Investigations into the Causes of Amphibian Malformations in the Lake Champlain Basin of New England

Executive Summary

I. Project Description

The overall objectives of this project were to conduct a series of coordinated field and laboratory tasks that would: **1) characterize the occurrence of *R. pipiens* abnormalities in both sub-samples and broad population level samples over time; 2) evaluate the potential of site sediment and water to induce developmental abnormalities of a type and at a frequency of occurrence observed in the field through laboratory developmental limb bud assays; 3) investigate potential causal agents through field developmental observations; 4) characterize the chemistry of water and sediment from test sites, and 5) evaluate limb development and response to environmental sample exposure in laboratory assays.** The five sites chosen within the Lake Champlain Basin of Vermont support breeding populations of *R. pipiens*, and show a range of gross hind limb abnormality incidence indicative of impact and reference conditions.

In order to address these objectives collaborators monitored the breeding and development of *R. pipiens* at all sites through adult metamorphosis. Representative sub-samples of developing and metamorphosed *R. pipiens* were collected and examined for the purpose of determining abnormality incidence rate and the relationship of observed anomalies to selected physical characteristics, including evidence of physical trauma. Intensive monitoring of amphibian populations was conducted at the Ward Marsh site to describe multi-species population-level abnormality incidence. Site water and sediment were evaluated by analyzing representative sediment and water samples for a range of agricultural pesticides and metabolites in common use throughout the Lake Champlain Basin. Developmental limb bud assays were conducted using both *Xenopus laevis* and Ranid species in order to evaluate the test response relationships between the two species and to evaluate endpoints relevant to Lake Champlain findings, specifically hind leg reduction malformations.

II. Methods and Results

a) Site Selection

Five sites within the Lake Champlain Basin were selected as primary study sites. The sites selected were established sites with a minimum of three years of data (VTDEC) describing the frequency of occurrence of abnormalities among *R. pipiens*. Two of the five sites (Mud Creek, North Hero) with monitoring data demonstrating a low incidence of abnormality (<3%) were designated "control" sites. The remaining three sites with monitoring data demonstrating a high incidence of abnormality (>8%) were designated test sites.

In addition to the five primary study sites, four supplemental sites were surveyed to provide additional data on the frequency and occurrence of abnormalities among newly metamorphosed *R. pipiens*. All of the sites are located in the Lake Champlain Basin and are close to Lake Champlain; with the exception of Cornwall Swamp they are all affected by the lake's annual fluctuations.

Preference has been given to sites where previous research activities have been conducted. One of the primary study sites, the Ward Marsh-Poultney River site, was selected for intensive drift fence sampling. Sampling sites have been selected with the goal of representing *R. pipiens* breeding grounds across a range of geographical locations within the Lake Champlain Basin of Vermont.

b) Northern Leopard frog (*Rana pipiens*) Metamorph Surveys

Rana pipiens was chosen as the target species for several reasons: it has been the most reported species with abnormalities in Vermont (and within the Lake Champlain Basin); it is primarily a terrestrial species as an adult; and it can be very abundant locally.

At the five primary study sites, a minimum sample size of 50 (maximum 150 per sampling event) *R. pipiens* metamorphs (<4cm) were targeted for collection using the search and seize method. Frogs were collected in open grassy areas adjacent to breeding areas using hand held nets. The frogs were placed into buckets that held some water and vegetation. Holding the frogs prevented recapture bias and helped keep the frogs in good condition. Frogs collected were measured and weighed to determine overall body condition and examined in the field for gross external abnormalities and characterized using standard nomenclature (Meteyer, 2000a). Uniform classification of malformations using universally accepted terminology allow for better comparison and insight into these syndromes. Two collections were made during the summer of 2001, the first collection occurred in early July as the metamorphs were transforming, the second collection occurred 4 - 6 weeks later. Digital photographs were taken as warranted to detail and catalog specific abnormalities observed.

Results

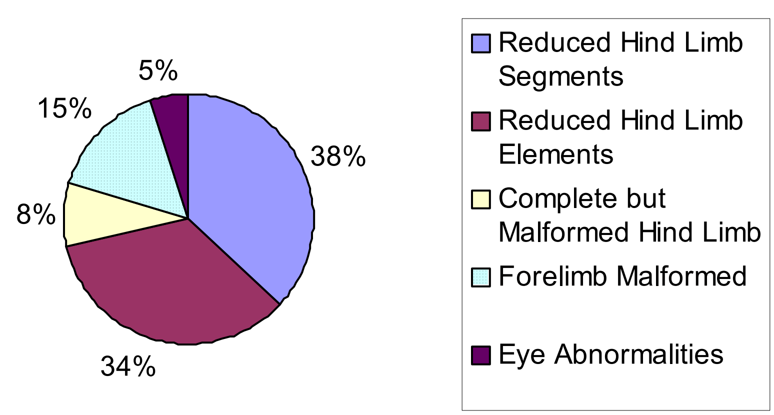
A total of 2959 *R. pipiens* metamorphs (juveniles) were collected and characterized from the five primary study sites (n = 2439) and the four supplemental sites (n = 520) in 2001. Overall abnormality rates at the five primary sites and the supplemental sites were 4.2% and 4.0%, respectively. Percent abnormalities ranged from 0% – 8.8% for the primary sites and 0% – 6.5% for the supplemental sites.

Categories of Metamorph Abnormalities

We characterized 120 abnormalities affecting 103 metamorphs at the 5 primary sites. Metamorph characterization was restricted to external visual examinations and the use of a head mounted 2x magnifier. It should be acknowledged that many abnormalities could go undetected without the use of radiography and histopathology. Previous Vermont studies revealed malformations of the hip that would not have been detected without the use of high detail radiography (Meteyer,1997). Radiographs, necropsy and histopathology are also important in differentiating trauma from malformation.

Figure E1 presents the categories of *R. pipiens* metamorph abnormalities observed at the five primary study sites in 2001. Hind limb abnormalities comprised 79% (n = 95 abnormalities). Forelimb abnormalities and eye abnormalities affected 15% and 5% of the specimen's respectively.

Figure E1. Major Categories of Metamorph Abnormalities at Five Primary Sites – 2001 (n = 120).



Almost 2/3^{rds} of the abnormal metamorphs were obtained from 2 of the 3 test sites: Otter Creek (33%) and Alburg Dune (32%). North Hero (reference site) metamorphs comprised 23% of the abnormalities observed. The Alburg and Otter Creek site abnormalities were dominated by missing hind limb segments (ectromelia), whereas the North Hero site was dominated by missing hind limb elements (ectro/brachydactyly). In fact 45% (n = 11) of the abnormalities at North Hero were brachydactyly, which could be considered the least severe of abnormalities encountered. Mud Creek (reference site) and Ward Marsh (test site) represented only 4% and 8% of the metamorph abnormalities observed, respectively.

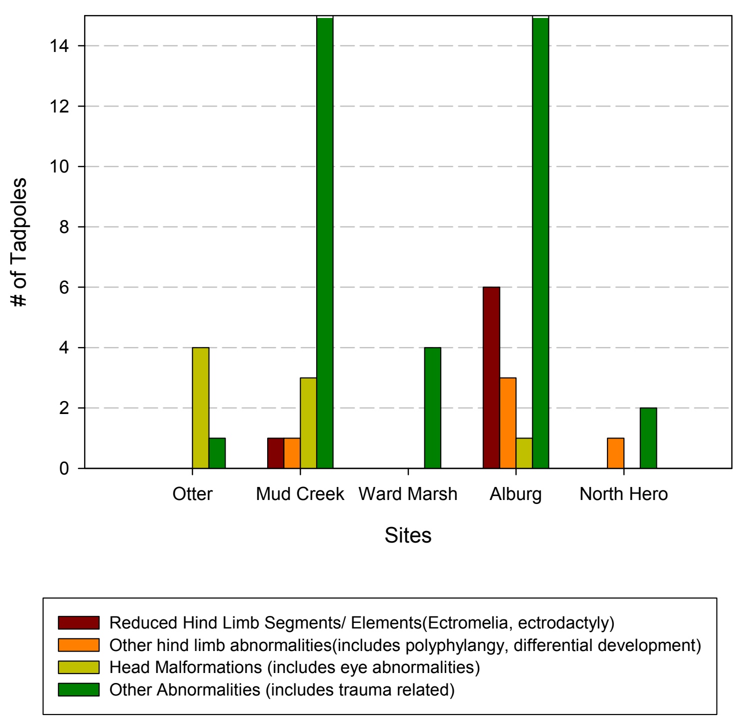
c) Tadpole Surveys

Developing *R. pipiens* were also observed at the five primary sites, from early limb development to metamorphosis. Larval stage tadpoles, Gosner stage 30-45, were collected and examined for gross abnormalities. A minimum of 100 tadpoles were examined from each site, using a dissecting scope (7-60X). Developmental characteristics (e.g. stage) were described and all abnormalities were characterized using a standardized descriptive format. Of particular interest were observations on the occurrence and character of hind limb abnormalities. A portion of abnormal tadpoles were brought back to the Laboratory for continued observations and rearing. Digital photographs were taken as warranted to detail and catalog specific abnormalities observed.

Results

A total of 1254 *R. pipiens* tadpoles (Gosner Stage 26- 45) were collected and characterized from the five primary study sites in 2001. Tadpole collections were not performed at the 4 supplemental sites. Overall tadpole abnormality rates at the five primary sites was 4.4% (55 of 1254); abnormalities ranged from 0% – 16.8%. Mud Creek had the highest observed tadpole abnormalities, 16.8 percent. By contrast, sampling events at the other four sites resulted in less than 5% abnormalities (0% – 4.3%). Figure E2 presents the major categories of tadpole abnormalities by site. The categories of greatest interest are the hind limb abnormalities, reduced hind limb segments (ectromelia) and elements (ectrodactyly) comprised 9.3% (n = 7) of the total abnormalities observed. Only Alburg Dune and Mud Creek represented this category (n = 6 and n = 1, respectively).

Figure E2. Major Categories of Tadpole Abnormalities by Site – 2001.



Conceivably the most captivating observations made during the tadpole study occurred while rearing a portion of the "abnormal" tadpoles in the laboratory. Digital photographs captured early stage progression of polydactyly, differential development, ectrodactyly, delayed development, and epidermal healing of limb trauma from known capture injury. The progression documented provides evidence that at least a portion of the hind limb abnormalities observed in the metamorphosed frogs appear to be manifest at early tadpole development and are not a result of late stage trauma from predation or other environmental insult.
These observations may directly address a data gap in the testing of the hypothesis that observed abnormalities are primary errors in development (malformations) rather than abnormalities caused by mechanical or non-developmental means (deformities).

d) Drift Fence Monitoring at Ward Marsh 1998-2001
Seven drift-fences were put into the ground around the southernmost bays of Ward Marsh in West Haven, Vermont, in 1998. The marsh is an extensive cattail marsh located next to the Poultney River at the southern tip of Lake Champlain. Most of the fences (six of the seven) were placed between the fields and the marsh in the narrow strip of uncut old-field. These fences were built in three side-by-side pairs.

All fences were made of 15.2-meter lengths of 51 cm aluminum flashing, buried 10 cm in the ground. This left a 41 cm barrier to amphibian feeding and migratory movement. Tangential with the flashing and buried flush with the ground surface were a series of cans and buckets with lids that were opened prior to periods of expected amphibian activity. On each side of a fence, there were two 22.7-liter buckets (one on each end), and one 6.2-liter stainless steel can placed halfway between the buckets. The can was 15.5cm in diameter, and 33cm in depth. Each can had a funnel placed into the top of it that has an opening diameter of 10.5cm. This was to prevent escape of animals that could crawl up the sides of the cans. Both the cans and the buckets have holes drilled in them 2.5cm from the bottom, in order to hold some water and prevent dehydration of amphibians. There was a 1-meter strip on each side of the fence cleared of shrubs and tall grasses.

Fences were opened before noon on warm days with rain, or when rain was expected, three times per month from April through October. Occasionally the fences were opened in anticipation of a rain that did not materialize or during which very little rain fell. When this happened the fences were opened an additional time during the month. If the fences were opened more than three times per month, data for population indices are used from only the three most productive nights (greatest number of amphibians caught).

Results
Over the four years of monitoring, eight species of amphibian have been captured in the drift-fences. Of these, the great majority are Northern Leopard Frogs (*Rana pipiens*) (91%, Figure 52). Green Frogs (*Rana clamitans*) are also caught in relatively large numbers (5%, 245 captures over four years). Eastern Newts (*Notophthalmus viridescens*,

3%, 147 caught) and Spotted Salamanders (*Ambystoma maculatum*, 0.4%, 21 caught) are the only two species of salamander were captured, but this is not surprising considering that the fences were not set in appropriate habitat to catch most salamander species. Newts and Spotted Salamanders are not caught in large numbers, but generally the number of captures is large enough (with the exception of adult Spotted Salamanders) to accurately monitor their populations.

Abnormalities

Abnormality data was calculated for the entire year of trapping, including days that were not considered successful and therefore not included in the abundance indices. Overall abnormality rates were very low for all species caught in significant numbers over the entire four-year period. Since *R. pipiens* was the species most frequently caught at the site, the vast majority of abnormalities seen were in this species, however, the relative percentage of abnormalities was essentially the same for Green Frogs (*Rana clamitans*). *R. pipiens* had a total abnormality rate of 0.88% (38 out of 4313) versus 1.22% (3 out of 245) for Green Frogs.

Since 1998 had the highest numbers of *R. pipiens* overall, it also had by far the most abnormalities. Still, the percentage of abnormalities was no greater than in 1999 (tied with 1999 at 1.1%). The abnormality rate of 1.0% in 2000 was just barely less than the 1.1% of 1999. The majority of these abnormalities occurred in the legs (ectromelia of the tibiafibula and amelia being the two most common abnormalities). Interestingly, amelia only occurred in frogs in 1998, and has not reappeared since during the drift fence surveys. Similarly, ectrodactyly has not reappeared since 1998 in our trapping efforts. In 2000 we had both the lowest number of abnormalities and the lowest percent abnormal (3, 0.4%). One of these was a trauma-related deformity, so the percent abnormal was only 0.3%. In two of the four years, the adult percentage of abnormalities was slightly higher than the juvenile percentage. Despite being low to begin with, the percentage of abnormal adult *R. pipiens* has declined slowly but steadily over the four years and although slightly more variable the trend in abnormal juveniles has also been toward lower percentages.

Comparisons with sub-sampling surveys

In comparing the drift fence abnormality percentages to those observed during *R. pipiens* metamorph sub-sampling efforts, the drift fence observed slightly lower percentages every year, but follow a similar trend. In 1998, sub-sampling surveys observed 3.4% abnormal; in 1999, 2.7%; in 2000, 0%; in 2001, 1.17%. Drift fence results for abnormalities were highest in 1998 (1.1%), and our lowest in 2000 (0.4%), coinciding with the sub-sampling high and low. This suggests that the sub-sampling method is comparable to the drift fence monitoring. However, if abnormal frogs are dispersing a shorter distance (from breeding site), the drift fence monitoring may be biased to normal frogs.

e) Limb Bud Assays

The limb bud assay study was designed to evaluate potential effects on limb development in frogs exposed to site-specific environmental waters and sediments. This study attempted to determine the effects of site-specific environmental water and sediment exposure to larval stage amphibians by evaluating mortality, morphological development (with emphasis on limb development), and teratogenesis (malformations) between the five sites and the three species of frogs; *Rana pipiens*, *Rana catesbeiana*, and *Xenopus laevis*. The Gosner Index (Gosner, 1960) was used to standardize developmental staging and facilitate comparisons between test species. The study was divided into two phases, early stage development and limb development. Phase I consisted of short-term (4-10 days) embryo-larval exposure (Frog Embryo Teratogenesis Assay—*Xenopus* [FETAX]) described in ASTM E1439-98 (ASTM, 1998), modified to accommodate ranid species. Phase II comprised a long-term exposure assay (ca. 90 days) designed to observe hind limb and forelimb development during pre-metamorphosis.

Water and Sediment samples

Three sets of five surface water and sediment samples from the 5 primary sites were collected over a 2 month period and shipped overnight via commercial carrier to the laboratory at 4°C. Three sites labeled ALB, WM, and OTC (Alburg,Ward Marsh, Otter Creek) were designated as test sites and two sites labeled MDC and NH (Mud Creek, North Hero) were designated as reference sites. These samples were stored at 4°C upon receipt and throughout the testing period. Each sample was thoroughly mixed before testing. Each set of water samples received was initially analyzed for pH, dissolved oxygen (DO), conductivity, hardness, alkalinity, ammonia-nitrogen, and chlorine. Temperature, pH, and DO were routinely monitored throughout the study.

Test Culture Care and Breeding

Adult *X. laevis* were acquired from *Xenopus* I (Dexter, MI). *Xenopus* adult care, breeding, and embryo collection were performed as described in ASTM E1439-98 (ASTM, 1998), at Fort Environmental Laboratories, Inc. Adult *X. laevis* were fed Salmon Starter pellets, purchased from Zeigler Bros. (Gardners, PA), once per day on alternating days, ad libium. *R. pipiens* adult care, breeding, and embryo collection were performed at Carolina Biological Supply Company (Burlington, NC). The collected embryos were shipped overnight via commercial carrier to Fort Environmental Laboratories, Inc. Larval stage specimens were fed Salmon Starter Mash (Zeigler Bros., Gardners, PA) three times daily, ad libium.

Early Embryo-Larval Stage Development (Phase I)

The larvae were exposed to each of the five water samples plus a laboratory negative control of FETAX solution (ASTM E 1439, 1998) until Gosner stage 26-28 (approximately 4-6 days). Daily mortality was determined for each replicate dish and an average (representative) stage of development was determined for larvae in each water exposure. At the end of each incubation period (free-swimming stage), all surviving larvae were scored for abnormalities, digitally photographed, and transferred to larger test vessels for the limb development phase of the study.

Limb Development (Phase II)
Each replicate dish of stage 26-28 free-swimming larvae were transferred from the Petri dish to an identically labeled 4-L plastic test vessel containing approximately 750 g sediment sample and 3 L of water sample approximating a 1:4 ratio. Prior to adding larvae, each vessel was fitted with a mesh screen insert positioned at the sediment and water interface to prevent the larvae from becoming lost in the sediment. Dechlorinated (aged) tap water and sterilized sand were used as the negative control, replacing the FETAX solution used in Phase I. FETAX solution was designed specifically for the needs of short-term bioassays and extended periods of exposure to the higher salt concentrations in the FETAX solution was a concern. Each test vessel received continuous aeration throughout Phase II. Mortality and developmental stage was recorded daily for each replicate test vessel. As the test specimens reached the end of limb development (stage 40-42), each was scored for abnormalities and digitally photographed. The study was concluded at the end of 3 months.

Results
Results from the limb bud assays indicated that samples from Alburg, Ward Marsh, and Otter Creek were capable of inducing early embryo-larval mal-development, as well as malformation at limb development stages in *X. laevis* and *R. pipiens*. Samples from North Hero were weakly capable of inducing abnormal development and samples from Mud Creek (reference site) did not induce malformation at either developmental stage

Malformation
No malformation in any of the species exposed to the Mud Creek reference samples was detected at either the early embryo-larval stage or the limb development stage. The North Hero samples induced 4 % malformation in *X. laevis* and no malformation in *R. pipiens* or *R. catesbeiana* during the early embryo-larval development phase. The North Hero samples induced 6.1 %, 5.3 %, and 0 % in *X. laevis*, *R. pipiens*, and *R. catesbeiana*, respectively, during the limb development phase. Samples from Alburg, Ward Marsh, and Otter Creek induced malformation rates of 46.0, 37.8, and 26.8 % in *X. laevis*, respectively, at the early embryo-larval development stage. Alburg, Ward Marsh, and Otter Creek samples caused malformation in 42.0, 32.0, and 18.8 % of *R. pipiens* tested during early embryo-larval development. At the limb development stage, Alburg, Ward Marsh, and Otter samples induced malformation in 54.6, 43.2, and 47.6 % of the *X. laevis*, and 35.7, 43.8, and 26.7 %, of the R. *pipiens* exposed, respectively.
Common malformations included mal-development of the mouth, tail, forelimb, hind limb, face, eye, brain, fin, notochord, and gut. Hemorrhaging and edema were also noted, but at a lesser rate. Overall, the malformation syndromes induced by each test site sample were reasonably consistent between *X. laevis* and *R. pipiens*.

Comparisons with Field Observations
Site sediment and water did induce developmental abnormalities of a type observed during the tadpole and metamorph field surveys. The frequency of abnormalities did not always correlate very well, most notably the high frequencies of *R .pipiens* hind limb abnormalities observed during field surveys were not observed within the Gosner stage 46 limb bud assays.

f) Water Quality Measurements

Field Water Quality Measurements

Field water quality measurements and collections were made prior to sediment collections and any other sampling activities just below the surface of the water where the water depth was less than one foot (30cm). Water column measurements performed on site with field meters included: temperature, pH, conductivity and dissolved oxygen. Water collections for VTDEC Laboratory analysis included: (13) priority pollutant heavy metals, calcium, magnesium, sodium, potassium, ammonia and nitrate.

Pesticide Analysis

Two water and sediment samples were evaluated for the occurrence of current use pesticides and metabolites, the first was collected in late April and the second were collected in mid-June. All samples were analyzed utilizing methods developed in the Vermont Department of Agriculture and Food Markets, (VDAFM) laboratory, generally adapted from published methods. Analytes were subdivided by a combination of analytical methods used and host group category.

Results

Field Water Quality Measurements:

Dissolved Oxygen

Dissolved Oxygen ranged from extremely low concentrations of less than 1.0 mg/l (Mud Creek, North Hero) to greater than 7.0 mg/l (Otter Creek, Ward Marsh and Alburg Dune). Many of the sites showed a steady decrease in dissolved oxygen as the season progressed (water levels dropped, temperature increased). Alburg Dune had the most pronounced seasonal decrease in dissolved oxygen, from 8.4 mg/l to 1.6 mg/l.

Temperature

Temperature ranged from a high of 37^{o}C (96^{o}F) to a low of 10^{o}C. Like dissolved oxygen, temperature values increased as the season progressed. The Alburg study site, a small tannic pond, had the highest value recorded (37 ^{o}C) in early August.

pH

The lowest pH recorded was 6.50 (Alburg), the highest value recorded was 7.71 at Ward Marsh. Many of the study sites are influenced by Lake Champlain water (average pH 7.4) especially during high water events.

Conductivity

Conductivity values ranged from 96 – 742 us/cm at the five study sites. Otter Creek had the lowest value and North Hero had the highest value. Most sites showed an increase in conductivity as the season progressed. Sites that exhibited the greatest decrease in water level had the highest conductivities.

Color

Alburg Dune had the highest and most consistent color readings (>500pcu). The color values at the North Hero and Mud Creek sites increased as the season progressed. The evaporation of site water coupled with the leaching of leaf material most likely contributed to the increased color at these shallow sites.

Major cations

Most of the sites showed an increase in the major cations (Na^+, K^+, Ca^{2+}, and Mg^{2+}) measured from early May to mid-June. This would be expected as the site water evaporates the concentration of total dissolved salts would increase. Calcium concentrations ranged from 11.9 – 30.8 mg/l, magnesium 2.8 –20 mg/l, potassium 1.1 – 3.53 mg/l, sodium 2.6 –9.9 mg/l.

Nitrate and Ammonia - Nitrogen

Nitrate and ammonia - Nitrogen levels ranged from trace to < 1.0 mg/l at all the sites except for North Hero. Nitrate + nitrite (NOX) levels at North Hero ranged from <0.05 to 4.62 mg/l, and ammonia (NH_3) levels ranged from <0.05 –1.36 mg/l.

Alkalinity

Alkalinity values ranged from a low of 36 mg/l recorded at Mud Creek to a high of 101 mg/l at Ward Marsh. All five sites showed a significant increase in alkalinity as the season progressed.

Heavy Metals

Water samples were collected and analyzed for 13 priority pollutant metals.
None of the water samples analyzed exceeded the Laboratory detection limits for any of the tested metals.

Pesticide Analysis:

Of the 32 pesticides and metabolites analyzed during this study, the only compounds detected in water were atrazine and it's desethyl metabolite, and metolachlor along with its Ethanesulfonic acid (ESA) and Oxanilic acid (OA) metabolites. No pesticides or metabolites were detected in any of the sediment samples.
Atrazine was detected in three out of ten water samples with one detection above 0.1 ppb, at Otter Creek in the June 12, 2001 sample. Metolachlor was detected in four of the ten water samples analyzed, again with the only sample above 0.1 ppb being the Otter Creek June sample. Desethyl atrazine was detected in four of ten samples, all below 0.1 ppb. Desethyl atrazine was the only metabolite of atrazine detected during this study, and the major metabolite detected during other surface water investigations (Kolpin et. al., 1999 and Thurman et. al., 1992). Metolachlor ESA was the major metolachlor metabolite detected, being found in seven of ten water samples, with one at 0.1 ppb. The metolachlor OA metabolite was also detected in five of ten water samples, all below 0.05 ppb.

These levels are far below any current regulatory aquatic life criteria, although some studies have reported effects on algae at the low ppb concentration range. For instance Torres et al.1976, reported a Lowest Observable Effect Level (LOEL) for algae of 1.0 μg/L (Tierney et. al., 1999). The Canadian aquatic life criteria for atrazine is 1.8 ppb and for metolachlor is 7.8 ppb, while the EPA has a draft ambient aquatic life criteria of 12 ppb for atrazine. With no pesticide detections above 0.13 ppb, the data from this study indicates levels well below any regulatory criteria.
However there is little data available on the relative toxicities of the metabolites. Recent studies have found concentrations of atrazine (0.1ppb) similar to our findings capable of endocrine disruption, inhibiting testosterone and inducing estrogen secretion in amphibians (Hayes et. al., 2002). Although the Hayes study did not relate to hind limb abnormalities, another study has linked limb malformations with abnormal sex hormone concentrations in New Hampshire frogs (Sower et al., 2000).

III. Discussion

This report marks the culmination of a five-year investigation into the causes of *Rana pipiens* abnormalities. Numerous multidisciplinary collaborations have contributed to this study. We have collected and examined over 10,000 *R. pipiens*, and have found hind limb truncations at more than 20 sites, spanning 120 miles of Lake Champlain. The common denominator of these sites is the Lake Champlain Basin and more specifically, the Lake Champlain lowlands. The rates of abnormalities have been highly variable, both seasonally and annually. However, only a few sites have consistently maintained low rates of abnormalities (less than 4%).

This investigation conducted a series of coordinated field and laboratory tasks that were designed to provide data that would help point the way to the cause of the northern leopard frog abnormalities. The life cycle of *R. pipiens* helps illustrate the complexity of this endeavor. The leopard frog encompasses three environments: the breeding wetland, the feeding landscape of the adult frogs and the overwintering site.

The potential for biological and chemical exposure is as plentiful as the environments of the leopard frog. Being a lowland species only adds to the elixir. Breeding in the floodplains of the Champlain lowlands exposes *R. pipiens* to large drainage areas and associated pollutants. *R. pipiens* populations breeding near the mouth of the Otter Creek are developing at the bottom of a 900 square mile drainage area. Their overwintering habitat may be in the river, lake or nearby permanent pond.

Perhaps one of the most significant variables of the breeding success and development of *R. pipiens* in the Lake Champlain lowlands is Lake Champlain's fluctuating water levels. Water level in the adjacent floodplains can affect temperature, tadpole densities, predator densities, predator-prey relationships, ionic composition, contaminant concentrations, parasite densities, ultraviolet radiation penetration, and disease. All of the primary sites, and three of the four supplemental sites are directly affected by Lake Champlain water levels. The annual variability in abnormalities we have observed may very well be connected to these fluctuating water levels.

Utilizing the data and observations obtained during this investigation, we will address the three main hypotheses for amphibian malformations: (1) trematode infestation, (2) xenobiotic chemicals and (3) UV-B radiation exposure. Each of these causes has been determined to produce amphibian malformations in laboratory studies. The question at large is, can these causes produce the types and frequencies of malformations that have been recently observed in Vermont?

Parasite infestation:

1) parasite infestation causes limb and pelvic malformities only
2) multiple limbs or mirror image limbs predominate
3) there is a correlation between parasite infestation and the presence of a malformity (within older tadpoles and newly metamorphosed animals).

The following Vermont observations would appear to exclude this hypothesis: Forelimb abnormalities were observed at all of the study sites, eye and pigment abnormalities were observed at many of the sites (2001). Over 10,000 *R.pipiens* metamorphs examined (1997-2001) have yielded only 1 specimen with a supernumerary limb. Several radiographs of ectromelic frogs have revealed spongiforme bone, which is not consistent with parasite infestation (Lannoo, 2000b). A subset of frogs examined by the NWHC showed no correlation between those frogs that had malformations and those that had metacercaria. (Meteyer, 1997).

UV-B Radiation:

1) limb malformity consisting of bilaterally symmetrical ectromelias (Ankley, 1998)
2) animals exposed to UV-B do not show spongiforme bone (Lannoo, 2000b)

This hypothesis can be excluded based on the "symmetrical" morphological signature of UV-B radiation. To date there have been no symmetrical limb truncations observed in Vermont. Furthermore many of the unilateral ectromelic frogs radiographed in Vermont do show spongiforme bone.

Xenobiotic chemical:

This hypothesis predicts a wide suite of malformities involving the limbs, a variety of organ systems, and a number of biochemical/physiological processes (Lannoo, 2000a). This range of abnormalities associated with this hypothesis is consistent with the abnormalities we have observed in the Lake Champlain Basin.

Our findings from the 2001 Vermont investigation show a strong dissociation between the parasite hypotheses, the UV-B radiation hypotheses and the failed predation hypotheses. However the xenobiotic chemical hypotheses has been reported to produce the range of abnormalities that we have observed in Vermont.

It should be noted, the exclusion of the hypotheses above: parasite infestation, UV-B radiation and failed predation; does not mean that these hypotheses do not explain any of the observed abnormalities. What we are saying is that it is highly unlikely that these hypotheses are the sole causation of the observed hind limb abnormalities.

Malformation vs. Deformation
The characterization and interpretation of *R. pipiens* (metamorphs and tadpoles) has provided evidence that malformations are occurring in metamorphs and tadpoles. In contrast, there has been a general lack of evidence supporting deformations.

If we are observing a predominance of malformations, we will still need to determine whether these malformations are due to anthropogenic causes or natural causes. Even within the volume of this report we recognize that there are many biotic factors such as high water temperatures or deficient ionic concentrations that can play a role in malformations.

Summary

In summary we feel that this multidisciplinary approach is on target with regards to providing a greater understanding of the biotic and abiotic factors that are playing a role in the *Rana pipiens* abnormalities. While a definitive answer is still at large, we feel that we are significantly closer to understanding this phenomenon.

Future work should include continued monitoring at study sites, characterizing *Rana pipiens* metamorphs and tadpoles during early developmental stages, drift fence monitoring at the Ward Marsh site, and pesticide monitoring at study sites. Laboratory amphibian developmental toxicity test with atrazine and its metabolites should be carried out to limb development. Field specimens should also be examined for gonadal malformations. Field studies utilizing enclosures would be helpful in providing more information on the role parasites; predators and other environmental factors play in the observed abnormalities.