condition (n=1): 1

2. Poultney River



POULTNEY RIVER (14895)

Total Frogs with malformed rear limbs: 22

Total malformations: 24 (two bilateral)

Amelia: 3 =13% of all malformations from Poultney River

Hemimelia (one frog was bilateral): 11 =46% of all malformations from Poultney River and 55% of these were femur

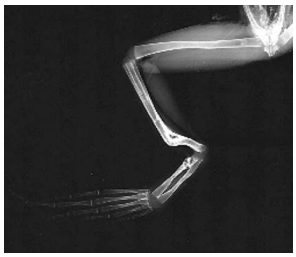
Abnormal feet or digits: 7 = 32% of all malformations from Poultney River(1 bilateral)

Abnormal limb but all bone components present: 3 = 13% of all malformations from Poultney River

Trauma: 1

Primary Site Malformation: **hemimelia/ectromelia** (6 femur, 3 tibiafibula, 1 tibiale fibulare, 7 digits or phalanges).

Frogs from this site represented 50% of all Vermont frogs submitted with no obvious limb development (amelia) on one side, and 45% of all long bone hemimelia (femur or tibiafibula) and 25% of missing digits or phalanges. With the exception of one from Missisquoi with a uniformly short leg, Poultney River was the only site that had frogs submitted with limb malformations representing abnormal bone growth but with all components of the limb represented. These frogs had rotation of the limb with bone bridging as the only abnormality(as shown in 14895-010); cutaneous fusion between digits 1 and 2 but the bones of these digits were normal; and (14895-017) had a large distorted region of femur with mid-shaft disruption of the trabeculae and cortex with the distal limb unremarkable.


14895-010

Average measurements for frogs with limb abnormalities:

Snout to vent length (n=20): 3.6 cm

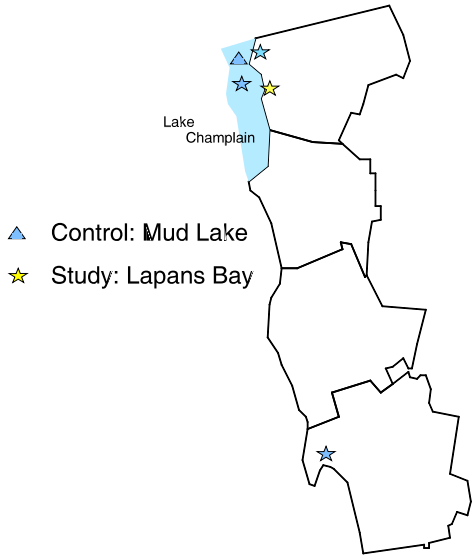
Weight (n=21): 2.92 gm

Body condition (n=10): 1.3

Average measurements for normal control frogs: (n=0)

Abnormal limbs were truncated at a more proximal site potentially suggesting that developmental errors began earlier in tadpoles at Poultney River. This site had the most apparent errors in initiation (amelia) as well as the most truncations involving the femur. Although no control frogs were submitted from this site for comparison, the average body condition of malformed frogs from this site is poor to emaciated.

3. Lapans Bay



LAPANS BAY (14898)
Total Frogs with malformed rear limbs: 12
Total Malformations: 13; one bilateral
Amelia: 1 = 8% of all malformations submitted from Lapans Bay
Hemimelia/ectromelia: 7 = 54% of all malformations submitted from Lapans Bay
Abnormal feet or digits: 5 = 38% of all malformations submitted from Lapans Bay
Trauma: None

Primary site feature: **hemimelia/ectromelia**. (1 femur, 6 tibiafibula, 1 foot, 3 digits or phalanges). Amelia at Lapans Bay represented 16% of all of the '97 Vermont frogs submitted with no obvious limb development on one side; 32% of all long bone hemimelia (femur or tibiafibula) and 17% of missing digits or phalanges. (one of the frogs (14898-010) had a tibiale or fibulare missing as well as missing 4 digits so that frog was represented twice in the above malformation count)

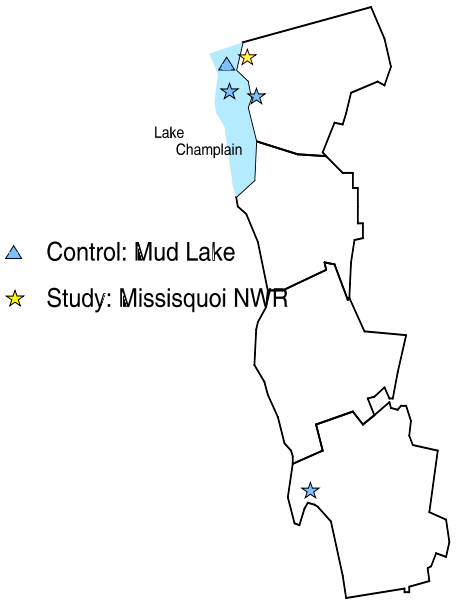
Frogs with rear limb malformation (14898- 004, 013 not included as these were forelimb malformations):

Snout to vent length (n=12): 3.08 cm
Weight (n=12): 2.46 gm
Body condition (n=9): 1.89

Normal control frog: (n=1)
Snout to vent length: 2.6 cm
Weight: 1.4 gm
Body condition: not reported but the weight/snout to vent ratio suggests an emaciated frog.

Frogs submitted from Lapans Bay had malformations that represented truncations of all bones of the limb. One frog had an apparent error in limb initiation. The single control frog from Lapans Bay and the 12 malformed frogs all had an average of poor to emaciated body condition. 14898-004 had a forelimb malformation.

4. Missisquoi NWR



MISSISQUOI (14897)
Total frogs with malformed rear limbs: 21
No Bilateral
Amelia: 0
Hemimelia/ectromelia: 7 =33% of all malformations submitted from Missisquoi
Abnormal feet or digits: 13 =62% of all malformations submitted from Missisquoi
Abnormal limb but all bone components present: 1 (all bones are proportionally short) =5%
Trauma: 2

Primary site feature: **hemimelia/ectromelia**
Rear leg **ectromelia** (0 femur, 6 tibiafibula, 1 tibiale fibulare, 2 foot, 11 digits or phalanges). It is interesting that all of missing bone components in frogs submitted from Missisquoi were distal to the femur. Missisquoi had 32% of all long bone hemimelia (most of these involved tibiafibula) and 54 % of missing digits or phalanges.

Average measurements for frogs with limb abnormalities for which measurements were taken:
(n=12; accession 002 not included as not a rear limb abnormality, 003 was not included because it was classified as trauma):

Snout to vent length: 3.27 cm
Weight: 2.34 gm
Body condition: 1.33

Average measurements for normal control frogs: (n=0)

Abnormal limbs were truncated at a more distal site potentially suggesting a malformation that began later in development of the tadpoles at Missisquoi. Even though the frogs from Missisquoi had less severe malformations than some of the other sites, they were still in poor body condition. 14897-002 had a forelimb malformation.

C. DETERMINATION OF BODY CONDITION

General body condition assessment (good, fair, poor, emaciated) performed at necropsy seemed to most accurately reflect the nutritional status of malformed frogs. This subjective assessment at necropsy was independent of the affect that the abnormality or frog size might have had on weight. When major body parts of the frog were all present, body weight to body length ratios reflect nutrition status of frogs more accurately than body weight alone or snout to vent length alone as a large frog (length) may be emaciated and have the same weight as a smaller frog with good body fat stores. For this study, it was assumed that sex was not a factor in the body condition or size of these immature, recently metamorphosed frogs. Frogs necropsied very close to the time of tail bud resorption (metamorphosis just completed) may still have fat reserves stored during the tadpole stage. These frogs would not have experienced the survival pressures placed on a frog that had been required to actively capture food for a longer period of time and the fat stores in these recently metamorphosed frogs could be misleading if used as an indicator of success as a frog.

D. HISTOPATHOLOGY FINDINGS

Histopathology was performed on 19 frogs (Appendix F). Histopathology on malformed bones showed two general types of bone changes. One of the characteristics of bone at the termination of the malformed limb was very disorganized and hyperplastic cartilage that was not ossifying normally and interfered with the formation of bone cortex. The second type of bone change at the point of termination was the formation of small nests of endosteal type cartilage matrix with some organization but no obvious maturation or ossification. None of the malformed limbs had inflammation and they often had very poor skeletal muscle development. Traumatic amputations did have microscopic evidence of skeletal muscle necrosis and inflammation. No consistent microscopic changes were seen in internal organs (heart, lung, liver, stomach, intestine, pancreas, kidney, bladder, gonad, spleen, thyroid gland, thymus, lymphoid aggregates) that could be related to malformations. The brain and spinal cord of the frogs submitted often had dilated ventricles and we are in the process of consulting with a neuropathologist to determine the significance of this finding.

E. RADIOGRAPHIC FINDINGS

Radiographs were extremely helpful in determining the degree of malformation in the limbs as well as the type of termination in both long bones and digits.(Appendix D) Malformations of the hip were also seen in some of the Vermont frogs and these would not have been detected without the use of high detail radiography. Radiographs were also helpful in differentiating trauma from malformation, although necropsy observations and histopathology were also important in ruling out trauma.

F. RESULTS OF MICROBIOLOGICAL TESTING

Lab tests were performed on the frogs sent into our lab so that information involving infectious agents could be looked at in relation to malformation types.

1. Parasitology

In an attempt to look for significance of metacercariae in the frogs, parasitology examined the frogs, cleared them and made an estimate of metacercarial load. Metacercarial load in frogs from Vermont were relatively heavy, compared to Minnesota frogs; the primary malformations in all Vermont sites were reduction defects such as hemimelia or truncation of a limb which has not been discussed as a direct sequelae of metacercarial infection.

Parasitologic examination was performed on 14 frogs; with at least 1 normal frog from each site following procedures outlined in the Study Plan (Appendix C). Skin, subcutaneous tissue, coelom, oral cavity, esophagus, intestinal serosa, kidney, urinary bladder, fat body, heart/pericardium, and skeletal muscle from the rear leg, back, abdomen, tail bud area were examined for metacercaria. Metacercariae were most consistently seen in the kidney, however this metacercaria was morphologically distinct (most likely Echinostomatae) from the metacercaria seen in other locations. Skin, tail bud, liver, tissue adjacent to eyes, epaxial muscles were also common sites where metacercariae (most likely Diplostomatidae) were found. The metacercariae table in Appendix H is organized by malformation and, although statistical analysis was not meaningful due to the small numbers, an association between metacercariae and malformation was not obvious. Metacercariae were present in both normal and malformed frogs.

Appendix H also contains a table for the adult helminths that were found in these frogs but these are listed for interest and are thought to be incidental findings as there was no pathology associated with these parasites.

The hip region of a cleared frog pictured above shows three metacercariae.

2. Bacteriology:

Cultures of liver and kidney from 30 abnormal frogs and 15 normal frogs (45 frogs) were conducted following the protocol outlined in the study plan (Appendix C). 29 different bacteria were isolated and identified from these frogs; most of them were in the group Enterobacteriaceae and are not usually considered to be primary pathogens. One important exception to this was *Aeromonas hydrophila* which was most commonly isolated from frogs collected at Lapans Bay. *Aeromonas hydrophila* is a known pathogen of amphibians and reptiles. None of the frogs with *Aeromonas hydrophila* isolated had evidence of trauma. The most common isolates from all sites were *Alcaligenes xylosidans* and *Pseudomonas cepacia* and these bacteria were also isolated from normal control frogs. No isolates could be related to malformations.

3. Virology

Attempts to isolate virus from livers, kidneys, hearts and occasionally skin sampled from 32 abnormal frogs and 19 normal frogs (51 frogs) followed the protocol for virus isolation outlined in the study plan. No viruses were isolated or identified in negatively stained preparations for electron microscopic in any of the samples from any of the frogs in this study.

Poultney River
14895-001-005 liver, kidney
-003-005 gut
-008,010 heart
-026-028 liver, kidney

Alburg Dune
14896-001-005 liver, gut, kidney
-008 foot skin
-008-010 heart
-017,018 liver, kidney, heart

Missisquoi
14897-002-005 liver, gut, kidney
-003 skin
-008-010 heart
-024-029 liver, kidney, heart

Lapans Bay
14898-001-005 liver, gut kidney
-008-010 heart
-017-020 liver, kidney, heart

Mud Creek
14899-001-003 liver, kidney
-002-004 gut
-002 lung
-004 heart

V. FUTURE WORK

Assessment of early tadpole development at selected Vermont sites.

APPENDICES

APPENDIX A: DEFINITIONS

Description of frog malformations in this report are based on terminology used in human literature (Bolande, Robbins), developmental biology (O'Rahilly; Carlson), and teratology (Wise). Many of these terms, defined in Appendix B, were originally used to describe defects that are present in the mammalian fetus at birth. Direct application of these terms to frogs that metamorphose in a free-living postembryonic state may or may not prove to be appropriate. However, common terminology may allow recognition of similar conditions by individuals in related specialties involving developmental anomalies across species and bring new interest and collaboration to the issues involving malformed frogs. The terminology in reference to possible points of error in development (mesodermal versus ectodermal) may be simplistic, but allows an initial attempt at looking at the pathogenesis of some of these malformations.

Case number: National Wildlife Health Center (NWHC) reference number given to a group of frogs submitted from the same site and usually collected in a similar time frame.

Accession number: NWHC reference number given to individual frogs within a case.

Body condition (as defined for this report by Meteyer): Body condition reflects the nutritional state of the frog and is determined by the pathologist by subjective assessment of relative quantity of muscle and body fat present in the frog at the time of necropsy. Assessment of body condition is independent of absolute weight or snout to vent length. Body condition has been scored in the following way so that averages can be made for comparison between sites and abnormalities; good = 4, fair = 3, poor = 2, emaciated = 1.

Good body condition: Adequate skeletal muscle and abundant fat in the fat bodies.
Fair body condition: Adequate skeletal muscle with reduced fat in the fat bodies.
Poor body condition: Adequate skeletal muscle with scant or no body fat in the fat bodies.
Emaciated body condition: Skeletal muscle wasting and no body fat in the fat bodies.

Weight to length ratio (as defined for this report by Meteyer): The weight (gm) of the frog divided by the length (cm) of the snout to vent length of the frog which provides an approximation of nutritional status or body condition as a numerical index value which correlates with body condition as assessed at necropsy.

Morphogenesis: Developmental process which gives rise to highly organized and specialized tissues through cell division and proliferation, cell migration, cell differentiation, and programmed cell death.

Pathogenesis: Cellular events, reactions and other pathologic mechanisms occurring in the development of disease

Mesoderm: The embryonic layer from which connective tissue, bone, cartilage, muscle, blood, vasculature, notochord, pleura, pericardium, peritoneum, kidney, and gonads are derived.

Ectoderm: The embryonic layer from which epidermal tissues (skin, hair, etc.), mucous membranes, nervous tissue, and external sense organs (eye, ear, etc.) are derived.

Malformations: Primary errors in any phase of morphogenesis including cell proliferation, cell migration, differentiation, programmed cell death or regression of larval structures.

Deformations: Deformations arise later in fetal life and represent alterations in form or structure resulting from mechanical factors.
There is no intrinsic defect in morphogenesis.

Malformation sequence: cascade of events leading to malformation (IE urethral obstruction may cause secondary affect of renal morphogenesis and may lead to defects in the lower limbs due to compression of blood vessels).

Appendix A: Definitions Continued.

Malformation syndrome: single etiologic agent that simultaneously affects several tissues (IE viruses) RCK.

Teratogenesis: Abnormal development that gives rise to malformations

Teratogen: An agent or factor that causes the production of physical defects in the developing organism (embryo)

Hypoplasia: Incomplete development of an organ

Aplasia (agenesis): Lack of development of an organ or tissue often resulting from failure of appearance of the primordium of an organ in embryonic development. For example, amelia is aplasia or agenesis of a limb.

Ectromelia: Hypoplasia or aplasia of one or more long bones of limbs. Types of ectromelia are amelia, hemimelia, and phocomelia.

Ectrodactyly: Absence of a digit (toe or finger)

Brachydactyly: Absence of phalanx (a bone that comprise the digits)

Amelia: No obvious limb development

Hemimelia: Developmental anomaly characterized by absence of all or part of the distal half of a limb; fibular hemimelia, tibial hemimelia

Phocomelia: Absence of the proximal portion of a limb, IE feet attached to the body by a single small irregularly shaped bone

Polymelia: A developmental anomaly characterized by the presence of supernumerary limbs.

Sympodia: Fusion of lower extremities

Skin Web (as defined for this report by Meteyer): The skin covering the leg traverses at least one joint restricting motion. Dorsal/ventral pigmentation maintained. Malformation or deformation of bone is often associated with restricted limb development and is seen as rotation of the involved bone or joint, foreshortening of the bone, or fusion of joint.

Rotation (as defined for this report by Meteyer): Deviation of bone or joint causing distal limb to have abnormal orientation.

APPENDIX B:

INVESTIGATION OF DEFORMITIES IN THE NORTHERN LEOPARD FROG (*RANA PIPIENS)* AT SELECTED SITES IN VERMONT

Background and Justification

In the fall of 1996, abnormalities in northern leopard frogs (*Rana pipiens*) were observed by the public at 12 sites adjacent to Lake Champlain in Vermont. Incidences of abnormal leopard frogs were verified at four of these sites by staff of the Vermont Agency of Natural Resources (VTANR) on October 9, 1996. The incidence of deformities averaged 16.5 %, ranging from 5-23 % at the four sites (230 frogs observed). Several of the sites where deformities were observed were near the borders of the Missisquoi National Wildlife Refuge in Swanton, Vermont. Most of the abnormalities observed were missing legs or feet, but also included eye abnormalities. Similar observations have been made in at least seven states and two Canadian provinces. The most extensive work to date has been conducted in Minnesota and in the St. Lawrence Valley of Quebec. The mechanism(s) by which these deformities are occurring have not been identified, however, the predominant theories include: UV-B radiation; xenobiotic contaminants; and viral, bacterial, fungal, or parasitic diseases. The purpose of this investigation is to take the first steps in examining the abnormalities in the Vermont frogs. Specifically, this investigation will examine affected frogs from four sites in Vermont, catalogue the external and internal abnormalities, and compare to frogs of an unaffected site in Vermont, as well as abnormal frogs collected from Minnesota in 1995 and 1996. Select samples will be examined for virology, bacteriology, and parasitology. The overall information should verify whether the frogs in Vermont have developmental abnormalities, whether the abnormalities observed in Vermont are the same as those observed in Minnesota, and to begin to determine whether viruses, bacteria, and/or parasites could be responsible for the abnormalities.

Purpose

The purposes of this investigation are several fold: (1) to determine whether the abnormalities observed in northern leopard frogs in Vermont are developmental in nature; (2) to compare abnormal frogs to normal-appearing frogs collected from an expected control site to verify that there are differences; (3) to compare abnormalities found in Vermont to abnormalities found in Minnesota to determine if they are the same phenomenon; and (4) to determine if a subsample of frogs harbor viruses, bacteria, or parasites which could be responsible for the abnormalities observed in Vermont.

Methods

Forty northern leopard frog breeding sites have been identified using recent data from reptile and amphibian atlas work in Vermont. Northern leopard frog metamorphs will be collected from these sites in July, 1997 as part of a larger study to determine the distribution of deformed leopard frogs in Vermont. The 40 sites include Missisquoi National Wildlife Refuge and the four sites where VTANR staff observed deformities in 1996. Fifteen abnormal-appearing frogs from up to four sites, and five normal-appearing from a site expected to be unaffected will be shipped live to the National Wildlife Health Center (NWHC) in Madison, Wisconsin. Frogs will be examined to characterize abnormalities and catalog them using standardized nomenclature and morphologic diagrams. Routine microbiological samples will be run as necessary on a subset of tissues. Catalogued data will be used to compare abnormalities in Vermont frogs to those recorded during 1995 and 1996 Minnesota frogs. Tissue samples will be archived for later use if funding becomes available.

Budget

Field trip for NWHC staff to document collection sites, demonstrate and coordinate methodology for classification of gross abnormalities and collection and shipment of frogs.$1,000

Radiography, external examination, necropsy examination, microscopic evaluation of tissues, classification and cataloging of abnormalities. Virology, bacteriology and/or parasitology on selected samples.$7,000

APPENDIX B:

USGS/BRD QUICK RESPONSE GRANT PROPOSAL

December 16, 1997

Title: Radiography of Abnormal Frogs at Select Sites in Vermont

Background

Although scientific reports of amphibian deformities have been noted by naturalists and scientists since at least the 1700s (Van Valen 1974), the concurrent geographic extent and rate of deformities appear to have markedly increased in recent years (DuBois 1996; Hoppe 1996; Tietge 1996). The majority of deformities have involved multiple, fused, or missing hind limbs, however, some recently reported developmental deformities have also included vestigial tails, missing, misplaced or supernumerary eyes and ossification abnormalities (R. Brannian, National Wildlife Health Center, pers. comm., 1997; J. Helgen, Minnesota Pollution Control Agency, pers. comm., 1997).

The causes of the rise in amphibian deformities remain unclear, however, a number of hypotheses are presently being researched including: xenobiotic chemicals, parasites, and UV-B light. Some researchers have indicated that classifying the types of abnormalities on a site will be helpful in isolating the causal agent or agents (C. Meteyer, NWHC, pers. comm., 1997).

In 1997, 19 sites in the Lake Champlain Basin in Vermont were surveyed for frog deformities. Rates greater than three percent were observed at 17 of the sites. Rates overall ranged from 0-45% and averaged approximately 8% (R. Levey, State of Vermont, pers. comm., 1997). A BRD Quick Initiative Grant of $8000 awarded to this project allowed the National Wildlife Health Center to conduct a workup of the abnormal frogs collected in Vermont. The workup included classification of abnormalities, histopathology, necropsy, virology, bacteriology, and parasitology. The results of this work suggested that there was no correlation between viruses, bacteria, or parasites and the frog abnormalities observed in Vermont (C. Meteyer, NWHC, pers. comm., 1997). What the work did not include, due to insufficient funds, was skeletal analysis through radiology. This additional work should clarify whether the abnormalities observed are deformities, caused by trauma or disease, or malformations, caused by a disfunction in development. The work should also serve to diagnose any skeletal abnormalities not externally observable.

Objective:

To determine through radiology of frogs collected in Vermont in 1997 whether the abnormalities observed are developmental in nature or trauma induced, as well as to diagnose skeletal abnormalities not externally observable.

Expected Products:

A written report of the project findings will be prepared. The work will be presented at professional meetings and will be incorporated into peer-reviewed literature.

Contact:

Laura Eaton-Poole, USFWS, New England Field Office, 22 Bridge Street, Concord, NH 03301. Tel. 603-225-1411, fax 603-225-1467, email laura_eaton@fws.gov.

BRD Science Center:

Dr. Katherine Converse of the National Wildlife Health Center is the recommended investigator. Dr. Converse has been involved in evaluating abnormal frogs in Minnesota, and she participated in the frog survey in Vermont in 1997. She is still in the process of evaluating abnormal frogs collected in Vermont. Some of the findings were presented at the workshop sponsored by the National Institute of Environmental Health Sciences on December 4, 1997.

Amount Requested:

70 frogs at $25.00/frog $1,750.00
Analysis and report .....$ 250.00
Total $2,000.00

Literature Cited

Dubois, R. B. 1996. Recent observations of deformed anurans in Wisconsin. NAAMP III - the North American Amphibian Monitoring Program Third Annual Meeting: A Meeting to Present, Evaluate, and Discuss Amphibian Monitoring Techniques for North America, World Wide Web Conference, November 14, 1996-February, 1997.

Hoppe, D.M. 1996. Historical observations and recent species diversity of deformed anurans in Minnesota. NAAMP III - the North American Amphibian Monitoring Program Third Annual Meeting: A Meeting to Present, Evaluate, and Discuss Amphibian Monitoring Techniques for North America, World Wide Web Conference, November 14, 1996-February, 1997.

Tietge, J. 1996. National reporting center for amphibian deformities. NAAMP III - the North American Amphibian Monitoring Program Third Annual Meeting: A Meeting to Present, Evaluate, and Discuss Amphibian Monitoring Techniques for North America, World Wide Web Conference, November 14, 1996-February, 1997.

APPENDIX B:
PROGRESS REPORT FOR USGS-BRD PROJECTS FUNDED BY
EASTERN REGION QUICK RESPONSE MONEY

PROJECT LEADER: Dr. Carol U. Meteyer, Dr. Kathryn Converse

USGS-BRD ORGANIZATION: National Wildlife Health Center - Madison

PROJECT TITLE: Radiographic Characterization of Abnormal Frogs from Selected Sites in Vermont

PROJECT COSTS: $ 2,000

Malformations of Vermont frogs collected during the summer of 1997 were characterized during necropsy examination, and lab tests were performed to determine if there were associations between diagnostic findings and malformations. Following that study, we obtained ultradetail radiographic equipment which provided very detailed and magnified images of bone changes in the frogs. Given this new capability, quick response money was requested and provided in 1998 for a retrospective radiographic study of these frogs to see if there were specific bone changes unique for frogs collected in Vermont or at specific sites in Vermont. Radiographs of 33 frogs collected in 1997 from 4 Vermont sites have been performed and interpretation of these radiographic changes is complete. Results of these findings are in the attached data table which includes a unique classification system developed through insight gained from radiographic images. This table combines description of necropsy and radiographic findings. At this time we are determining significance of the radiographic findings based on malformation type and geographic distribution of the frogs. A final report will be sent by December 31, 1998 to the USGS-BRD Eastern Regional Office, USFWS Region 5, and the Vermont Agency of Natural Resources after the radiographic findings are incorporated into the overall final report for the Vermont study. We are currently looking for funding to extend this study to include microscopic characterization of bone changes with comparison to gross and radiographic findings in an effort to more completely delineate factors that might be contributing to the production of malformations at the cellular level.

APPENDIX C:

U.S. Geological Survey
Biological Resources Division
NATIONAL WILDLIFE HEALTH CENTER
6006 Schroeder Road
Madison, Wisconsin 53711
Phone: 608-271-4640

STUDY PLAN

Study Plan: Investigation of deformities in the northern leopard frog *(Rana pipiens)* at selected sites in Vermont.

Background and Justification: In the fall of 1996, deformities in northern leopard frogs (*Rana pipiens*) were observed by the public at 12 sites adjacent to Lake Champlain in Vermont. Several sites were near the border of the Missisquoi NWR in Swanton, Vermont. The presence of deformed leopard frogs were verified at four of these sites by staff of the Vermont Agency of Natural Resources (VTDEC) on October 9, 1996. The prevalence of deformities averaged 16.5 %, ranging from 5-23 % at the four sites (230 frogs observed). Most of the frogs observed had missing legs or feet and some had eye abnormalities. Similar observations have been made in at least seven states and two Canadian provinces. The most extensive surveys and identification of deformities in frogs and environmental sampling to date has been conduced in Minnesota and the St. Lawrence Valley of Quebec. The mechanism(s) by which these deformities are occurring has not been identified; however, the popular theories include: UV-B radiation; xenobiotic contaminants; and viral, bacterial, fungal, or parasitic diseases. The purpose of this investigation is to take a first step by completing a clinical examination of deformed frogs collected from four sites in Vermont to complete the following objectives:

Objectives:

1. Characterize and catalog the external and internal deformities in leopard frogs in Vermont;
2. Compare deformed frogs to normal-appearing frogs from an expected control site;
3. Compare morphometric descriptions of abnormalities in Vermont leopard frogs to abnormalities confirmed in Minnesota frogs to determine whether any similarities exist; and
4. Determine the presence or absence of viruses, bacteria, or parasites in deformed frogs.

Methods and approach: Forty site where northern leopard frogs breed are identified in the recent Vermont reptile and amphibian atlas (Andrews, 1995).
). In 1996, deformed frogs were observed by the public on 12 of these sites; VTDEC staff confirmed the frog deformities on four of these sites in 1996. A least 100 frogs will be collected from each of the 40 sites. Four sites with at least 10% of the frogs appearing abnormal will be selected for this study. Fifteen abnormal-appearing frogs will be collected from each of these four sites and five normal-appearing frogs will be collected from a control site where no deformities were present in a sample of at least 50 frogs.

Recently metamorphosed frogs will be collected and examined for the presence and type of deformities. Frogs with deformities will be placed in 11 x 32 cm plastic boxes with lids containing 1 air-hole/inch in a chilled cooler or shady location. Approximately 3 cm of water from the site will be placed in the boxes along with sphagnum moss. At the completion of frog collection, the plastic boxes will be placed in hard-sided coolers, surrounded on four sides with frozen blue ice containers. The boxes will be held in place by a washable metal frame. Eight frogs will be placed live in each container and shipped via overnight service to the National Wildlife Health Center (NWHC). Upon arrival, frogs will be examined to deter ermine how the frog responds to this abnormality, photographed, the deformities will be characterized and cataloged using nomenclature and morphologic diagrams developed at NWHC based on frogs submitted to NWHC, photographs by the Minnesota Pollution Control Agency (MPCA, Helgen), and a Quebec study (Ouellet, 1997). Routine histopathological and microbiological samples will be collected and processed on a subset of tissues. Catalogued data will be used to compare abnormalities in Vermont frogs to those recorded during 1995 and 1996 Minnesota frogs. Tissue samples or whole frogs will be archived for later use if funding becomes available or a potential agent causing the deformities is identified.

Characterization of abnormalities and Necropsy Examination

1. Describe clinical condition/function or movement of any deformed limbs, multiple limbs or other grossly apparent deformities..
2. Photograph abnormalities before the frog is anesthetized or euthanized if possible.
3. Euthanize one frog at a time using inhalation of Halothane (see NWHC SOP).
4. Necropsy will be performed with the use of a dissecting scope and 2X head loop. Frogs are opened with an ventral midline incision. The heart is collected and a blood smear made. Tissues for virology will be placed in viral transport media, tissues for bacteriology in vials of broth and tissue for histopathology in formalin. Very small tissues will be places in embedding bags in formalin.

5. Necropsy set-up and lab distribution of frogs is as follows:

Sites 1-4 Case #'s 14895 - 14898

Accessions 001-005, Pathology:
- 2 blood smears
- Multiple tissues in formalin and embedding bag
- Tissues for bacteriology, virology

Accessions 006-007, Parasitology:
- Description of gross pathology
- Submit whole carcasses in whirl-paks, in saline

Accessions 008-010, Virology:
- Description of gross pathology
- Blood smear
- Submit tissues in viral transport media
- Carcass saved in formalin jar - Hold

Accessions 011-013, Bacteriology:
- Description of gross pathology
- Blood smear
- Submit tissues bact broth vial
- Carcass saved in formalin jar - Hold

Accessions 014-015, Hold Frozen:
- Description of gross pathology
- Freeze back in whirl-paks

Normals, Case # 14899

Accession 001: Pathology (necropsy as above)
Accessions 002-003: Virology (necropsy as above)
Accessions 004-005: Bacteriology (necropsy as above)

Virology

1. Virus isolation will be attempted in amphibian and fish cell culture. Liver, spleen, kidney, gut and heart were pooled by like tissues (liver with livers, spleen with spleens) with a maximum of three frogs per pool.

2. Viral isolates will be identified and characterized.

3. Electron microscopy will be performed on the pooled tissues in an attempt to detect any viruses that cannot be isolated in cell culture.

Bacteriology

Frog tissues consisting primarily of liver, heart, kidney and others will be placed directly into appropriately labeled tubes of tryptic soy broth (TSB) at necropsy and submitted to diagnostic bacteriology. The following procedure will be followed:

1. The 10 ml tubes of TSB with tissue will be vortexes for 5-10 seconds and subcultured immediately onto blood agar (BAP), eosine-methylene blue agar (EMB), (or MacConkey agar), Lowenstein-Jensen agar (LJ), sabouraud-dextrose agar (SAB), thioglycollate broth (THIO), and cooked-meat broth (CM).

2. Media (except LJ and SAB) will be examined at 18-24 hour intervals for up to 72 hours before being discarded as negative. Positive cultures will be subcultured and identified to at least genus level. TSB will be subcultured after 24 hours if growth appears. LJ and SAB will be held as appropriate until sufficient growth develops or there is no growth.

3. Identification of bacterial isolates will be consistent with current capabilities and will include the use of commercial identification systems; predominately the API system, bioMerieux Vitex, St. Louis, Missouri. Reference laboratories, primarily the National Veterinary Services Laboratory, Ames, Iowa will be used as needed.

Parasitology

To determine the presence and species of parasites or evidence of parasite infections, live or freshly euthanized frogs will be sent to the parasitology laboratory for examination under a dissection scope. The following procedures will be followed:

1. Frogs will be skinned and the muscles and fascia examined under the dissecting microscope. Hind limb muscles will be teased apart and examined for metacercaria.

2. Metacercaria in the subdermal layers will be dissected free and placed temporarily in ½ strength Locke's solution and refrigerated. In some cases, this will cause the metacercaria to encyst so that fixation and staining can proceed. Notes will be kept and vial labeled as to collection site for the metacercaria.

3. A ventral incision will be made from vent to lower mandible to expose internal organs.

4. The heart will be removed and a blood smear made according to DPL SOP.

5. All organs will be examined grossly, any parasites removed, prepped for fixation and fixed in AFA or 70% depending on the class of parasite. Most organs are small enough to examine via a squash preparation. Fat bodies will be examined via squash preparation for mesocercariae.

6. The body cavity will be examined for immature helminths. If found they will be removed, relaxed and fixed.

7. Eviscerated carcasses will be placed in 10% buffered formalin and later cleared according to Hanken ans Wassersug (1981). Following clearing frogs will be examined and the presence or absence of metacercaria noted.

Critical Data: 1. Specific characterization of external deformities,

2. Documentation of internal deformities,

3. Histopathological findings for Minnesota frogs for comparison with Vermont frogs.

Statistical Treatment: The research sites will be selected from areas with at least 15% of the frogs affected in a sample of 100 frogs. No specific statistical methods will be applied the data from this study. Routine statistical methods may be applied to determine the frequencies of certain lesions and establish similarities between sites.

Assessment Criteria: Frogs will be examined for external and internal morphological and histological lesions and these lesions characterized and cataloged. The lesions present in Vermont frogs in this study will compared to lesions in Minnesota frogs described by NWHC in 1996.

Funding and FTEs:

Operational Costs:		
Salaries and benefits: (NWHC staff)		$ 5,000.00
Wildlife Disease Specialist	FTE .05	
Pathologist	FTE .05	
Travel for field work		1,000
Sample shipment		200
Field equipment/supplies		350
Laboratory equipment/supplies		450
Histological preparation and radiography		1,000

Funding Source: $8,000 from USGS-BRD Quick Response Funds. Salaries are from NWHC base funding.

Project Investigators:
Principal Investigators;
Kathryn Converse, Ph.D., NWHC, Wildlife Disease Specialist
Carol Meteyer, D.V.M., NWHC, Pathologist
Laura Eaton-Poole, USFWS, Biologist
Rick, Levey, Vermont Dept. Environmental Conservation Biologist

Special Safety Requirements: All necropsies and laboratory analysis will be conducted in a Level 3 bio-containment facility to protect sample purity, quality, and eliminate the potential for exposure to any human pathogens. Protective clothing will be worn during examinations and goggles when handling formalin. Follow standard operating procedure for use of euthanasia solution.

Completion Date: January 15, 1998

Schedule and milestones:

General schedule:

6/9-13/97	Visit sample sites in Vermont and review collection protocol with USFWS and VTDEC.
7/7/97	Ship coolers and supplies to Vermont
7/21-30/97	Collect frogs, ship to NWHC and begin diagnostic evaluation
9/1/97	Complete diagnostic tests
12/15/97	Distribute first draft
1/15/98	Distribute final report

Study Milestones:

1. Complete evaluation of field key to amphibian deformities
2. Start key to internal deformities
3. Complete comparative evaluation of Vermont and Minnesota frog deformities
4. Document sites in Vermont with deformed frogs for use in this study.
5. Evaluate effectiveness of diagnostic procedures and sample size and type for amphibians

Relationship to Other Work:

This study is supported by ongoing surveys of frog populations in Vermont conducted by the Environmental Agency of Vermont, Middlebury College (Middlebury Vermont), the US Fish and Wildlife Service and the US Environmental Protection Agency.

Expected Products: Key to internal deformities to allow for standardization of diagnostic findings. Publication, BIB,

completion report and proposal for additional studies.

Literature Cited:

Andrews, S. James. 1995. A preliminary atlas of the reptiles and amphibians of Vermont. Vermont Department of Fish and Wildlife, Natural Heritage Program. 64pp.

Hanken, James and R. Wassersug. 1981. The visible skeleton. Functional Photography 16:22-26, 44.

Ouellet, M., J. Bonin, J. Rodrique, J-L DesGranges and S. Lair. 1997. Hindlimb deformities (ectromelia, ectrodactyly) in free-living anurans from agricultural habitats. J. Wildlife Diseases 95-104.

Submitted by: _____

Kathryn Converse, Wildlife Disease Specialist Date
Principal Investigator

Approved: _____

Christopher Brand, Branch Chief Date
Field Investigations

Concurrence: _____

Milton Friend Director, NWHC Date

Appendix D. Classification of Gross and Radiographic Findings in VT Frogs - 1997

Title	CASE/AC	Deformity	Side L/R/B	Deformity Comment
Initiation Appears Absent				
	14895-002	Amelia	Left	
	14895-006	Amelia/No right ischium/no right pubis	Right	The ilium is displaced caudally. The distal ilium has a rough, irregular cortex, flocculent trabeculae traversing the marrow and an abnormal distal border.
	14895-015	Amelia/No ischium/No pubis	Right	Trabecular pattern instead of acetabulum/ No R ischium or pubis & L ischium not fused with pubis/ Abnormal distal Right ilium with disruption of trabecular pattern and cortex/articulation of distal right ilial cortex with left ischium and left distal ischial fracture.
	14896-007	Amelia/No left ischium/No left pubis	Left	Very similar to 14895-15 w/no left ischium or pubis and R ischium not fused with pubis
	14896-009	Amelia	Right	All pelvic elements present
	14898-003	Amelia	Right	
Initiation Incomplete Pattern				
Missing Distal Segments				
	14895-004	Hemimelia femur	Left	
	14895-005	Hemimelia femur	Left	with small narrow termination
	14895-005	Brachydactyly/Skin Web	Right	Digits 1 and 5 short malformed (curled) brachydactyly (also left hemimelia)
	14895-007	Hemimelia Tibiale Fibulare	Right	Abnormal tibiale fibulare region/bones are abnormally wide cortices are very thin, the trabeculae give a light honeycomb appearance that is also present in a short region of proximal shaft of both tibiafibula bones.
	14895-010	Hemimelia tibiafibula	Right	Proximal cortex may be normal then cortical & trabecular disruption at termination
	14895-011	Hemimelia Tibiale Fibulare	Left	With mild trabecular and cortical disruption
	14895-011	Hemimelia tibiafibula	Right	With moderate trabecular disruption and mild cortical disruption
	14895-012	Hemimelia femur	Right	Similar to 14895-20 /mid-shaft with mild trabecular and cortical disruption with abrupt termination w/out disruption of cortical/trabecular pattern
	14895-013	Hemimelia femur/no left pubis	Left	Terminal trabecular hypertrophy hyperplasia possible pelvic abnormality, severe cortical disruption.
	14895-019	Hemimelia femur	Right	Trabecular hypertrophy/ hyperplasia/ moderate cortical disruption/ nice normal pelvis
	14895-020	Hemimelia femur	Left	Midshaft trabecular hypert hyperplasa/mild cortical disruption/abrupt termination without disruption of cortical/trabecular pattern similar to 14896-03.

Title	CASE/AC	Deformity	Side L/R/B	Deformity Comment
Initiation Incomplete Pattern				
Missing Distal Segments				
	14895-021	Hemimelia tibiafibula	Left	Similar to 14895-20 /mid-shaft with mild trabecular and cortical disruption with abrupt termination w/out disruption of cortical/trabecular pattern
	14896-002	Hemimelia femur	Left	
	14896-003	Hemimelia femur/no right pubis	Bilateral	No cortical or trabeculae disruption
	14896-005	Hemimelia tibiafibula	Left	
	14896-006	Hemimelia tibiafibula	Right	Pubis missing/distal femur with wide cortical shadow/short tibfib segment with very short proximal element & disrupted cortical and trabecular pattern distally
	14896-010	Hemimelia femur	Right	Femur short with disruption of terminal bone/cortex thing with delicate spherical bone shadow around original line of cortex/increased trabecular density and disorganization.
	14896-014	Hemimelia femur	Left	Very similar to 14895-20 with midshaft trabecular hypertrophy hyperplasia mild rotation cortical disruption then distal abrupt termination without cortical/trabecular change
	14897-006	Adactyly	Left	Tibiale and fibulare are both abnormal/short with mildly irregular cortex and flocculent trabecular appearance with abrupt symmetrical end to both bones. Proximal limb is unremarkable/similar to 14898-017
	14897-008	Hemimelia tibiafibula/Rotation femur	Right	Femur rotated with mild trabecular disorganization cortex unremarkble/ Tibiafibula very short with disruption of trabecular and cortical pattern
	14897-009	Hemimelia tibiafibula	Right	Trabecular & cortical disorganization/ a midshaft fracture with periosteal reaction & 2 areas (one proximal/one distal) that look
	14897-010	Hemimelia tibiafibula	Right	Trabeculae and cortical disorganization
	14897-011	Hemimelia tibiafibula/Rotation tibiafibula/bone bridge tibiafibula/Skin Web	Left	Lateral rotation with increased cortical thickness along lateral bone along lateral bend/ very small bone base bridge at bend with duplicate cortical pattern distally suggesting unfused tibiafibula (grossly looks complete as need rads at time to see digits
	14897-012	Hemimelia tibiale fibulare/Rotation tibiafibula	Right	Very abnormal & short bones w/trabecular & cortical disorganization/ Abnormal tibiafibula short w/distal 1/3 trabecular & cortical disoranization/ medial rotation of tibiafibula at distal bulbous thickening & duplicate cortical images at the end suggesting unfused bones-possible attempt at single digit?

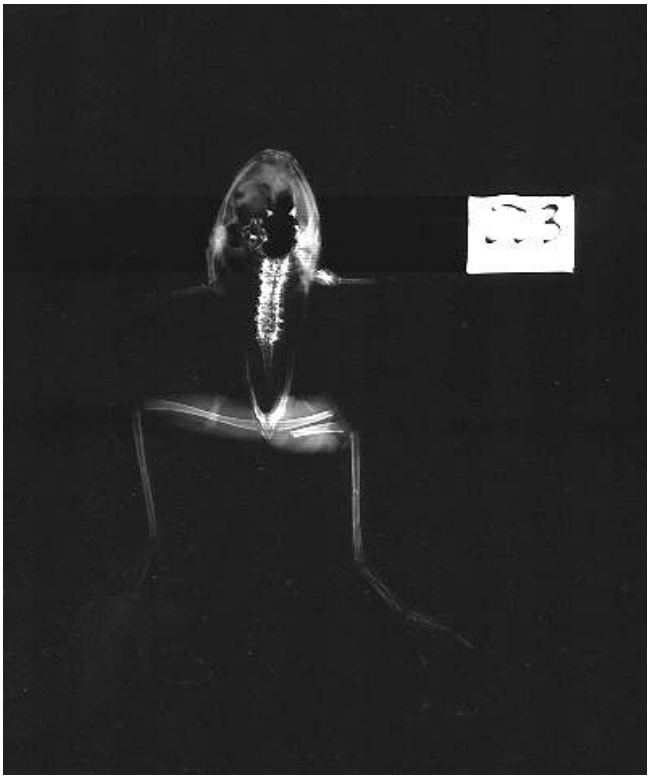
Title	CASE/AC	Deformity	Side L/R/B	Deformity Comment
Initiation Incomplete Pattern Missing Distal Segments				
	14897-014	Hemimelia tibiafibula/Rotation tibiafibula	Right	Lateral rotation & mild increase cortical density laterally but trabecular pattern is not significantly changed. Possible superimpose terminal dorsal rotation of distal 0.1 cm of
	14897-017	Adactyly	Right	Tibiale and fibulare are both abnormal/short with mildly irregular cortex and flocculent trabecular appearance with abrupt symmetrical end to both bones. Proximal limb is
	14897-022	Hemimelia tibiafibula	Left	Only very short (unfused?) tibiafibula with no cortical or trabecular disruption.
	14898-002	Hemimelia tibiafibula	Left	
	14898-005	Hemimelial tibiafibula	Right	Terminal bulge that may be bone structure
	14898-007	Hemimelia tibiafibula	Right	Short remaining segment is abnormal/normal proximal cortex with disorganization of terminal cortex and tibiafibula
	14898-008	Hemimelia tibiafibula	Left	Short thick remaining segment like 14898-07 with normal proximal cortex and terminal disorganization-but mild.
	14898-009	Hemimelia femur	Right	Periosteal reaction along shaft with distal widening with delicate sphere of bone similar to 14896-010, (do histo)
	14898-011	Hemimelia tibiafibula	Right	Much like 14898-08 radiographically.
	14898-012	Adactyly	Bilateral	Tibiale and fibulare bones are short and end abruptly without cortical or trabecular disruption. Fractures in tibiafibula and tibiale fibulare are thought to be artefacts.
	14898-014	Hemimelia tibiafibula	Right	Short segment of tibiafibula very short normal bone. Silhouette proximally and then the terminal cortex became disorganized with increased trabecular pattern/distal femur is folded dorsally with increased disorganized trabecular pattern and a delicate dorsal bone
Initiation Pattern Complete but Missing Distal Elements				
	14895-001	Ectrodactyly/Brachydactyly/Skin	Left	Like 14895-018. Abnormal tibiafibula (1/2 length) Abnormal tibiale fibulare (1/2 length), dorsal rotation probably triangular tib fib; complete web hip to hock; 3 incomplete digits
	14895-008	Brachydactyly/ Rotation/Skin web	Left	Like 14895-18 w/abn distal-mid-tibfib/skin web hip to hock/ tibfib 1/2 length w/midshaft mild disruption of trabeculae & double cortical image suggesting nonfusion of tibfib distal to midshaft /rotation (dorsal hock) mshaft. Tibiale fibulare short, but no trabecular disruption/ tibiale now appears lateral. No function hock joint w/medial dorsal rotation 180o

Title	CASE/AC	Deformity	Side L/R/B	Deformity Comment
Initiation Pattern Complete but Missing Distal Elements				
	14895-009	Ectrodactyly	Left	Tibiale and fibulare seem to begin normally w/abrupt disruption of trabeculae & cortex; single bony projection from tibiale fibulare
	14895-014	Ectrodactyly/Brachydactyly	Right	No obvious tibiale fibulare/short&thick ossified/ abnormal digits, digit 1 missing/ digit 2-5 missing all but the terminal phalan/ like
	14895-016	Ectrodactyly/Brachydactyly	Left	Abnormal tibiale fibulare - short thick / 3 abnormal digits like 14895-09
	14895-018	Ectrodactyly/Brachydactyly	Left	Abnormal tibiale fibulare/ only 2 digits & they are abnormal & w/cutaneous fusion/ No functional hock joint/ tibiafibula short w/midshaft trabecular & cortical disruption/ tibiale distal disruption of trabecular & cortex/ fibulare midshaft disruption.
	14896-001	Ectrodactyly	Right	Abnormal tibialefibulare, one abnormal digit much like 14895-009
	14896-004	Brachydactyly	Right	Digit 4 missing distal phalanges/2-4 also missing right hand
	14896-009	Ectrodactyly	Left	Digit 1 missing. Tibiafibula with disorganised trabecular pattern
	14897-001	Ectrodactyly	Right	Abnormal tibiale fibulare/abnormal digits
	14897-004	Ectrodactyly/Brachydactyly/Rotation/Skin Web	Right	Abnormal tibiafibula w/lateral rotation proximal to hock w/increase assymetrical cortical density along lateral side of lateral rotation, not defined as "bony bridge" no ossified tibiale fibulare/Abnormal digits with fusion of 3-4.
	14897-005	Brachydactyly	Right	Abnormal digits
	14897-007	Ectrodactyly/Brachydactyly/Rotation/Skin Web	Left	Abnormal tibfib/with distal rotation/either tibiale or fibulare missing; the other is short and rotated/Digit #1 has abnormal short irregular M-1 with indistinct Ph-2; Digit #2 and 3 are unremarkable. Digit #4 has only short M-1 and there is no 5th digit.
	14897-015	Ectrodactyly/Brachydactyly	Left	5 metatarsals present, but thick, short on digits 5, 4, 3. Digit 2 only has metatarsus. Digit 1 is unremarkable and remainder of limb is unremarkable.
	14897-016	Ectrodactyly	Right	Only 4 metatarsal present and the center 2 are fused. Digit #1 has M-1 with short Ph-2 growing at a 90 degree angle medially with a short terminal Ph 2; Digit #3 has M-1 fused with Ph1, 2 and terminal 3; Digit 4 has M-1 Ph-1 and terminal Ph2. Proximal limb is
	14897-018	Brachydactyly	Right	Metarsals all seem normal and digits 1, 2, 3 have normal phalanges. Digits 4 and 5 have only metatarsus Ph1 and a terminal, poorly ossified Ph 2.

Title	CASE/AC	Deformity	Side L/R/B	Deformity Comment
Initiation Pattern Complete but Missing Distal Elements				
	14897-019	Ectrodactyly	Right	Only 3 digits ar present and these are abnormal/digits 1 and 3 have short M-1 and short terminal Ph 1; and the middle digit has a short M-1 and short Ph 1 and a short terminal
	14897-020	Ectrodactyly	Right	Digits 4 and 5 are unremarkable; digit 2 has small indistinct M-1 and small poorly ossified terminal Ph-1; the second digit has a split M-1 which appears to be webbed with skin covering digit 1; the lateral split of digit 2 M-1 has distinct Ph-1 and terminal Ph-2.
	14897-021	Ectrodactyly	Right	Resembles 14895-018. Abnormal tibiale and fibulare with distal 2/3 thick with flocculent trabecular shadow filling marrow and very thin cortex distally. Two digits are present, but resemble only thin splices of bone; the lateral bone may have a terminal phalanx.
	14897-023	Ectrodactyly	Left	There are only 4 metatarsal bones and the center 2 are fused. Digit #1 has a short, terminal Ph 1; The center fused metatarsi lead to two fused Ph 1 that split and each has a Ph 2 and a terminal Ph 3. Digit 3 has a M-1, Ph 1 and a terminal Ph 2.
	14898-001	Ectrodactyly	Right	Abnormal tibiale fibulare and digits; attempt at 3 small buds
	14898-006	Ectrodactyly	Right	Abnormal tibiale fibulare and digits; attempt at 3 small buds like 14898-06
	14898-010	Ectrodactyly	Right	Abnormal tibiale fibulare; attempt a 1 digit like 14895-09. Either tibiale or fibulare missing but no cortical disruption in remaining bone
Initiation with Completed Pattern All segments present and elements present but Abnormality				
	14895-010	Abnormal/Rotation/Bony bridge	Left	Distal tibfib sharp medial bend with medial bony bridge that looks like it may have originaly have been persistent periosteal band/possibly "folding" had start of "some triangle" with bridging of a bend/Skin Web/dramatic bend with traversing boney
	14895-012	Abnormal	Left	Cutaneous fusion between normal digits 1 and 2
	14895-017	Abnormal	Left	Mid-femur large balbous cortical trabecular disruption all else normal
	14897-013	Micromelia	Left	with periosteal reaction around stifle (distal femur proximal tibiafibula) suggesting trauma (need histo).
Runting/Body Composition				
	14896-011	Runted	Both	Vertebrae possibly compressed

Title	CASE/AC	Deformity	Side L/R/B	Deformity Comment
Trauma				
	14895-003	Trauma	Left	Femur overiding fracture - no callous
	14896-008	Trauma	Left	Leg soft tissue swelling, but development appears OK-radiographs show no abnormalities in pelvis or rear limbs.
	14897-003	Trauma	Right	Rear limb traumatic amputation
	14897-013	Trauma	Left	Increased reaction around distal femur & proximal tibiafibula at stifle. No function to distal limb suggesting trauma. The entire left limb with micromelia

APPENDIX E: ENLARGEMENTS OF REPORT TABLES AND RADIOGRAPHIC IMAGES



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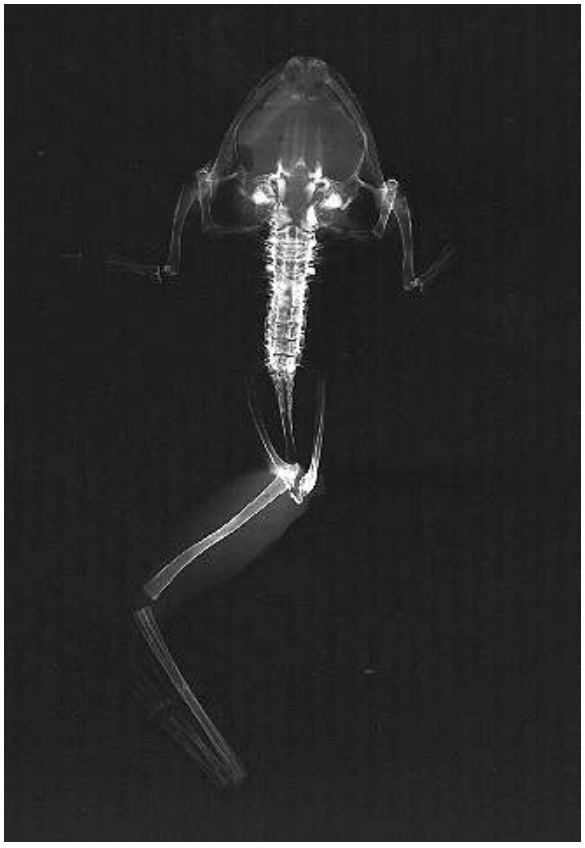
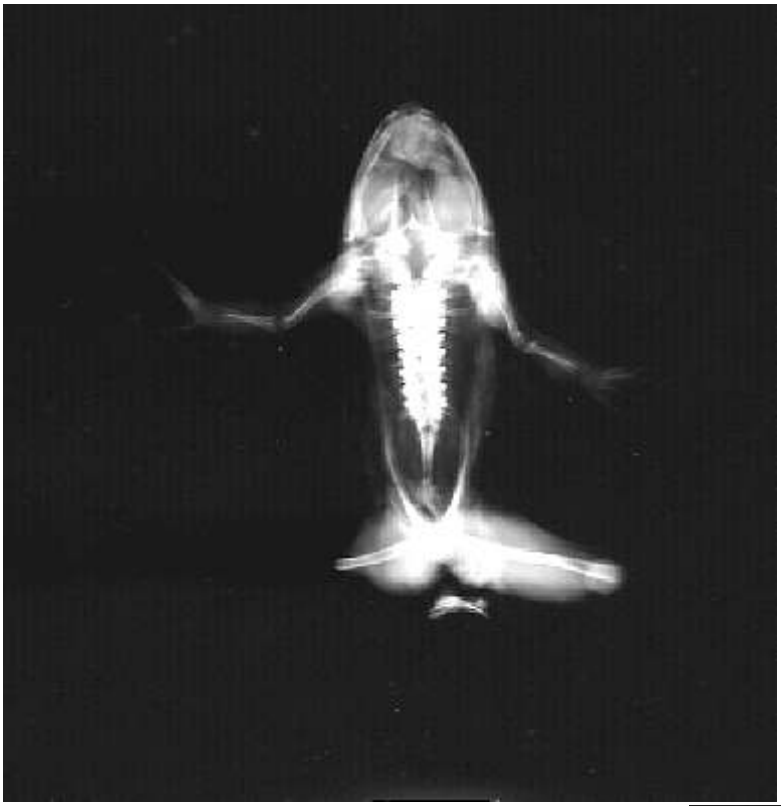
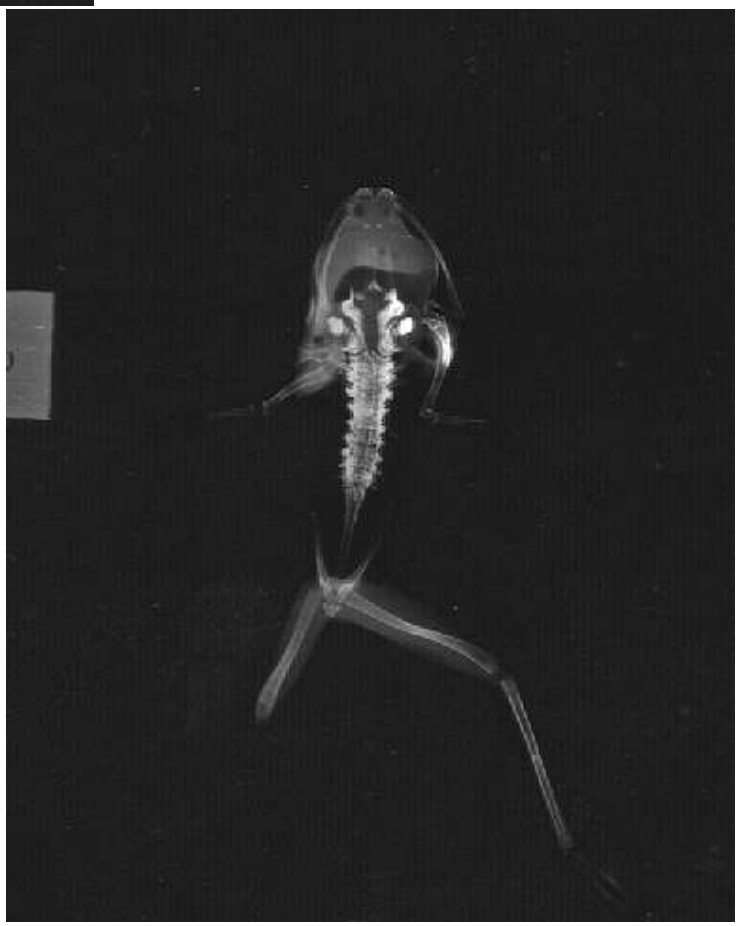


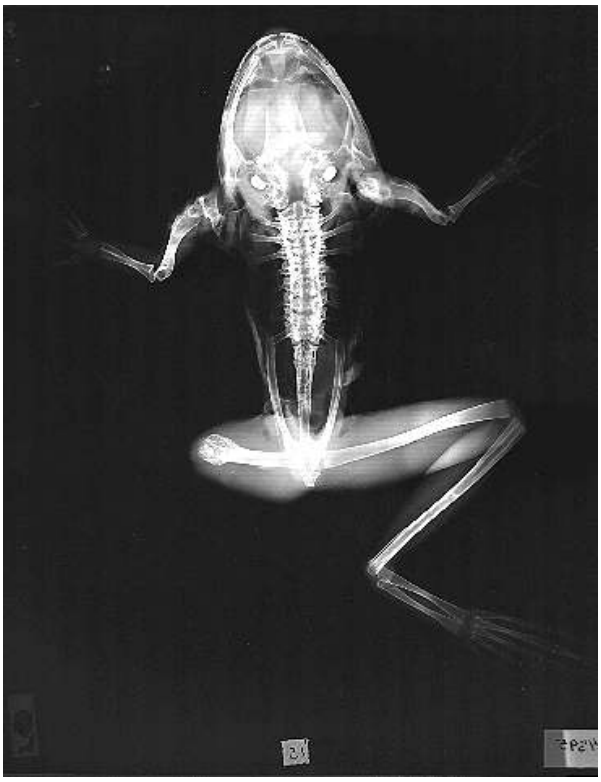
14895-015



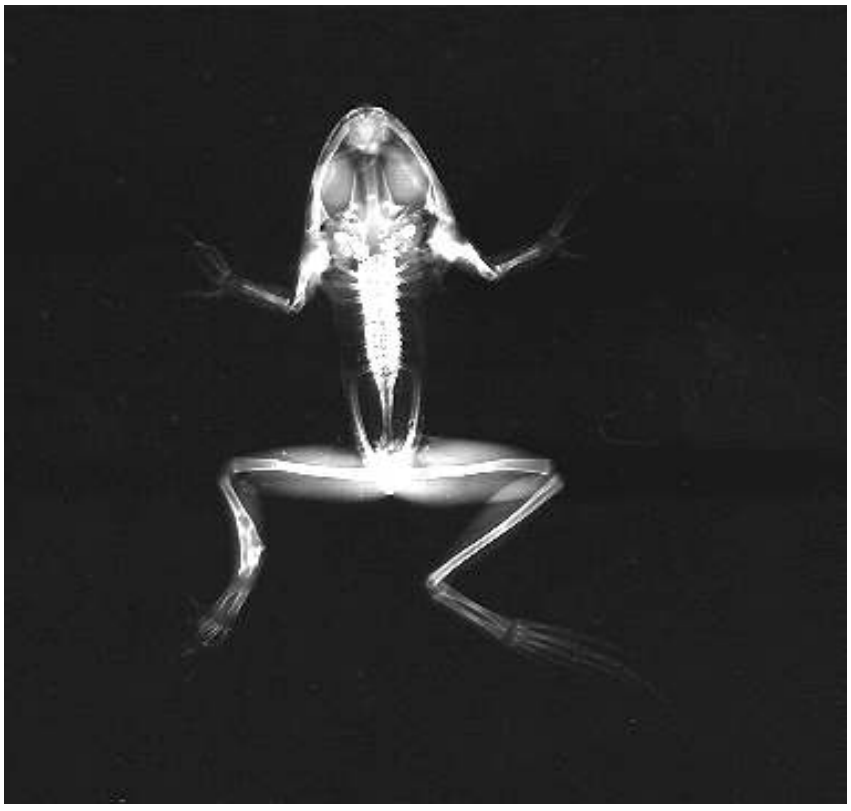
14896-003



14896-014



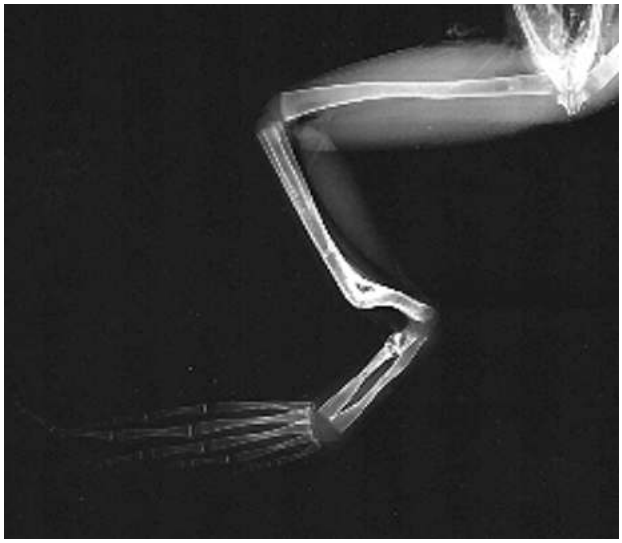
14896-013



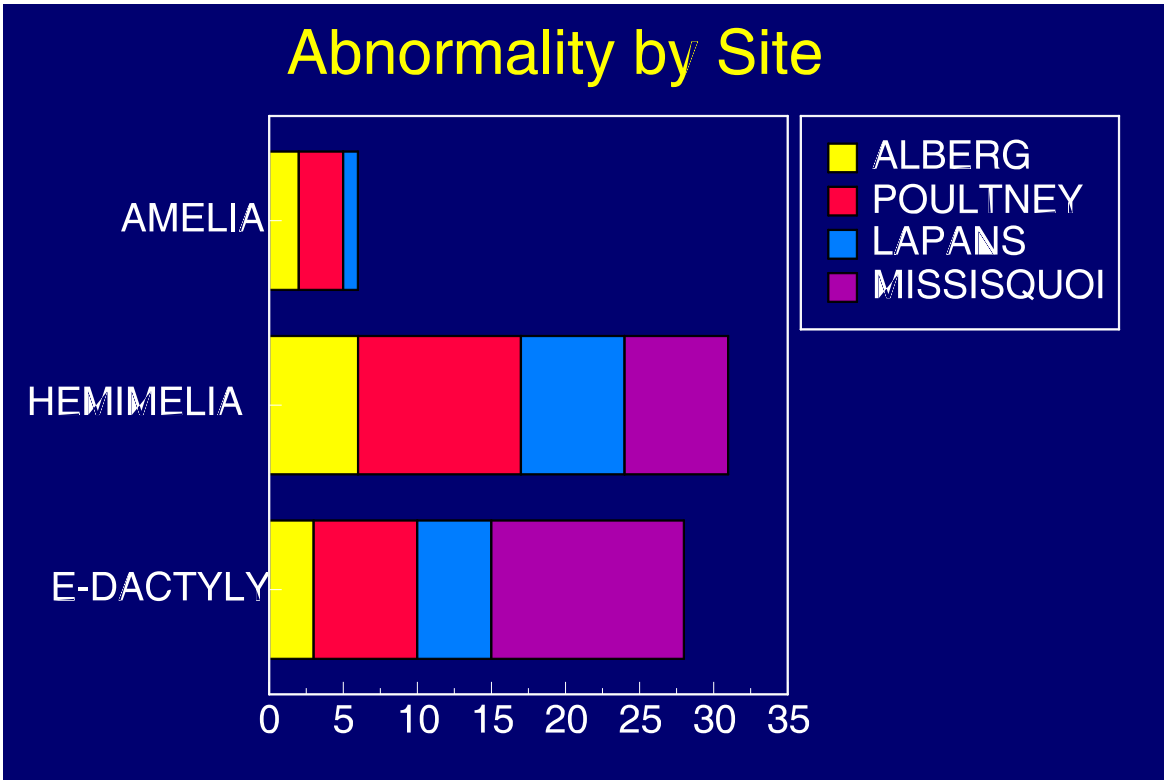
14895-008

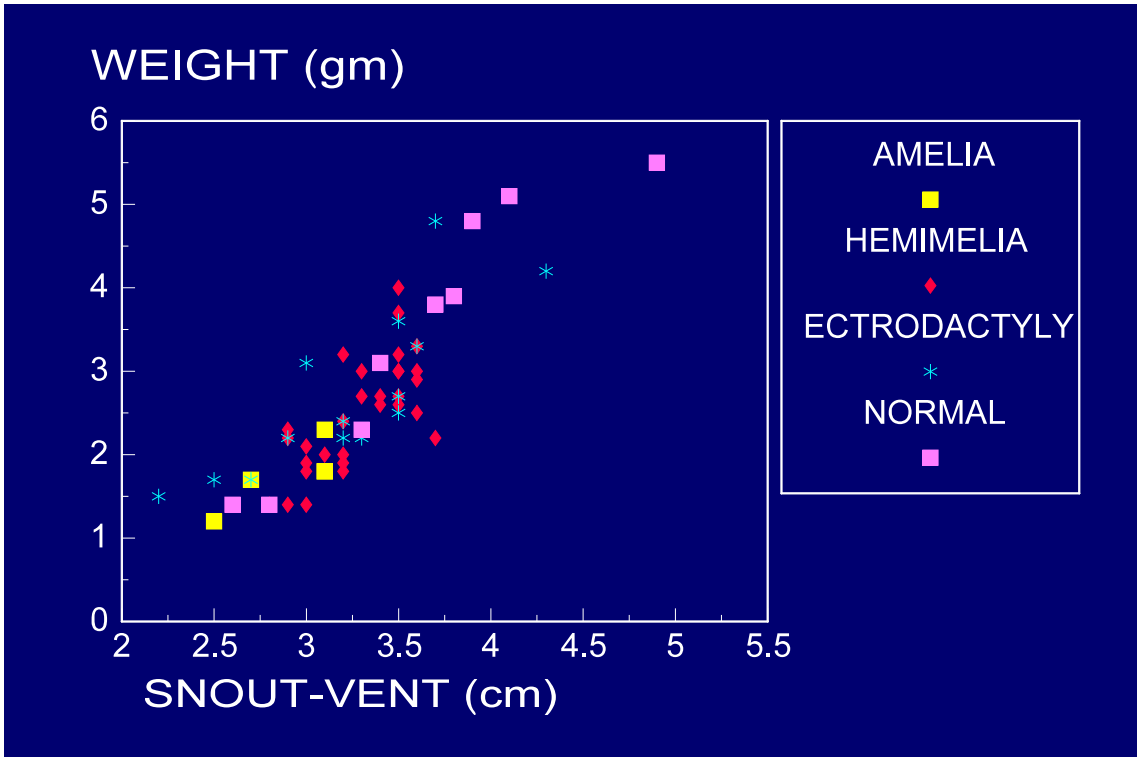
14895-018

14895-010



14895-010 Inset


Abnormality by Site
AMELIA
HEMIMELIA
E-DACTYLY
0 5 10 15 20 25 30 35
ALBERG
POULTNEY
LAPANS
MISSISQUOI


WEIGHT (gm)
6
5
4
3
2
1
0
2 2.5 3 3.5 4 4.5 5 5.5
SNOUT-VENT (cm)
AMELIA
HEMIMELIA
ECTRODACTYLY
NORMAL

APPENDIX F: TABLE #1 Frog Body Condition/Average by Deformity
TABLE #2 Frog Body Condition/Average by Collection Site

Appendix F is available by contacting Dr. Carol Meteyer at:

National Wildlife Health Center
6006 Schroeder Road
Madison, WI 53711
608-270-2400

APPENDIX G: HISTOPATHOLOGY FINDINGS

Appendix G is available by contacting Dr. Carol Meteyer at:

National Wildlife Health Center
6006 Schroeder Road
Madison, WI 53711
608-270-2400

Appendix H. Bacterial Isolates from Liver and Kidneys VT 97

Isolation	*Alburg Dune*	*Lapans Bay*	*Missisquoi NWR*	*Mud Creek Control Area*	*Poultney River*	*Total Frogs*
Acenitobacter baumanii		1		3		4
Aeromonas hydrophila		4	1		2	7
Aeromonas sp.		2	2		1	5
Aeromonas veronii		1				1
Alcaligenes sp.			1			1
Alcaligenes xylosidans	8	3	3	1		15
Bacillus cereus	1					1
Bacillus sp.				1		1
Citrobacter freundii		1	2	1	2	6
Enterobacter agglomerans				2		2
Enterobacter cloacae			1	1		2
Enterobacter intermedium			1			1
Enterobacter taylorae		1				1
Enterococcus sp.	1				1	2
Escherichia coli					1	1
Escherichia sp.	1				1	2
Escherichia vulneris		2				2
Hafnia alvei		1	1	2	1	5
Klebseilla pneumoniae				1		1
Klebsiella group 47					2	2
Klyvera sp.			1			1
No Growth			1		3	4
Pseudomonas cepacia	8	3	4	3	2	20
Pseudomonas putida			1			1
Pseudomonas sp.				2	3	5
Serratia sp.			1			1
Staphylococcus sp.					1	1
Vibrio fluvialis		2				2
Xanthamonas maltophilia	1					1

Appendix H. Metacercariae Identification in '97 Metamorphs

	Pancreas	*Head*	*Eyes*	*Intestinal Contents*	*Lung*	*Liver*	*Kidney*	*Muscle*	*Muscle/ skin interface*	*Skin*	*Tail bud Muscle*	*Rear Leg Muscle*	*Back Muscle*	*Belly Muscle*	*Body Cavity*	*Stomach or Intest Serosa*	*Urinary Bladder*	*Esophagus*	*Heart /Pericardial*	*Hard Palate*	*Fat Body*
Amelia/No right ischium/no right pubis																					
14895-006 Poultney River					✔	✔	✔	✔		✔		✔		✔			✔				
14896-007 Alburg Dune			✔			✔	✔				✔		✔								✔
Ectrodactyly/Brachydactyly																					
14895-016 Poultney River			✔		✔	✔	✔		✔		✔	✔	✔	✔	✔	✔			✔		
14897-007 Missisquoi NWR	✔	✔	✔		✔	✔	✔			✔	✔		✔		✔				✔		✔
14898-006 Lapans Bay		✔	✔		✔	✔	✔			✔	✔		✔	✔		✔		✔	✔		
Hemimelia Tibiale Fibulare																					
14895-007 Poultney River				✔	✔	✔	✔		✔		✔		✔	✔	✔						
14896-006 Alburg Dune		✔	✔		✔	✔	✔	✔	✔	✔	✔				✔						✔
14898-007 Lapans Bay		✔	✔			✔	✔	✔		✔			✔	✔							
Normal																					
14895-022 Poultney River		✔	✔		✔	✔	✔			✔	✔				✔						
14896-016 Alburg Dune	✔		✔		✔	✔	✔			✔	✔	✔	✔	✔	✔	✔	✔				
14897-024 Missisquoi NWR							✔								✔						
14896-016 Lapans Bay	✔		✔			✔	✔			✔	✔		✔				✔	✔			✔
14899-006 Mud Creek WMA	✔		✔			✔	✔			✔			✔				✔				✔
14899-007 Mud Creek WMA	✔	✔	✔				✔			✔	✔		✔	✔	✔						

Appendix H. Adult Helminth Identification in '97 Metamorphs

Isolation	*Alburg Dune*	*Lapans Bay*	*Missisquoi NWR*	*Mud Creek Control Area*	*Poultney River*	*Total Frogs*
Gorgoderidae						
	2	2			1	**5**
Nematode						
	1	2	1		1	**5**
Oxyurid						
					2	**2**
Rhabdias ranae						
	1		1			**2**
Trematode						
	1			1	2	**4**

APPENDIX I: LIMB MEASUREMENTS

1. REAR LEG MEASUREMENTS

2. FRONT LEG MEASUREMENTS

Appendix I is available by contacting Dr. Carol Meteyer at:

National Wildlife Health Center
6006 Schroeder Road
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APPENDIX K: NECROPSY REPORTS

Necropsy reports are available by contacting Dr. Carol Meteyer at:

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