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Contaminants as Contributing Factors to Wood Frog Abnormalities on the Kenai National Wildlife Refuge, Alaska

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ABBREVIATIONS**:**

AIC	Akaike's Information Criterion
CCC	criterion continuous concentration - chronic limit for the priority pollutant in fresh water
CERC	Columbia Environmental Research Center
DO	dissolved oxygen
DNA	deoxyribonucleic acid
metamorph	frog between Gosner stage 42 and 46
MSCL	Mississippi State Chemical Laboratory
NWRC	National Wetlands Research Center
OC	organochlorine
OR	odds ratio
PAH	polycyclic aromatic hydrocarbon
PCA	principal components analysis
PCB	polychlorinated biphenyl
PEL	probable effects level - concentration or exposure level at which significant adverse effects become likely
pH	negative log of the hydrogen ion concentration, a measure of acidity
Refuge	National Wildlife Refuge
SpC	specific conductivity
SPMD	semipermeable membrane device
SVL	snout to vent length
TDS	total dissolved solids
TEL	threshold effects level - the concentration or exposure level below which a significant adverse effect is not observed

UCR	University of California at Riverside
UET	Upper effects threshold
USFWS	U.S. Fish and Wildlife Service
USGS	U.S. Geological Survey
UVB	ultraviolet-B radiation
UWL	University of Wisconsin LaCrosse

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EXECUTIVE SUMMARY

BACKGROUND: Amphibian abnormalities and diseases are not well understood, and appear to be increasing while global populations decline.

OBJECTIVES: The goals of this study were to identify stressors associated with amphibian abnormalities on the Kenai Refuge and assess whether anthropogenic factors contributed to these abnormalities.

METHODS: Between 2004 and 2006, we assessed 38 breeding sites for prevalence of abnormal wood frogs. We chose 21 ponds for more intensive study, measuring the following variables known to cause abnormalities in amphibians: UVB, temperature, basic water quality, contaminants, and abundance of predatory invertebrates. On a subset of frogs, we assessed gonadal structure, DNA integrity, and biomarkers of genetic damage, and identified and enumerated parasites. We analyzed field data with logistic regression, using AIC to compare competing models.

RESULTS: Of 5,716 metamorphic wood frogs examined, 450 (7.9%) had skeletal or eye abnormalities. We documented 558 abnormalities in these 450 abnormal frogs because frogs often had more than one abnormality. Over 25 abnormality types were seen. The four most common were micromelia (small limb), ectromelia (truncated limb), amelia (no limb), and unpigmented iris. We found evidence for two diseases of conservation concern, *Batrachochytrium dendrobatidis*, a fungal pathogen responsible for global amphibian population declines, and an undescribed protozoan, quite virulent in Kenai study populations. We also observed intersex frogs, 41 of 163 frogs (25%) examined had abnormal gonadal morphology. None of the 448 frogs assessed for parasites were infected with the abnormality-inducing trematode, *Ribeiroia ondatrae*. We quantified predatory invertebrates in study sites, including dragonfly larvae, damselfly larvae, water beetles, leeches, and spiders. Organic and inorganic contaminants exceeded toxic thresholds in study site sediments. PCBs were found in every pond, and DDT was higher than toxic thresholds in four sites. Arsenic, iron, selenium, cadmium, copper, and nickel were all higher than toxic thresholds in sediments, and barium, iron, cadmium, and copper surpassed thresholds in water. In statistical analyses, we identified dragonflies, toxic metals, and temperature as predictors for skeletal abnormalities and malformations. Metals that correlated with skeletal abnormalities included arsenic, cadmium, copper, and selenium in sediment and barium, iron, potassium, and lead in water. Environmental factors predictive of disease were temperature, acidity, metals, and total dissolved solids. Controlled experiments showed toxicity but not teratogenicity from abiotic site media.

DISCUSSION: We propose the ultimate cause of skeletal abnormalities in Kenai wood frogs is amputation injury, probably by dragonfly larvae. The significant effects of metals and temperature in our statistical analyses suggest one or both of these factors may be disrupting the normal predator-prey relationship between dragonflies and wood frogs. Contaminants in sediment may slow development or interfere with normal predator detection and avoidance strategies. Warmer temperatures may increase abundance of dragonfly larvae or change the timing of dragonfly presence relative to tadpole growth. Higher temperatures and poor water quality were positively associated with disease. Two initial hypotheses for the intersex condition are high temperatures and PCBs, both previously shown to cause endocrine disruption in amphibians. Anthropogenic disruption of climate and consequent high temperatures appear linked to three of the four abnormality types we documented. These temperature effects may be particularly significant in the face of further predicted global change.

INTRODUCTION

Amphibians are considered sentinels of ecological health and early indicators of environmental change (Cohen 2001; Van der Schalie et al. 1999). Permeable skin renders them vulnerable to environmental pollutants (Hayes 2004) and development in water increases the probability of injury by ultraviolet radiation (Ankley et al. 1998, 2002), invertebrate predators (Viertel and Vieth, 1992), and parasites (Johnson and Sutherland 2003).

Amphibian populations are declining (Houlahan et al. 2000; Stuart et al. 2004; Wake 1991), concurrent with an apparent increase in morphological abnormalities (Gray 2000; Hoppe 2000; McCallum and Trauth 2003). In Minnesota, Hoppe (2000) reported that "frog abnormalities [were] more frequent, more varied, more severe, and more widely distributed in 1996-97 than in 1958-93." In Arkansas, the prevalence of abnormal frogs increased from 3.3% in 1957-1979, to 6.9% in the 1990s, to 8.5% in 2000 (McCallum and Trauth 2003).

Documented causes of amphibian abnormalities include parasites, ultraviolet-B radiation (UVB), predation or other trauma, and chemical exposure (Blaustein and Johnson 2003; Loeffler et al. 2001). The trematode parasite *Ribeiroia ondatrae* induces malformations (predominantly extra, missing, or misshapen limbs and skin webbings) by infecting the developing tadpole limb bud with its metacercarial life stage (Johnson et al. 2001a; Sessions and Ruth 1990). At ponds in the western United States infested with *R. ondatrae*, abnormality prevalence of up to 90% has been documented (Johnson et al. 2002). Laboratory and microcosm studies have established that exposure to UVB can cause limb reductions or deletions in amphibians, but there is much discussion about the relevance of UVB in nature, where it is attenuated by organic carbon dissolved in water and the diagnostic bilateral limb abnormalities are rare (Ankley et al. 2004; Blaustein et al. 1997; Diamond et al. 2002). Predator attacks can also cause skeletal deformities such as missing limb elements (Brodie and Formanowicz 1983; Henrikson 1990), and early injury to the tadpole limb bud can cause developmental malformations, such as shrunken limbs (Forsyth 1949; Fry 1966). Finally, chemicals such as thiosemicarbazide, organochlorines, carbamate and organophosphate insecticides, and retinoids and their environmental mimics have all caused skeletal abnormalities in laboratory studies (Alvarez et al. 1995; Gardiner et al. 2003; LaClair et al. 1998; Riley and Weil, 1986; Schuytema and Nebeker 1998; Snawder and Chambers 1989). Causes of eye abnormalities are less well understood, but authors have suggested chemical contaminants and early season temperature extremes (Vershinin 2002) or a recessive genetic mutation (Nishioka 1977).

Abnormal amphibians have been documented on the Kenai National Wildlife Refuge in multiple years of sampling (Reeves et al. 2008). In an analysis of data from five National Wildlife Refuges in Alaska, these authors found that proximity to roads increased the probability of skeletal abnormalities, but not eye abnormalities, in Alaskan wood frogs (*Rana sylvatica*, also *Lithobates sylvaticus*). The goal of the present study is to measure stressors associated with the abnormalities Reeves et al. (2008) observed, and to evaluate which of the possible causes of abnormalities are best supported by field data.

The Kenai National Wildlife Refuge juxtaposes human disturbance with wilderness areas, enabling us to concurrently study the abnormal frog problem and the role humans play in it. The refuge has 345 km of roads, including the only major highway bisecting the Kenai Peninsula. Roads have been shown to release contaminants by changing the biogeochemistry of disturbed areas, mobilizing metals from the roadbed, especially during storm events (Sansalone and Buchberger 1997). Roads also carry traffic, which releases hydrocarbons and metals through exhaust (Wheeler et al. 2005). Many Kenai Refuge roads were developed to support the two operating oil and gas fields in the refuge, the first of which began drilling in the 1950s. The oil and gas development and other road-associated human activities have led to release of contaminants including pentachlorophenol, petroleum products, and polychlorinated biphenyls, mercury from historic mining, and historic herbicide applications (Parson 2001). Yet, the 797,200 ha refuge also harbors four designated wilderness areas, and vast stretches of habitat that by all global measures is pristine and difficult to access.

There has never been a landscape-scale study that concurrently measures all the stressors known to cause amphibian abnormalities. Moreover, most studies do not combine controlled experiments with field observations to test proposed hypotheses. Most often, authors examine one or two stressors, or assess associations between abnormal frogs and human disturbance (Bacon et al. 2006; Gurushankara et al. 2007; Hopkins et al. 2000 Linzey et al. 2003; Ouellet et al. 1997; Reeves et al. 2008; Taylor et al. 2005). Yet, the results of these studies can only be correlative, leaving the authors unable to identify which environmental variables are causing the abnormalities.

The goal of this study was to determine which environmental factors are correlated with amphibian abnormalities in the Kenai Refuge and use experimental methods to test initial hypotheses about how these stressors lead to the abnormalities we observe in nature. This study was also designed to address the issue of road-based human disturbance and whether it contributes to amphibian abnormalities in this system.

OBJECTIVES

1. Evaluate the occurrence of frog abnormalities from ponds located in developed areas and wilderness areas of the Kenai NWR.

2. Measure contaminants in abiotic media from selected frog ponds.

3. Evaluate the effects of contaminants on development of *R. sylvatica*.

4. Evaluate sublethal contaminant effects on tadpoles and changes in predator densities as they may contribute to observed Kenai NWR frog abnormalities.

5. Evaluate the role of parasites in explaining the high wood frog abnormality rates in Kenai NWR, and evaluate the interaction between contaminants and parasites.

6. Evaluate the interaction between contaminants and UV radiation.

7. Measure chromosomal damage in frogs from Kenai NWR ponds.

METHODS

FIELD METHODS

Study Area and Selection of Sites

Before this study, we had no information about abnormalities in remote wilderness areas in the Kenai Refuge. All ponds sampled for abnormal amphibians between 2000 and 2003 were adjacent to roads or other industrial areas. We had no information about frog abnormalities from ponds farther from the road. Therefore, one objective of this study was to monitor additional ponds for abnormalities at remote sites (Figure 1).

To select new study sites, we identified areas of potential frog habitat on the Refuge using a combination of aerial photos, topographic maps, road surveys, and geographic information systems (ArcMap, ESRI, Redmond, WA, USA). Using these methods, we identified road accessible and remote areas of potential frog habitat. We stratified these areas based on whether they were developed (i.e. within 1 km of towns, roads, mines, areas of oil development, etc.) or remote (i.e. in designated wilderness and more that 1 km away from the above-listed areas). We selected developed and remote sites differently based on logistics.

Figure 1. Map of Study Site Locations

Developed Sites.
All areas of potential wood frog habitat within 1 km of a road and on refuge land were identified as potential study sites based on the following criteria. Sites were:

1. Wet in the breeding season,
2. Searchable – not too large or diffuse for monitoring or collections. We limited the maximum size of a breeding site to a continuous or interconnected body of water <1 km on the longest side, as measured with air photos and GIS,
3. Deep enough that a semipermeable membrane device (SPMD) could be deployed in June/July and pond would not dry on a normal year,
    a. Ponds identified in early-season driving surveys were compared to air photos to determine whether they retained water through the summer. (Air photos were taken in July).
    b. If they did not appear to retain water, they were classed as *Dry* and were ranked higher than all ponds that appeared to retain water throughout the summer,
4. All wetlands identified in the driving and air photo census were assigned an ID number,
5. The MS Excel™ random number generator was used to assign random numbers to each pond,
6. Ponds were then sorted by their random numbers and given a rank based on this number,
7. The lowest random number was assigned rank 1, the next lowest, rank 2, etc. until all ponds were ranked,
8. The ponds with the lowest ranks were selected for field evaluation in each area, monitored for frogs, then chosen for inclusion in the study if they had tadpoles when searched.

Some ponds in this study were monitored for the National Abnormal Amphibian Program (NAAP) between 2000 and 2003. Therefore, we had abnormality data for several Kenai Refuge sites, four of which were included in this study. These four ponds were classified as "developed" because of their proximity to the road system and industrial development.

Remote Sites.
By definition, all remote sites were farther than 1 km from a road or other human disturbance and located in an area formally designated as wilderness (Wilderness Act of 1964:16 U.S. C. 1131-1136). Four wilderness areas were chosen for study, based on location of available habitat and logistics of site access. Because the Refuge prohibits air travel over designated wilderness, we sampled only in areas we could reach on foot or by boat.

We sampled remote sites in four different wilderness areas: Mystery Hills, Swanson River, Skilak Lake, and Tustamena Lake. Although we tried to get equal numbers of sites in each location, the availability and density of wood frog habitat prevented this in both the Skilak Lake and Tustamena Lake areas. All sites within each wilderness were clustered within several km of each other because otherwise sampling would be too time intensive. Methods for remote site selection are described below.

Mystery Hills.
All sites in this area are within 100 m of the Fuller Lakes trail. We identified eight potential ponds via air photos, performed field surveys for tadpoles at six of these, found wood frogs at four of them, and selected these four for inclusion in the study as intensive study sites, based on abundance of wood frogs and ease of sampling.

Swanson River.
Wood frog habitat is dense and abundant in this wilderness, therefore, a block of land for study was selected randomly from all potential blocks, according to the following methods. All administrative sections of land with potential wood frog habitat and accessible by the Swanson River or Swan Lake Canoe Route were identified on topographic maps. Then, one section was selected randomly from all possible sections. This section was used as the center of a block of land, which included the eight sections abutting it. This block became our search area. It was located on the Swanson River Canoe Route. In this block, we identified 13 ponds via maps and air photos, searched them all for wood frogs, found frogs at eight, and found adequate numbers for collection at two of these in 2004. These two sites were included as intensive study sites. In later years, we sampled metamorphs for abnormalities at an additional three ponds in this area.

Skilak Lake.
This lake is approximately 15 km long and in bad weather, boating conditions are hazardous. Habitat is less dense and abundant here, so all ponds within 1 km of Skilak Lake were identified via maps and air photos. Here, we identified six potential sites, searched four of them for wood frogs, found frogs at three, and adequate numbers for abnormality sampling at two. These sites were both included as intensive sites in this study.

Tustamena Lake.
This lake is approximately 30 km long and boating conditions here can also be hazardous. Frog breeding habitat is also less dense and abundant in this area. We therefore limited our search for wood frog breeding sites to those accessible from the closest 15 km of the lake. In this area, we identified all potential study sites from maps and air photos. We identified nine potential study sites in this way. We found frogs at four of these, and adequate numbers for abnormality sampling at only one of them, which is a large wetland complex. We selected this site for intensive study.

In total, between 2004 and 2006, we assessed 105 potential study sites, identified from topographic maps and aerial photos. Of these, we collected adequate numbers of frogs for abnormality evaluation at 38. We chose 21 of these ponds for more intensive study; 12 ponds were classified as developed sites and nine were remote.

At these intensive study sites we measured the following variables: UVB, basic water quality, contaminants, temperature, and abundance of predatory invertebrates. We also performed the following analyses on frogs from each site: Abnormality assessment and classification, parasite identification and enumeration, gonad histological examination, DNA integrity, and biomarker testing. Methods are described below.

UVB

In 2004, the U.S. Geological Survey (USGS) staff measured UVB radiation at eight study sites during a single sampling period with a broadband UV meter (Macam Photometrics Ltd. Livingston, Scotland). This instrument measures total UVB with a peak spectral response at 311 nanometers (nm) and a bandwidth of 292 to 330 nm. It also measures total UVA with a peak spectral response at 369 nm and a bandwidth of 332 to 406 nm. The instrument was calibrated using standards traceable to the British Standard Institute. In 2006, the USFWS used the same instrument to take repeated measures of UVB at the surface and 10 cm depth in all study sites. We measured UVB at different times of the day, during changing weather conditions, and on different dates in all study sites.

Basic Water Quality

Each time we measured UVB, we concurrently measured basic water quality with a Hydrolab Minisonde 4.0 (Hach Environmental, Loveland, CO, USA). We measured pH, dissolved oxygen (DO), total dissolved solids (TDS), and specific conductivity (SpC), multiple times in variable weather and at different times of day. All measurements were taken at the same location within a pond, at 15 cm depth, adjacent to the temperature logger.

Water Sampling - Organics

Semi-Permeable Membrane Devices (SPMDs) are a passive sampling technology that sequesters organic contaminants from the water column (Huckins *et al*. 2002). In 2004 and 2005, SPMDs were deployed in all 21 study sites. SPMDs were deployed at eight sites in 2004 and 13 sites in 2005. The SPMDs were extracted at Environmental Sampling Technologies, Inc., because this process is patented for this type of device. The extracts were analyzed by Mississippi State Chemical Laboratory for total PCBs, PAHs and their alkylated homologues, and organochlorine compounds (OCs).

Water Sampling - Inorganics

Water samples were collected from all study ponds during abnormality assessments in June or July of 2004 or 2005, using standard field collection protocols (Csuros 1994). Water was analyzed for total metals by inductively coupled plasma/mass spectrometry at the Trace Element Research Lab (TERL) in College Station, Texas. Sample results were compared to water quality criteria presented in the National Oceanic and Atmospheric Administration, Screening Quick Reference Tables (Buchman 1999).

Sediment Sampling - Organics and Inorganics

In 2004 and 2005, sediments from all study ponds were sampled for total metals, total organic carbon, OCs, PCBs, PAHs and Dioxin/Furans. Samples were collected using the methods of Csuros (1994). Samples were homogenates pooled from three random locations in a pond. At each location, we sampled the top 30 cm of sediment. Shallow site samples were collected with hand-held scoops – stainless steel for organics and plastic for inorganics. Deeper sites were sampled with an Eckman dredge. Organic samples were homogenized in stainless steel bowls. Inorganic samples were homogenized in Ziploc® bags. All equipment was washed with Alconox

and water, rinsed with deionized water, then hexane, then acetone between sampling sites. All samples were sent to USFWS contract laboratories for analyses. Inorganic samples were analyzed at TERL. Organic samples were analyzed at the Geochemical and Environmental Research Group (GERG) in College Station, Texas. Sample results were compared to sediment toxicity thresholds presented in the National Oceanic and Atmospheric Administration, Screening Quick Reference Tables (Buchman 1999).

Temperature

We measured temperature at all study sites with data loggers (Hobo, Tidbit Loggers, Onset Data Corporation, Pocasset, MA, USA). During 2004 and 2005, temperature loggers were affixed to the SPMD canisters. In 2006, temperature loggers were placed adjacent to egg masses and attached to a float that kept them at a depth of 5 cm below water surface. Temperature loggers were deployed within several weeks of frog breeding (late April to early May, depending on the year) and retrieved when metamorphs were assessed for abnormalities.

Invertebrate Predator Assessments

We identified potential invertebrate predators and quantified their densities twice per year in all 21 study ponds for two years. Invertebrates were collected by continuously sweeping a 0.3 m X 0.3 m D-frame net (350 mm mesh net) at a depth of 1 m on a 10 m long transect through vegetative perimeters of the ponds, where tadpoles are often captured. Three 10 m transects were swept, to sample approximately 2120 l of water. Samples were removed from the net, placed in pre-labeled whirl-pak® bags, preserved with 70 percent ethanol, and identified by Dr. Peter Jensen at the University of California at Riverside (UCR). Each sample was then sorted in its entirety under 2X magnification. Invertebrates were placed in glass vials with fresh 70% ethanol and stored until identification. All individuals were identified and counted using appropriate taxonomic keys For additional details on methodology of this section, please see the reports by Jensen (2005 and 2006), which are included as Appendix A.

Species Selection

The wood frog, *R. sylvatica* (Hillis 2007) or *Lithobates sylvaticus* is the only amphibian common in most of Alaska, and the only amphibian in the Kenai NWR. Wood frogs breed explosively just after snowmelt, laying eggs in late April or early May and metamorphosing in late June, July, or early August depending on climatic factors like temperature (Herreid and Kinney 1967), timing of snowmelt, and hydroperiod of the wetland. After metamorphosis, young frogs migrate up to 2 km from breeding wetlands to adult habitat in adjacent woods (Berven and Grudzien 1990). This synchronous breeding and development cause larvae to metamorphose within a 5-7 day window at each breeding pond (Herreid and Kinney 1967). We examined frogs for abnormalities only during this time.

Abnormality Assessment

Between 50 and 100 metamorphic frogs, stage 42-46 (Gosner 1960), were assessed for abnormalities at each site. Stages 42-44 were mainly aquatic and were captured with dip-nets. Stages 45-46 were primarily terrestrial, and were caught by hand at the pond edge. Frogs were placed in buckets at the capture site until examined for abnormalities using standard protocols

(U.S. FWS 1999). Snout-to-vent length (SVL) and tail length were measured, and developmental stage was recorded. Abnormal frogs were euthanized with tricaine methanesulfonate (MS-222, Argent Chemical Laboratories, Redmond, WA), photographed, and sent to Ball State University or the USGS, National Wildlife Health Center for radiographs to aid in abnormality classification. A subset of normal and abnormal frogs (n=448) were examined for parasites, including *R. ondatrae*, at the University of Wisconsin, La Crosse (UWL). All normal frogs not collected for parasitology were released at the capture site after field examination. Equipment was disinfected with 5% bleach solution between sites to prevent disease spread. All animals were treated humanely with regard to alleviation of suffering.

Abnormality Classification

According to Johnson et al. (2001b) abnormality is a general term referring to "any gross deviation from the normal range in morphological variation," and includes both malformations (permanent structural defects resulting from abnormal development), and deformities (alterations, such as amputation, to an otherwise correctly formed organ or structure). Abnormalities were categorized for analysis using standard protocols (U.S. FWS 2007) and published guides (Meteyer 2000). Abnormalities were subdivided into the following categories: skeletal abnormalities, eye abnormalities, surface abnormalities (e.g., wounds, skin discolorations, cysts) and diseases. These categories suggest either different causes of the abnormalities or different timing of injuries that resulted in the abnormalities – an early injury might result in a malformation, like a shrunken limb, whereas an injury after a developing tadpole has lost its regenerative ability might result in a missing limb segment (Fry 1966). We separated eye abnormalities from skeletal abnormalities because they may have different causes. Skeletal abnormalities include three subcategories: malformations, injuries, and abnormalities of unknown etiology, which also suggest either different causes or different timing of injury (Table 1). A single researcher classified all frogs in this data set from pictures, radiographs, and field notes.

Additional Frog Diagnostics: Parasites, Gonad Structure, and Biomarkers

We selected both normal and abnormal frogs from each site to undergo the following additional diagnostics: Parasitology, DNA Integrity and biomarker analysis, and gonad histology. For these analyses, we collected 15 frogs from each of 12 ponds (four ponds per year) and sent them live to UWL for parasitology. However, not all frogs survived each trip. Frogs were coded by field personnel so that UWL researchers did not know their origin or whether frogs were viewed as "abnormal" or from developed or remote sites. Researchers at UWL identified and enumerated parasites, drew blood for DNA and biomarker analysis, extracted the gonads of the animals, then shipped the blood to USGS National Wetlands Research Center (NWRC) and the gonads to McNeese State University. Using NWRC Standard Operating Procedures, Dr. Jill Jenkins used flow cytometry to measure DNA damage in blood cells. In 2004, blood from 59 normal and abnormal frogs was preserved and analyzed by flow cytometry. In 2005, DNA analysis was conducted on blood samples from an additional 82 frogs. Assays to examine DNA repair enzymes were developed and performed on the 2005 samples. In 2006, 142 frogs from 15 field sites and 41 frogs from the USFWS toxicity test were shipped to NWRC for DNA integrity analysis. For more details on the methods used, please see Jenkins (2008 - included as Appendix

B). At McNeese State University, Dr. Connie Kersten examined gonads from each frog and determined sex initially by gross morphological examination under a dissecting microscope. Gonads were then dissected, sectioned, stained with hematoxylin and eosin, and prepared for histological examination by embedding in paraffin. Sections were prepared that spanned several layers of the tissue. A minimum of two slides were prepared from each gonad. Gross gonadal abnormalities (i.e., intersex) were determined as left/right intersex (testes on one side and ovary on the other), rostral/caudal intersex (testicular or ovarian characteristics clearly defined caudally and rostrally), or mixed intersex (testicular and ovarian tissues mixed within the gonad with no regional differentiation; Carr *et al*. 2003).

CONTROLLED STUDIES: TOXICITY TESTING AND PREDATOR EXCLUSION

In 2001 and 2004, the USGS and the USFWS deployed SPMDs specially-designed to sequester contaminants from the water column for experiments by USGS. During 2004, staff from the USGS Columbia Environmental Research Center (CERC) collected sediment, retrieved SPMDs at each of eight study sites, and measured UVB to calibrate their experiments. In 2005, USGS performed two laboratory toxicity tests, exposing wood frogs to SPMD extracts and UVB, then to site sediments without UVB. During summer 2007, USGS performed one final toxicity test to address data gaps from the prior study, and incorporated UVB testing into the sediment toxicity experiment. Detailed methods for these experiments are included in the USGS final reports (Bridges and Little 2002; Little et al. 2008 - attached as Appendix C).

In 2006, the USFWS conducted an additional controlled toxicity test. In it, we exposed tadpoles to site sediments and site water, rather than extracts, because SPMDs do not sequester inorganic contaminants. While collecting water for this study, we also gathered more complete data on ambient UVB during different weather conditions, which allowed USGS to better calibrate their UVB experiment in 2007. Finally, we submitted metamorphs from this sediment and water toxicity experiment to our collaborators for parasitology, gonad histology, and DNA analysis. For this, USFWS collected additional sediment samples from six study sites and additional Alaskan wood frog eggs for use in the USGS study. Detailed methods for the USFWS sediment and water toxicity study are included as Appendix D.

The USFWS also performed a test in 2007 to address whether the wood frog abnormalities are heritable, by evaluating development of tadpoles from affected breeding sites when reared under controlled conditions in clean water (Alaska's Best Water, Anchorage, AK, USA). This was an important mechanistic question that needed to be evaluated before data interpretation. The study plan for this experiment is included as Appendix E.

Finally, in 2005 and 2006, collaborators from the University of California at Riverside (UCR) performed predator exclusion studies in sites at which abnormal frogs were consistently found. Methods for these studies are detailed in two reports (Jensen 2005 and 2006 – Attached as Appendix A).

STATISTICAL ANALYSIS AND HYPOTHESIS EVALUATION

The four known causes of skeletal abnormalities in amphibians are parasite infection, predation injury, UVB radiation, and chemical contaminants (Blaustein and Johnson 2003). We performed a unifying statistical analysis to evaluate which of these hypotheses for amphibian abnormalities were most plausible and in greatest agreement with field data collected in Kenai NWR. To test hypotheses, we used the following abnormality categories as response variables: skeletal abnormalities, skeletal malformations, disease, unpigmented irises (the most common eye abnormality), and intersex.

Predictor Variables

Before we performed the regression analyses, field data were reduced to several variables that represented each hypothesis for abnormalities (i.e. parasites, contaminants, water quality, temperature, UVB, invertebrate predators, or interactions among these). Before we analyzed the data, we used correlation tables to examine collinearity between each combination of possible predictor variables. If two predictors were highly correlated ($r>0.7$), the collinear variables were altered or removed prior to model construction. For pairs of variables that could not be altered or removed to avoid collinearity, results are presented acknowledging the uncertainty caused by each internal correlation. The variables we entered into the statistical models are detailed under each research objective below, as are the methods used to reduce the dimensionality of field data.

UVB

We calculated percent penetration from repeated UVB measurements we took in 2006 at 0 and 10 cm depth adjacent to the temperature logger. This measurement represents the percent of surface UVB that reaches a 10 cm depth in site water, and was the only variable used to represent UVB.

Basic Water Quality

From repeated measurements we took adjacent to the temperature logger at 15 cm depth during the field season of 2006, we calculated the following:

1. Mean, minimum, and maximum acidity (pH)
2. Mean, minimum, and maximum dissolved oxygen (DO)
3. Mean, minimum, and maximum total dissolved solids (TDS)
4. Mean, minimum, and maximum specific conductivity (SpC)

We then examined pairwise correlations between these variables. Based on the correlation structure of the data, biological considerations, and ease of interpretation, we chose the following four variables to represent site water quality in analyses:

1. Average pH,
2. Minimum measured DO,
3. Maximum measured TDS, and
4. Maximum measured SpC.

Contaminants

Contaminants in each media (sediment, water, and SPMDs) were treated as separate groups for data reduction. These groups were kept separate for two reasons. First, there

were differences in the number of analytes detected (almost all inorganic analytes were detected in almost all samples, but few organic analytes were detected in all samples). Second, the different classes of contaminants may have different modes of toxicological action. Therefore, it simplified data interpretation to identify which specific classes of chemicals were correlated with abnormalities. So few organic contaminants were detected in the SPMDs (site water), they were not included in statistical analysis.
Data were reduced according to the following methods.

Non-detect Filter
Because a large proportion of the contaminants we measured were not detected in all study sites, we first applied non-detect filters to the data set. We did this in two separate analyses for organics and inorganics. For the organic data, detection limits varied by site. Therefore, we required each analyte to be present in at least 20% of the sites, at concentrations greater than the highest detection limit for that analyte in the data set. For example, with 21 study sites, total PCBs had to be greater than the highest detection limit in the data set for total PCBs, in at least five sites, for this analyte to undergo statistical analysis. Because inorganic contaminants were detected much more frequently, we applied a 70% filter to these data. Each analyte had to be detected at 70% of the sites sampled.

Principal Components Analysis
Contaminants that made it through the non-detect filters were subjected to principal components analyses (PCA) in the following groupings:

1. Inorganic contaminants in water
2. Inorganic contaminants in sediment
3. PAHs in sediment

Final Predictor Variables for Contaminants Data
After the data reduction described above, we used the following variables as predictors of the different classes of frog abnormalities:

1. 2 PCA vectors of inorganic contaminants in sediment
2. 2 PCA vectors of inorganic contaminants in water
3. All organic contaminants in sediment after the non-detect filters.
    A. Analytes found in all sites included:
        a. total PCBs,
        b. total petroleum hydrocarbons (TPH), and
        c. aliphatic hydrocarbons (which were positively correlated with TPH). Because of the correlation with aliphatics, TPH was used to represent aromatic and aliphatic hydrocarbons in sediment.

    B. Organic analytes remaining after the 20% filter included:
        a. chlorpyrifos*
        b. 1_2_4_5_Tetrachlorobenze*

c. DDT*
d. total PCBs *
e. TPH and aliphatic hydrocarbons
f. akylated chrysenes**
g. akylated fluorenes**
h. akylated phenanthrenes/anthracenes**
i. perylene**

*PCBs comprised the vast majority (by mass) of organochlorines in sediment. The concentration of PCBs was therefore highly correlated with the concentration of total OCs. Because we expect PCBs and other OCs to have similar toxicological properties, we summed these contaminants for a "total OC" variable.

**These compounds are all polycyclic aromatic hydrocarbons (PAH). A composite measure, the sum of PAHs in each sample, was calculated to represent these four compounds in sediment, but this measure was correlated ($r \geq 0.7$) with the early season abundance of dragonfly larvae. We therefore performed a separate principal components analysis, which yielded two vectors that we used to represent PAHs in site sediment.

Temperature

From data loggers deployed at each site in 2006 and set to record every 0.5 hours, we calculated the following:

1. Mean, minimum, maximum, and range in temperature during the entire season of egg, embryo, and tadpole development to metamorphosis,
2. Mean, minimum, maximum, and range in temperature during egg and embryo development (Gosner stages 0-25),
3. Mean, minimum, maximum, and range in temperature during tadpole development (Gosner stages 26-46),

We then examined pairwise correlations between these variables. Based on the correlation structure of the data, biological considerations, and ease of interpretation, we chose the following three variables to represent site temperature in analyses:

1. Average temperature,
2. Range in temperature during the egg and embryo stage, and
3. Range in temperature during the tadpole stage.

Invertebrate Predator Density

Predatory invertebrates found in study sites included nine genera of diving beetles, three genera of dragonflies, and two genera of damselflies. We also quantified predatory fishing spiders and leeches. For the statistical analysis, all predatory invertebrates were first broken down by individual species to examine correlations between pairwise combinations of each species' abundance in early and late season sweeps. To condense the data, we combined individual species based on taxonomy (eg. dragonflies versus water beetles) and size (large dragonfly nymphs versus smaller ones), then re-examined the correlation structure of the data. Data were then combined to eliminate correlations ($r \geq 0.7$) between pairs of invertebrate predators. This was

an iterative process, which eventually yielded uncorrelated predictor variables. Finally, we had to attach a single variable to each site to match the scale of other data, even though sweeps were done in two different years. Of the two years of data, we used the maximum abundance of each predator found in the pond during early season and late season sweeps to represent two variables: early season abundance and late season abundance. Abundance of leeches and spiders was so low we did not include them in statistical analysis. We also excluded *Colymbetes* spp. and *Neoscutopterus* spp., because we did not measure their abundance during both years of data collection. Ultimately, we used the following variables to represent "predators" in statistical models:

1. Number of taxa is a commonly used richness metric that indicates biological diversity and water quality impairment. We calculated this for early and late season sweeps.
2. Abundance of predacious individuals is the sum of all predacious individuals found in each sample. We calculated this for early and late season sweeps.
3. For the *Odonata* (dragonflies and damselflies), we used the following:
    a. The maximum early season abundance of *Aeshna sitchensis* (the largest dragonfly nymph)
    b. The maximum early season abundance of all small dragonflies (*Leucorrhinia glacialis, L. proxima,* and *Lebellulid* spp.*)*
    c. The maximum early season abundance of all dragonflies in *a* and *b* combined,
    d. The maximum early season abundance of all damselflies combined (*Lestes disjunctus* and *Coenagrion* spp.)
    e. The maximum early season abundance of all odonates combined (all species in *a-d* above)
    f. Each of variables *a-e* was also calculated for the late season sweeps.
4. For the water beetles, we calculated the following, which were not correlated with each other in these groupings:
    a. The maximum early season abundance of *Dytiscus* beetles (the largest beetles)
    b. The maximum early season abundance of *Graphoderus* beetles (medium sized beetles)
    c. The maximum early season abundance of *Rhantus* and *Ilybius* (also medium sized beetles, correlated with each other)
    d. The maximum early season abundance of *Agabus, Copelatus,* and *Corixid* spp. (the smallest beetles, also correlated with each other)
    e. The maximum early season abundance of all beetles
    f. The maximum late season abundance of all beetles (correlated with the small beetles in late season sweeps, so used in their stead)
    g. The maximum late season abundance of *Dytiscus* and *Acilius* (also correlated with each other in late sweeps, so combined)
    h. The variables *b* and *c* were also calculated for late season sweeps.

Parasites
Between 2004 and 2006, 448 metamorphs from 25 study sites were analyzed for parasites. Because of the low abundance of parasites in frogs, we did not include parasites in the statistical analysis.

Selection of Assessment Endpoints
We selected the following endpoints for evaluation in statistical analyses: Skeletal abnormalities, skeletal malformations, eye abnormalities, intersex, and diseases. We used unpigmented irises as a response variable for the eye abnormalities because they comprised the bulk (86%) of the eye abnormalities and we wanted to evaluate predictors for this abnormality type. The disease category is all one disease, an endoparasitic infection described in the results section.

Model Building
We used the Information Theoretic Approach (Burnham and Anderson 2002) to determine which explanatory models best fit the field data. In this method, we established *a priori* models with limited sets of predictor variables, and ran each of them in a logistic regression framework. We used Akaike's Information Criterion (AIC) to compare models and establish which model best predicted field data. The best fit model as evaluated by AIC is by definition parsimonious, because each model is penalized for additional parameters included as predictors. The ultimate goal of the model building exercise was to determine which of the hypotheses for skeletal and eye abnormalities were most plausible and in greatest agreement with observed patterns at the Kenai Refuge.

Experimental Data Analysis
Data from controlled studies were analyzed by general linear models (ANOVA and GLM), using predictor variables appropriate to each study design. Statistical methods for each study are detailed in the appropriate study plans and reports, included as appendices.

a. UVB and Water Extracts (Bridges and Little 2002; Little et al. 2008 – Appendix C)
b. UVB and Sediments (Little et al. 2008 - Appendix C)
c. Site Sediment and Water (Appendix D)
d. Heritable Abnormalities (Appendix E)
e. Predator Exclusion (Jensen 2005 and 2006 – Appendix A)

RESULTS – FIELD ASSESSMENTS

ABNORMALITY TYPES AND PREVALENCE

Between 2000 and 2006, a total of 5,716 metamorphs were examined at 38 breeding sites. Of the metamorphs, 450 were abnormal according to national protocols. We documented 558 separate abnormalities on these individuals, because abnormal individuals often had more than one abnormal body part. Over 25 types of abnormalities were seen (Table 1). Ectromelia (partial limb), micromelia (shrunken limb or limb element), amelia (limb totally missing), and unpigmented iris (eye totally black) were the four most common, collectively accounting for 73% of the abnormalities (Figure 2; Table 1). Skeletal malformations accounted for 36% of all abnormalities, skeletal injuries for 17%, and the remainder were classed as unclear etiology. Eye abnormalities comprised 137 (25%) of the 558 abnormalities, and 86% of the eye abnormalities were unpigmented irises. The more unusual abnormalities included anteversion (twisted long bones), microcephaly (shrunken head), scoliosis (curved spine), cutaneous fusion (skin webbing), and kinked tails (Table 1). The rarest abnormality type was polymelia (extra limb); only one specimen had an extra limb.

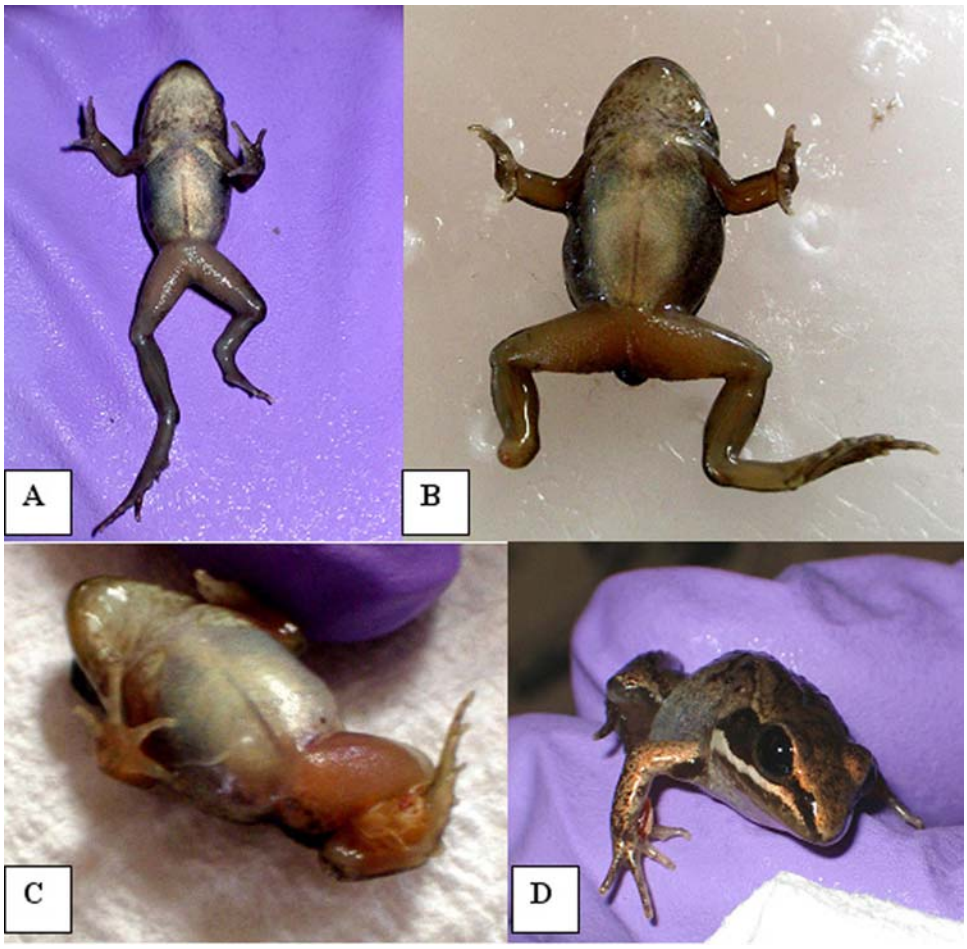


Figure 2. Pictures of the four most common abnormalities in Alaskan wood frogs. A. Micromelia, B. Ectromelia, C. Amelia, and D. Unpigmented Iris

The prevalence of skeletal and eye abnormalities at individual breeding sites ranged from 0 to 20%, with the highest prevalence found at a road-accessible site in 2005. The median breeding site abnormality prevalence was 7.6%. Abnormal frogs were found at most sites sampled; only four collection events out of 104 yielded frogs with no skeletal or eye abnormalities.

Field abnormality data from five Alaskan Refuges, including Kenai, have been analyzed and published in the journal, *Environmental Health Perspectives* (Reeves et al. 2008; Appendix F). In this larger data set, we found correlations between skeletal abnormalities and distance from the breeding site to the nearest road (Figure 3). We also found frog size and frog developmental stage to be significant predictors of the skeletal abnormalities (Figures 4 and 5). This manuscript includes all the field survey data for frog abnormalities on the Kenai NWR and four other Alaskan refuges.

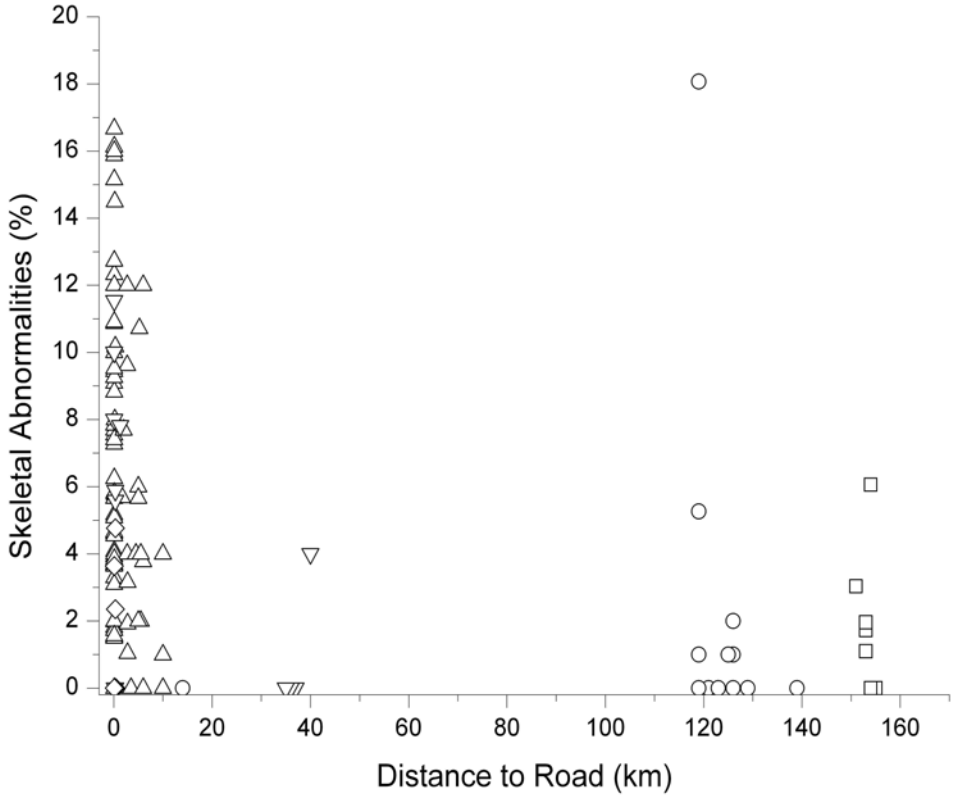


Figure 3. Skeletal abnormalities and malformations versus distance to the nearest road. Symbols are prevalence of frogs with skeletal abnormalities during single collection events at different refuges: Arctic (□) Innoko (○) Kenai (Δ) Tetlin(▼) and Yukon Delta (◊)

Table 1. Summary of abnormalities in Wood Frog populations at the Kenai Refuge Values are number of abnormalities in each category.

Abnormality Type		
Eye Abnormality	Anopthalmia (missing eye)	12
	Heterochromia (unpigmented iris)	118
	Micropthalmia (small eye)	1
	Other[1]	6
Skeletal Injury[2]	Brachydactyly (short digits)	7
	Ectrodactyly (missing digits)	4
	Ectromelia (partial limb)	44
	Limb crushed	14
	Other[3]	2
Skeletal Malformation	Amelia (missing limb)	31
	Anteversion (twisted longbones)	9
	Brachygnathia (short jaw)	6
	Microcephaly (shrunken head or blunt snout)	4
	Micromelia (shrunken limb or limb element)	126
	Polymelia (extra limb)	1
	Polydactyly (extra digits)	2
	Scoliosis or Lordosis (curved spine)	2
	Cutaneous fusion (skin webbing)	3
	Syndactyly (digits fused)	11
	Taumelia (bone bridge or triangle)	4
Skeletal Unknown Origin	Kinked tail	3
	Brachydactyly (short digits)	27
	Ectrodactyly (missing digits)	26
	Ectromelia (partial limb)	90
	Other[4]	5
Overall	Eye Total	137
	Injury Total	71
	Malformation Total	199
	Unknown Origin Total	151
Total no. abnormalities		558
Total no. abnormal individuals		450
Total no. individuals examined		5716
% Individuals Abnormal		7.9%

[1]Includes oversized eyes, abnormally shaped pupils, and cataracts.

[2]Either fresh blood or exposed bone must be noted for the injury category

[3]Includes dissociated and dangling limb

[4]Includes apparent dislocations

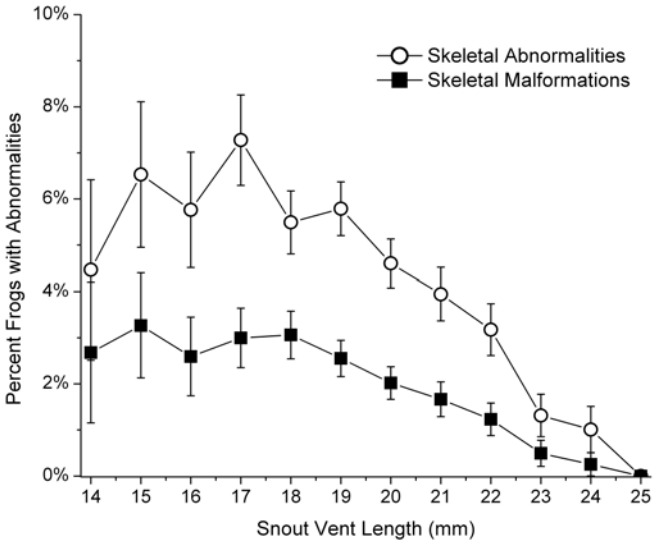


Figure 4. Skeletal abnormalities and malformations versus size. Values are proportion of frogs abnormal in each category.

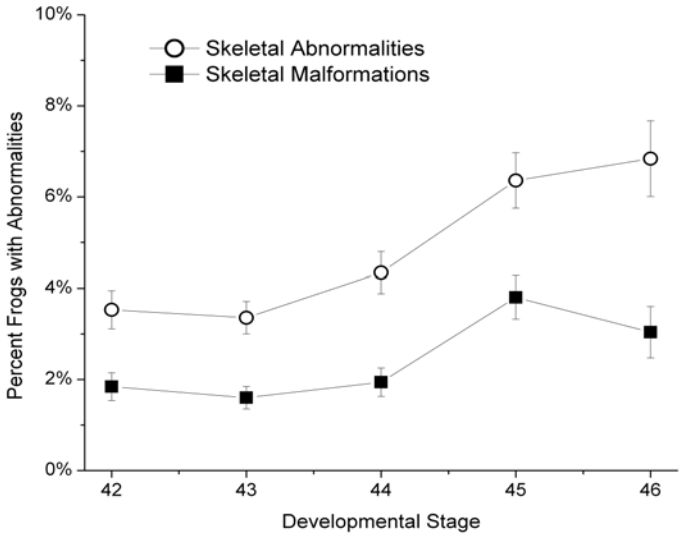


Figure 5. Skeletal abnormalities and malformations versus developmental stage. Values are proportion of frogs abnormal in each category.

DISEASES

During this investigation we found evidence for two diseases of conservation concern, *Batrachochytrium dendrobatidis* (Bd), a fungal pathogen responsible for global amphibian population declines, and an undescribed protozoan, quite virulent in Kenai study populations. We observed the latter pathogen kill entire cohorts of wood frogs, prompting us to submit diseased specimens for diagnostic testing. As a result, we helped identify a new anuran disease, a protozoan similar to the saltwater mollusk endoparasite, *Perkinsus marinus,* which causes die-offs in marine invertebrates. This disease has since been documented in frogs in Maine and Missouri, and is raising conservation concerns nationally. Diseased animals, 103 frogs with symptoms of infection by this organism (Figure 6) were observed at 18 of the 38 sites at which collections took place. At one intensive study site, we were unable to collect metamorphs during any of the three years of this study due to tadpole mortality from this disease.

During field work in 2006, we also sampled adult frogs for the fungal pathogen *Batrachochytrium dendrobatidis* (Bd), which has been implicated in amphibian extinctions worldwide. We had found this pathogen in one dead frog at a Kenai site in 2002 (Reeves and Green 2006) and performed additional sampling in 2006 to follow up on this earlier result. We found Bd at some Kenai sites, but not in any sites at the Innoko or Tetlin refuges (Reeves 2008 - attached as Appendix G). In the Kenai Refuge, Bd distribution followed a recreational access corridor.

Figure 6. Metamorphic wood frog infected with the Perkinsus-like protozoan organism

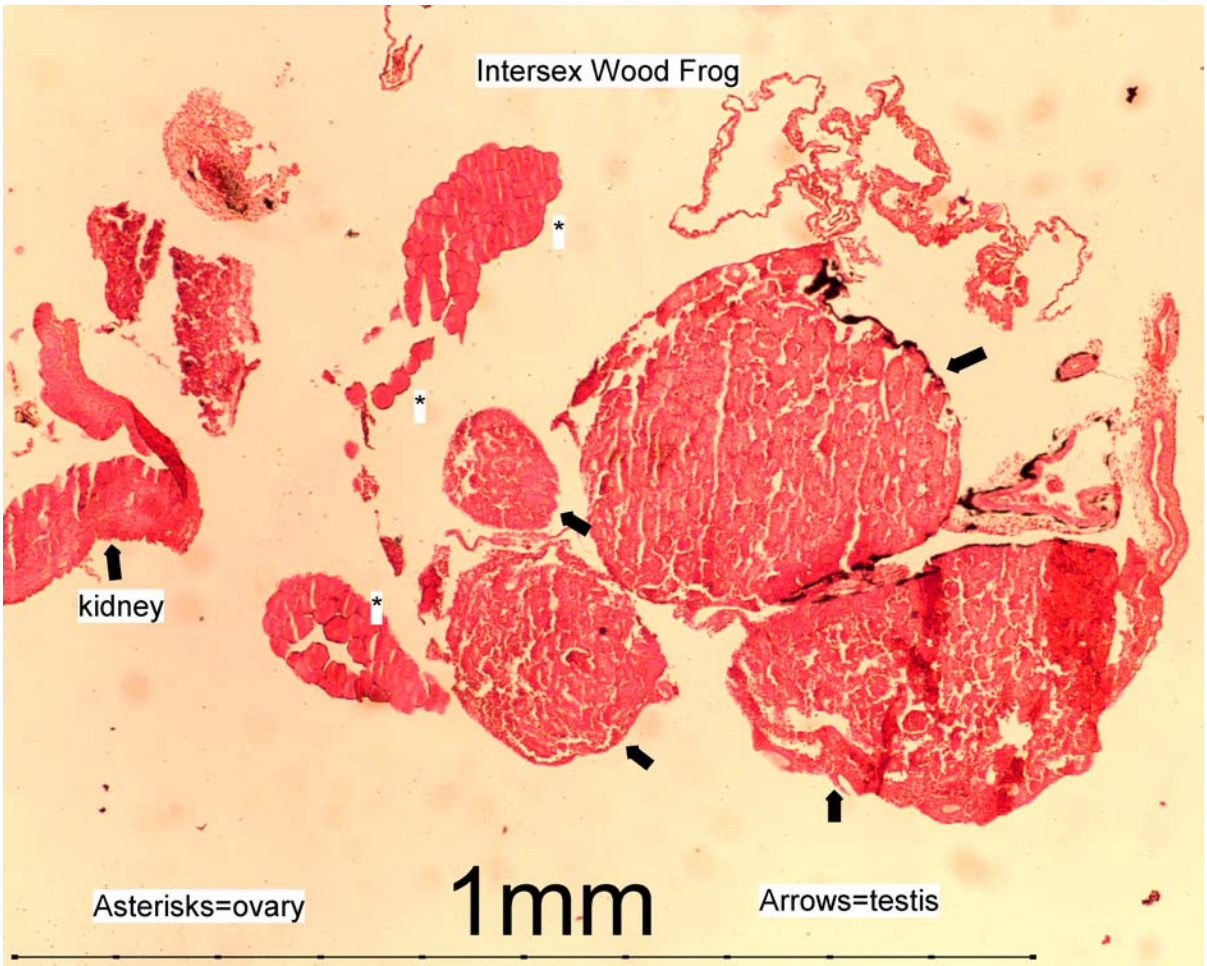
INTERSEX

Intersex frogs were also found in surprising proportions (Table 2). Of the 163 frogs sampled between 2004 and 2006, 25% had abnormal gonadal morphology. These animals had both ovarian and testicular tissue without directional organization, best described as mixed intersex (Carr et al. 2003; Figure 7). Sex ratios were also strongly biased toward males, with an average ratio of 1.5:1 over three years of sampling (Table 2).

Table 2. Summary of Intersex Frogs in Wood Frog Study Sites at the Kenai Refuge

SiteID	Female	Intersex	Male	Frogs Sampled	Proportion Intersex
KNA01	5	6	14	25	24.0%
KNA02		1		1	100.0%
KNA03	3	1	6	10	10.0%
KNA08	3	1	2	6	16.7%
KNA12		3	9	12	25.0%
KNA17	1	3	2	6	50.0%
KNA31		4	6	10	40.0%
KNA46	5	3	4	12	25.0%
KNA47	6	5	9	20	25.0%
KNA56	8		3	11	0.0%
KNA60	6	2	6	14	14.3%
KNA62	4	2		6	33.3%
KNA90	2	3	4	9	33.3%
KNA95	2			2	0.0%
KNA97	4	7	8	19	36.8%
Grand Total	**49**	**41**	**73**	**163**	**25.2%**

Figure 7. Histological slide of an intersex wood frog's gonad



PARASITES

The trematode parasite, *R. ondatrae,* is known to induce skeletal malformations in amphibians (Johnson and Sutherland 2003). To investigate whether *R. ondatrae* could be implicated in the abnormalities we identified, a subset of normal and abnormal frogs assessed for abnormalities were kept for parasite analysis. We examined 448 specimens for parasites. None of these frogs were infected with *R. ondatrae*, nor were planorbid snail hosts seen at any sampling site. Also notable was the lack of diversity and abundance of parasite flora in Kenai NWR frogs, relative to frogs in other parts of North America (D. Sutherland, personal communication).

ORGANIC CONTAMINANTS IN SEDIMENT

Organic contaminants were detected in study site sediment at levels exceeding threshold effects levels (TEL), the concentration or exposure level below which a significant adverse effect is not observed (U.S. EPA 1996). PCBs were detected in sediment from every pond, at concentrations ranging from 0.057 to 4.589 mg kg^{-1} dry weight (dw). At every site, total PCBs exceeded the TEL of 0.034 mg/kg dw for freshwater sediment (Figure 8). At 11 of 21 sites, concentrations exceeded the probable effects level (PEL) of 0.277 mg kg^{-1} dw. The PEL is the concentration or exposure level at which significant adverse effects become likely (U.S. EPA 1996). We also found DDT in site sediment; four of 21 sites exceeded the TEL of 0.00698 ppm total DDT. One of these exceeded the PEL of 0.0445 mg kg^{-1} dw. Some form of tetrachlorobenzene – a PCB degradation product - (either 1,2,3,4- or 1,2,4,5-) was detected at every site. There is no screening level for sediment toxicity for this compound. Sediment concentrations of individual tetrachlorobenzene congeners ranged from

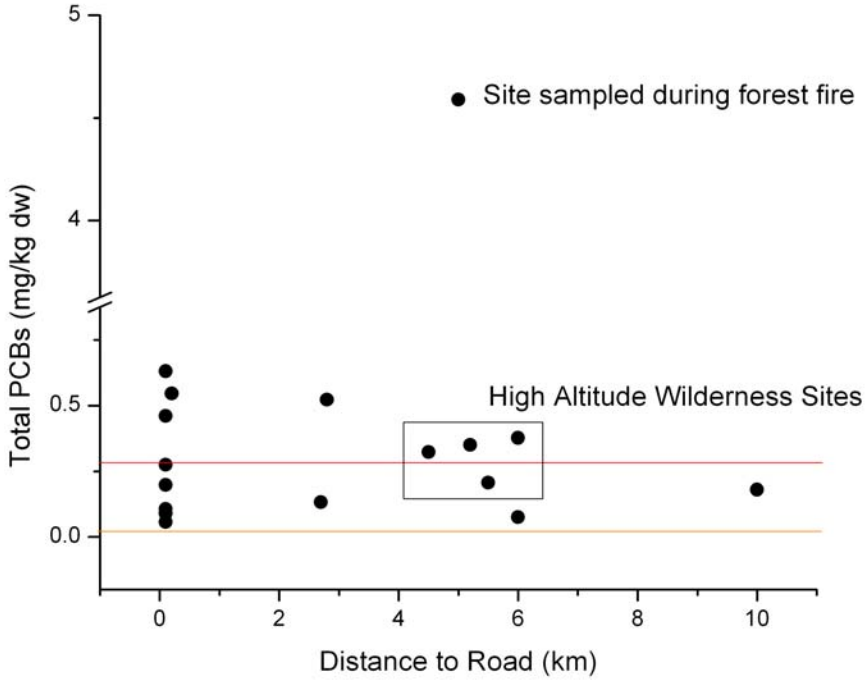


Figure 4. Total PCBs in study site sediment. Red line is PEL. Orange line is TEL

0.0000912 to 0.063 mg kg^{-1} dw. Concentrations of organic contaminants are presented in Tables 3 and 4 (Appendix H).

INORGANIC CONTAMINANTS IN SEDIMENT

Inorganic contaminants also exceeded both threshold and probable effects levels in study site sediment (Table 5 – Appendix H). Metals associated with skeletal abnormalities and malformations in this study are in ***italics***. Eight sites (38%) exceeded the TEL of 5.9 mg kg^{-1} dw

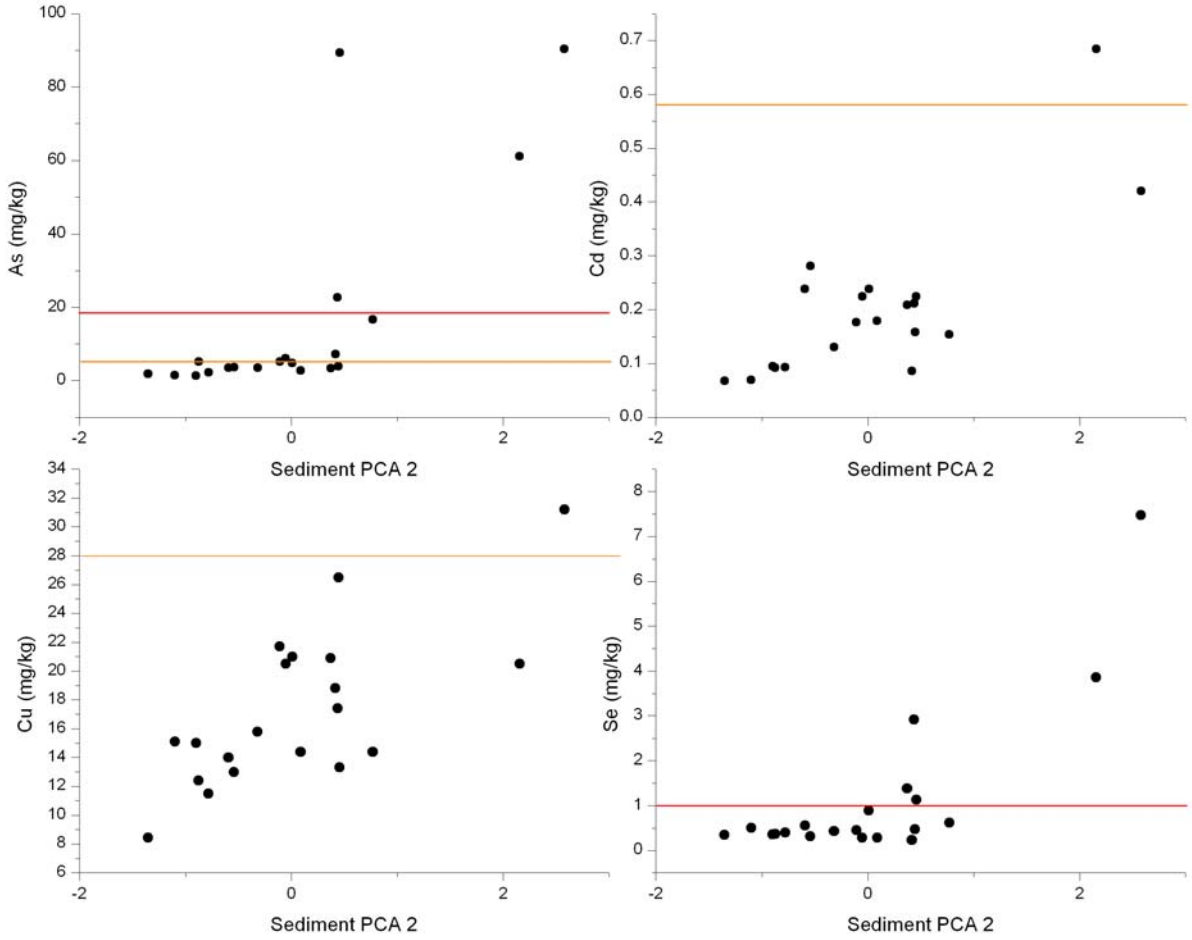


Figure 5. Sediment concentrations of As, Cd, Cu, and Se plotted against PCA vector 2, a significant predictor of skeletal abnormalities. Red lines are PELs. Orange lines are TELs for each element

for ***Arsenic***, and four (19%) of these exceeded the PEL of 17 mg kg^{-1} dw (Figure 9). Seventeen (81%) of the study sites had ***iron*** concentrations in sediment above the 4,000 mg kg^{-1} dw upper

effects threshold (UET) for infaunal community impacts (Buchman 1999). Sediment iron concentrations ranged from 2,380 to 21,100 mg kg^{-1} dw (Figure 10). Although there is no TEL or PEL for ***selenium*** for freshwater sediment, five sites (24%) exceeded the marine sediment apparent effects threshold of 1 mg kg^{-1} dw. One site had ***cadmium*** concentrations above the TEL of 0.596 (0.684 mg kg^{-1} dw). Two sites had ***copper*** concentrations above the lowest observable effects level for the *Hyallela azteca* bioassay, but below the designated TEL for ***copper*** of 36.2 mg kg^{-1} dw (site values were 29.3 and 31.2 mg kg^{-1} dw). Two sites had nickel concentrations above the TEL of 18 mg kg^{-1} dw. No other metals exceeded threshold effects levels for sediment.

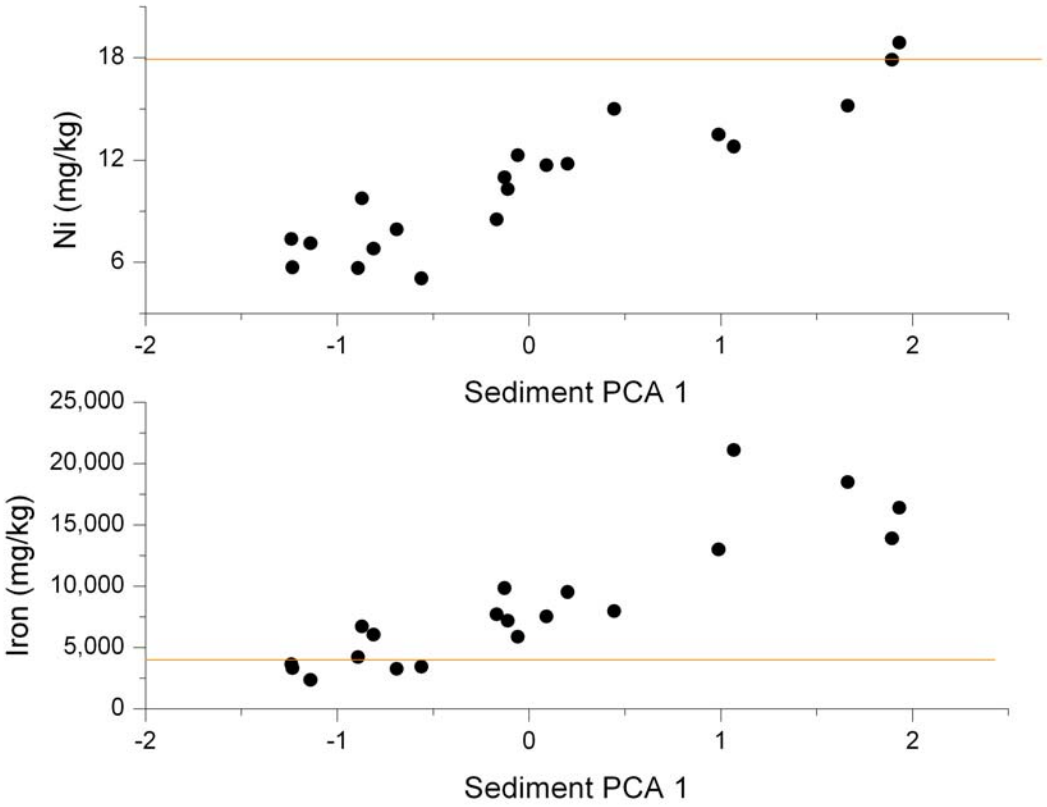


Figure 6. Sediment concentrations of Ni and Fe plotted against PCA vector 1, not a predictor of skeletal abnormalities. Orange lines are TEL for Ni and the UET for Fe

ORGANIC CONTAMINANTS IN WATER

Few organic compounds were detected in site SPMDs, used to measure organic contaminants in water. Napthalenes, phenanthrenes, and indeno(1,2,3-cd)pyrene were the only contaminants found in SPMDs. All napthalenes and phenanthrenes were detected in devices deployed in 2004, napthalenes in six sites at concentrations ranging from 0.012-0.024 (µg $SPMD^{-1}$) and phenanthrenes in the same six sites, from 0.024-0.054 (µg $SPMD^{-1}$). Indeno(1,2,3-cd)pyrene was found in four sites in 2005 at concentrations ranging from 0.014-0.033 (µg $SPMD^{-1}$). These compounds, when detected, were found in both remote and road-accessible sites.

INORGANIC CONTAMINANTS IN WATER

Metals and other elements also exceeded water quality criteria in study sites (Table 6). The criteria used for comparison are the criterion continuous concentrations (CCC), the chronic limit for the priority pollutant in fresh water (Buchman 1999). Only exceedences of this water quality standard, or metals relevant in statistical analyses, are noted here. ***Barium*** was above the freshwater CCC of 4 mg L^{-1} in 86% (18) of study sites, with concentrations ranging from 1 to 52 ppb (Figure 11). Seven (30%) of the 21 sites were above the CCC of 1,000 mg L^{-1} for ***iron***, with values above the limit ranging from 1,100-8070 mg L^{-1}. Five sites (24%) exceeded the CCC of 120 mg L^{-1} for manganese. ***Copper*** was only detected in water at one site, at 11 mg L^{-1}, which is above the CCC of 9 mg L^{-1}. ***Cadmium*** was also above the CCC of 0.25 mg L^{-1} at only one site.

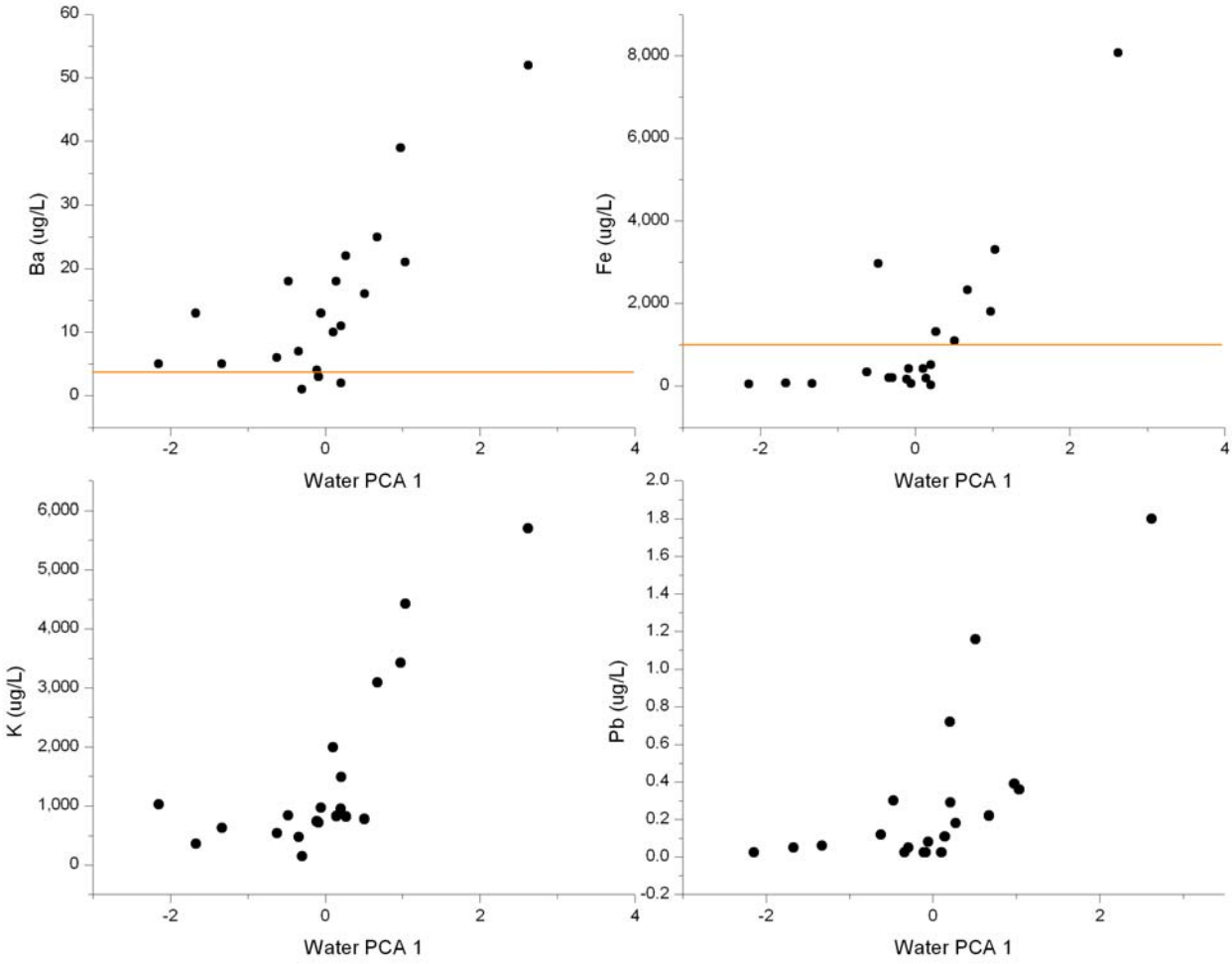


Figure 7. Inorganic contaminants in water plotted against PCA vector used to represent them in statistical analysis. Orange lines are CCCs

The maximum value for ***lead*** in site water was 1.8 mg L^{-1}, and no value exceeded the CCC of 2.5 mg L^{-1}. There is no CCC associated with ***potassium***, also included in the water PCA vector correlated with skeletal abnormalities.

RESULTS – STATISTICAL ASSESSMENT AND CONTROLLED EXPERIMENTS

The goal of this study was to evaluate risk factors for wood frog abnormalities in the Kenai Refuge. We did this by measuring environmental stressors known to cause abnormalities and evaluating correlations between these stressors and the abnormalities we observed in the field. To this end, a unifying statistical analysis and several controlled experiments were performed. The results of both the analysis and the controlled experiments are presented below in sections for each of the four different types of abnormalities: Skeletal abnormalities, eye abnormalities, disease, and intersex.

SKELETAL ABNORMALITIES AND MALFORMATIONS

A combination of metals in water and sediment, average water temperature, and the early season abundance of dragonfly larvae were the best predictors for skeletal abnormalities and malformations. Frog size and frog developmental stage were significant covariates.

We identified significant metals in water and sediment through a regression on the vectors from a principal components analysis (PCA). PCA reduces multidimensional data to vectors that describe similar variation in the data set. For example, PCA vector 1 for sediment explained 46% of the variance in the metals data and was positively correlated ($r \geq 0.6$) with the following elements: Aluminum, Beryllium, Cobalt, Chromium, Iron, Potassium, Magnesium, Manganese, Nickel, Titanium, Vanadium, and Zinc. PCA vector 2 explained 17% of the variance and was positively correlated ($r \geq 0.6$) with Arsenic, Cadmium, Copper, and Selenium (Figure 9). For the water analysis, PCA vector 1 explained 40% of the variance, and was positively correlated ($r \geq 0.6$) with Barium, Iron, Potassium, and Lead (Figure 11). Water PCA vector 2 explained an additional 36% of variation in the data and was correlated with Arsenic, Calcium, Magnesium, and Strontium. These PCA vectors were then used to represent the metals with which they were correlated in the regression analysis.

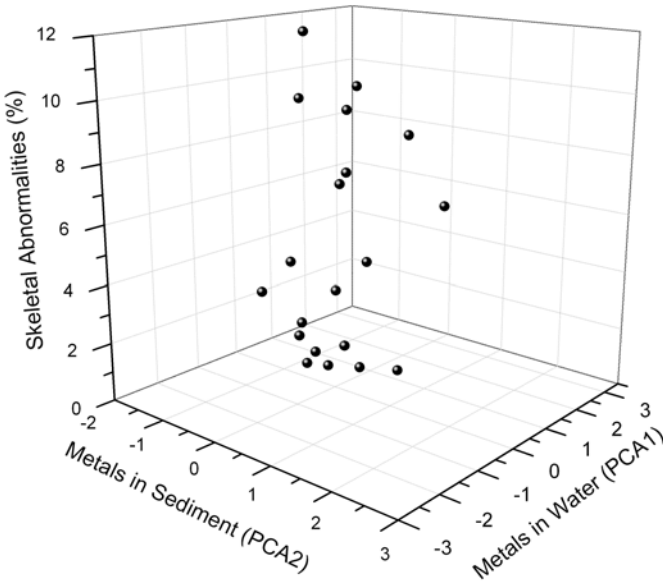


Figure 12. Skeletal abnormalities versus the PCA vectors for metals in sediment (As, Cd, Cu, Se) and water (Ba, Fe, K, Pb)

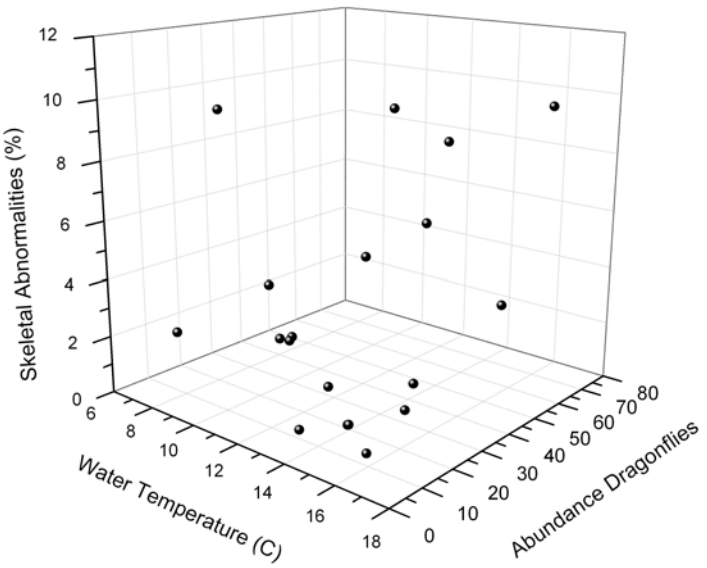


Figure 13. Skeletal abnormalities versus average pond water temperature and early season abundance of dragonfly larvae

Sediment PCA vector 2 (As, Cd, Cu, and Se) and water PCA vector 1 (Ba, Fe, K, Pb) were significantly correlated with skeletal abnormalities in the best-fitting model (Figure 12). For every 1 unit increase in Sediment PCA vector 2 (OR=2.087), the odds of having a skeletal abnormality increased by a factor of 2. An increase in metals in water had a similar effect. For each 1 unit increase in Water PCA vector 1 (OR=1.601), the odds of having a skeletal abnormality increased by a factor of 1.6.

The early season abundance of dragonfly larvae (OR=1.018) and pond temperature (OR=1.283) were also positively related to the risk of skeletal abnormalities (Figure 13). For every additional dragonfly nymph found in early season abundance sweeps, the odds of a frog having a skeletal abnormality increased by a factor of 1.02. For each 1º C increase in temperature, the odds of skeletal abnormality increased by approximately 1.3 times. Frog size and frog developmental stage were also correlated with skeletal abnormalities in this model. Smaller metamorphs (OR=0.871) and later stage metamorphs were more likely to be abnormal (OR=1.222).

Skeletal malformations followed a similar pattern (Figure 14), and the best predictive model for skeletal malformations included metals in sediment (OR=1.948), but not in water; early season larval dragonfly

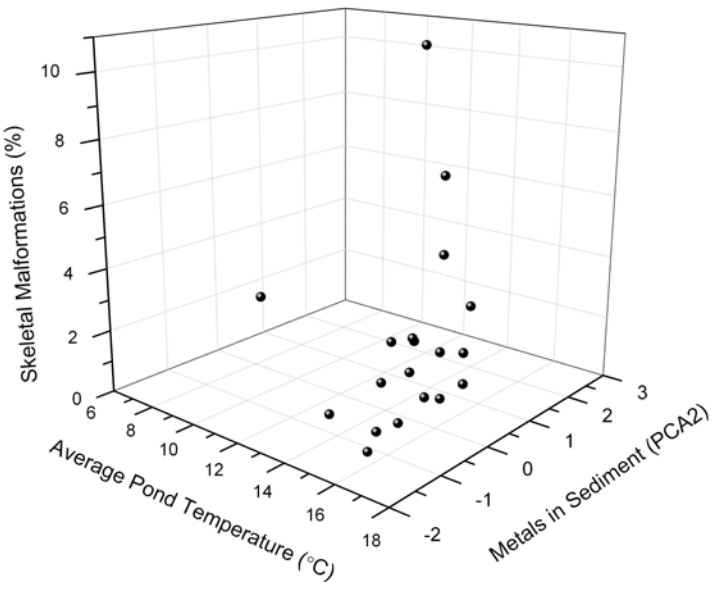


Figure 14. Skeletal malformations versus average pond water temperature and the PCA vector for metals in sediment (As, Cd, Cu, Se)

abundance (OR=1.016), average temperature (OR=1.227), frog size, and frog developmental stage.

We assessed model fit with Akaike's Information Criterion (AIC). When interpreting AIC, all models are compared to the best fitting model, which by definition has the lowest AIC. The rule of thumb for interpreting the differences among models is that a difference of 2 suggests substantial evidence for the better model, a difference between 3 and 7 suggests the worse model has considerably less support, and a difference greater than 10 indicates the worse model is substantially less likely (Burnam and Anderson 2002). The difference in AIC for the skeletal abnormality model that included only dragonfly larvae and the (size and stage) covariates was 26 units. The model that only included metals and covariates was 38 units, and the model that only included temperature and covariates was 36 units. ***This comparative analysis indicates that all three factors (metals, dragonflies, and temperature), produced a substantially better model than any of these factors alone. This is strong evidence that metals, dragonflies, and temperature are somehow working together to produce the skeletal abnormalities and malformations in Kenai Refuge wood frogs.***

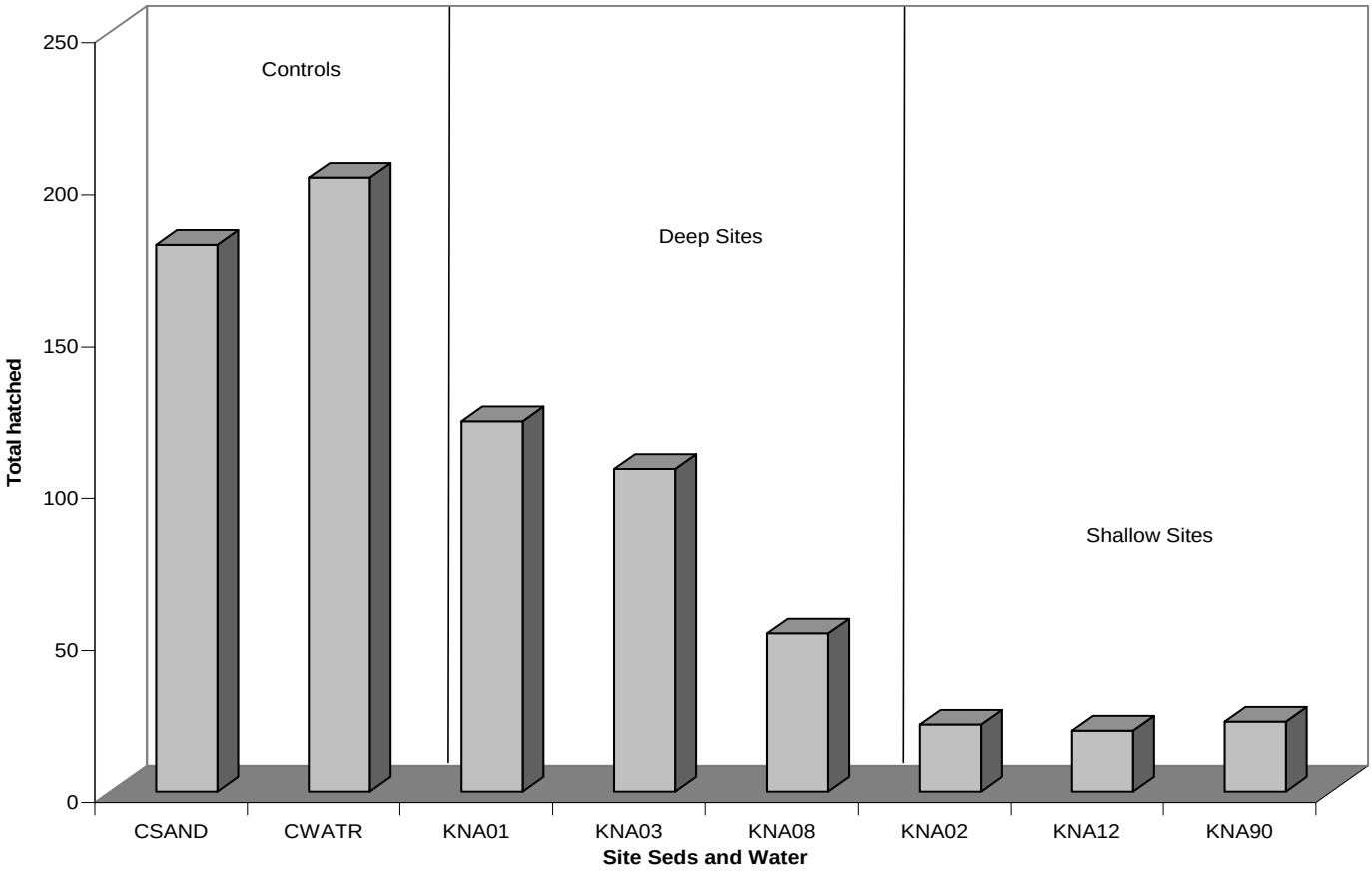


Figure 15. Differences in hatching success for controlled sediment and water toxicity experiment

Evidence from several controlled experiments provides further insight into the risk factors for frog abnormalities in natural settings. Most of the abnormalities we observed in the wild were asymmetrical abnormalities of the hind limbs, yet in all five of the controlled experiments done to assess toxicity of site water and sediment, we never observed an asymmetrical limb abnormality. Collaborators at USGS performed several experiments to assess toxicity and teratogenicity of site sediment and water, with and without exposure to UVB radiation, and consistently found toxicity, but not teratogenicity, due to exposure to abiotic site media (Bridges and Little 2002, Little et al. 2008; Appendix C) Toxic effects included differences in size at metamorphosis and time to metamorphosis attributable to water extracts and site sediment. We also performed a controlled experiment in Kenai to assess the toxicity and teratogenicity of site sediment and water, and found results similar to Bridges and Little (2002) and Little et al. (2008). In the USFWS experiment, we tested the effects of site sediment and water on hatching success, size at metamorphosis, and time to metamorphosis. We found significant differences in hatching success ($p<0.0001$; Figure 15), size at metamorphosis ($p=0.0052$), and time to metamorphosis ($p<0.0001$) at different sites and relative to controls. Eggs and tadpoles exposed to sediment and water from study sites suffered greater mortality, took longer to develop, and were smaller at metamorphosis than control frogs. Finally, we performed an experiment to assess the heritability of the asymmetrical limb abnormalities. Not one of the 261 tadpoles (from six parent pairs) that survived until the end of the experiment had an asymmetrical limb abnormality, providing evidence that these types of abnormalities are not heritable. ***In summary, the results of statistical modeling and controlled experiments together suggest that although abiotic conditions appear to play a significant role in the production of skeletal abnormalities, they seem unable to produce them independently in Kenai Refuge study sites.***

The significance of larval dragonfly abundance suggests that limb amputation from failed predation attempts is probably the ultimate cause of the asymmetrical abnormalities we observe in Kenai. Evidence for this statement comes from the correlation analysis we just completed, as well as the analysis done by the invertebrate collaborator on the project (Jensen 2006 and 2007; Appendix A). In the predator exclusion studies documented in these reports, no tadpoles manifested skeletal abnormalities, even though cages were placed in sites where we repeatedly measured high abnormality prevalence.

Factors without much explanatory power for skeletal abnormalities and malformations were water beetle abundance, UVB radiation, and organic contaminants (PCBs and other organochlorines, aliphatic compounds, and PAHs). Parasites are also an unlikely cause of the skeletal abnormalities because so few parasites were detected in Kenai frogs, and the abnormality-inducing parasite, *R. ondatrae,* was not found in any frog sampled. Damage to DNA, as measured by several biomarkers, was also not consistently correlated with any frog abnormalities, but here statistical power was limited due to relatively low sample number (Jenkins 2008; Appendix B). With the possible exception of DNA damage, there is no evidence that any of these factors are causing skeletal abnormalities in Kenai Refuge wood frogs.

EYE ABNORMALITIES

A subset of water beetle larvae provided the best model fit with eye abnormalities. In this model, we found positive associations between unpigmented irises and the early season abundance of *Graphoderus* spp. beetles (OR=1.109), and the combined early season abundance of *Rhantus* and *Ilbyius* beetles (OR=1.003). In this model we also found a negative association between the late season abundance of *Dytiscus* spp. and *Acilius* spp. beetles (OR=0.7795). This model should be interpreted with caution, however, because there is also a correlation ($r \geq 0.7$) between *Graphoderus* spp. and distance to the nearest road in our data; the abundance of these beetles increases in sites farther from roads.

The next best model, with an AIC value 3 points higher (indicating worse model fit), included a combination of water beetle abundance, contaminants, and water quality. This model included positive associations between the eye abnormalities and the beetle abundances, the average pH, metals in water (PCA vector 1), and total organochlorines (OCs). Again, this model should be interpreted cautiously, because the correlation with beetle larvae might also suggest a correlation with distance from the breeding site to roads.

The next best model, with an AIC value 5 points higher than the beetle model, included only metals in water (PCA vector 1), total OCs, and average pH. All three of these factors were positively associated with the eye abnormalities. For every 1 unit increase in metals in water (PCA vector 1; OR=1.448), the odds of having an unpigmented iris increased nearly 1.5 times. For every 1 unit increase in the total organochlorine concentration in site sediment (mg kg^{-1} dw), the odds of a frog having an unpigmented iris increased by about 25% (OR=1.243), and for every one-unit increase in average site pH, the frog's odds of having an unpigmented iris nearly doubled (OR=1.915).

Models with less explanatory power for unpigmented irises included site temperature, water quality, dragonflies, metals, and UVB. The temperature models had AIC differences of 11 (for the range in site temperature) and 14 (for the average site temperature), from the beetle-only model. The water quality model (pH, TDS, DO, SpC) was 13 AIC units higher. A metal model, which included all four PCA vectors for water and sediment metals, was 19 units higher and had an AIC value identical to a model with no predictors – indicating this model had no explanatory power. The dragonfly model had an AIC just 1 unit "better" than the metals model, again indicating a poor fit with the data. Finally, the UVB model had a worse fit with the data than a model that included no predictors, suggesting it is very unlikely UVB is causing these eye abnormalities.

Experimental data yielded little insight into these eye abnormalities. We observed three animals with this abnormality in the USFWS sediment and water toxicity experiment. Two of these had unpigmented left eyes and one had both eyes lacking pigmentation. Two were exposed to sediments from the same site, and perhaps more interesting, they were all siblings from the same parent pair. But, due to the low number observed, it is impossible to say whether either of these factors was important in causing the eye abnormality. We also observed a high instance of this abnormality during the heritable abnormality study, but it never occurred in any of the treatment

animals. We had "leftover" tadpoles and kept them in a separate tub of water during the experiment. These were kept at much higher density and with much less regard to water quality in their tank than the treatment animals were. Approximately 30% of these "extra" tadpoles showed reduced pigmentation in both eyes – none of them had a single-eye with reduced pigment, which is what we more often see in the field. Also, because we pooled sibling groups in the "extra" frogs, it is impossible to attribute this abnormality to different parents.

DISEASE

An AIC-based regression analysis was run to determine factors predictive of disease occurrence, because the protozoan organism had symptoms readily diagnosed in the field. The factors included in the best fit model were temperature, pH, metals in water and sediment, and total dissolved solids. Gosner stage was a significant covariate in all models, where fewer diseased frogs were found at later developmental stages, suggesting diseased frogs die as they go though metamorphosis. This result is consistent with field observations, and diseased animals were so weak they often died awaiting abnormality assessment. Higher temperature and more acid conditions increased the risk of this disease (Figures 16 and 17). Notably, the disease was not detected in any sites with average pH above 6.5. Also notable, the site with the highest observed incidence of disease had the lowest average pH of all study sites, 5.1 units. This site is not presented on

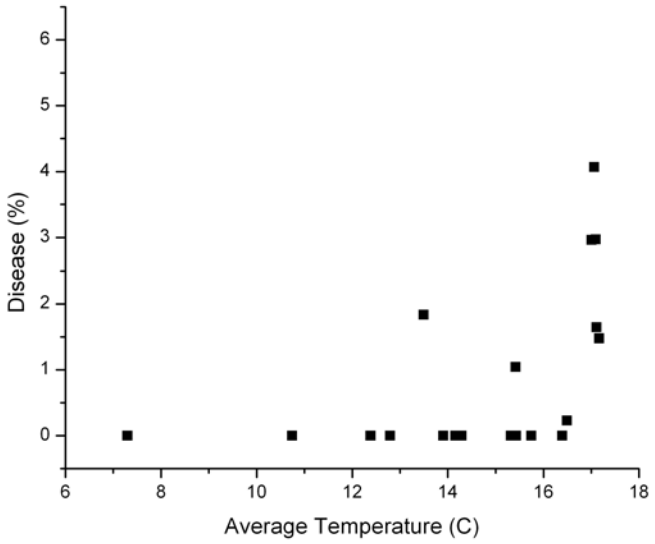


Figure 9. Prevalence of the Perkinsus-like organism in metamorphic wood frogs assessed for abnormalities versus average pond temperature

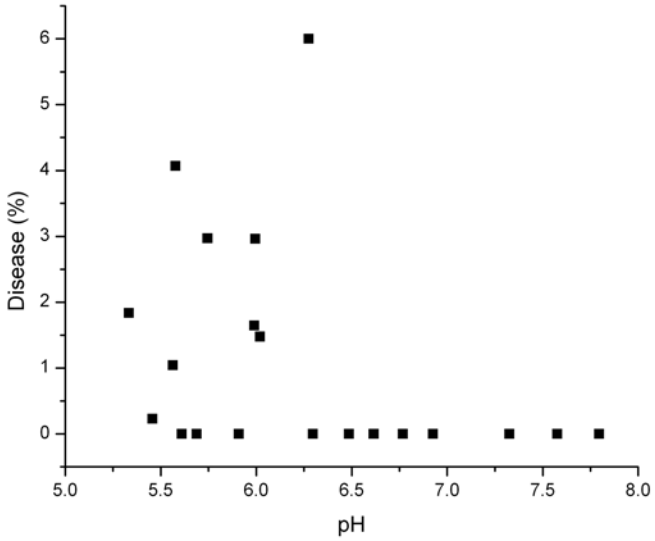


Figure 8. Prevalence of the Perkinsus -like organism in metamorphic wood frogs assessed for abnormalities versus average pond pH

Figure 17 because so many tadpoles died before metamorphosis that we could never catch enough to assess disease prevalence at metamorphosis. Higher TDS and more central values of water PCA vector 1, which is not the vector associated with skeletal abnormalities, seemed to bear some relationship to the disease (Figure 18) , yet the nature of this relationship is unclear. There were nonlinearities in the data, which make the model difficult to interpret because the odds ratios become unreasonable when nonlinear data are fit with a model that assumes linear relationships. Nevertheless, the combination of these factors provided a much better explanatory fit to the data than did any of these factors alone. If TDS is removed from the best model, the AIC increased by 13 points, and if just the metals are removed, AIC increased by 11. This disease clearly warrants further research and the field correlations we have identified in this analysis may be help us understand risk factors in Kenai frog populations. High temperature and low pH were associated with increased disease incidence, and the temperature effects may become particularly important as Alaska's temperatures warm with a changing global climate.

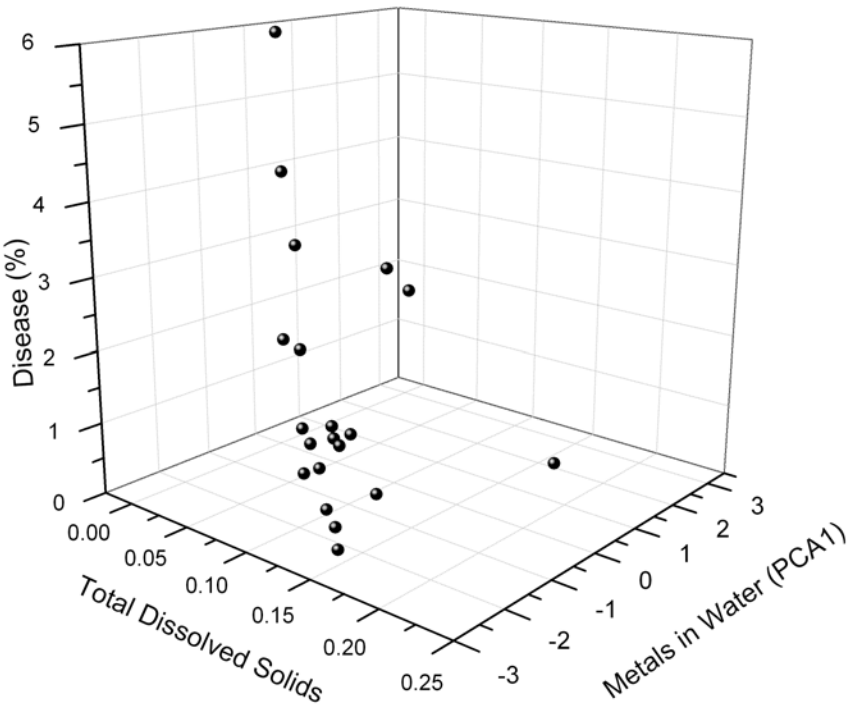


Figure 18. Prevalence of the *Perkinsus* -like organism in metamorphic wood frogs assessed for abnormalities versus average pond TDS and the PCA vector found to be associated with a decreased incidence of the disease (Al, Be, Co, Cr, Fe, K, Mg, Mn, Ni, Ti, Vd, and

INTERSEX

A regression analysis was also done on the intersex frogs to determine factors predictive of this condition. Nothing we measured in the field gave us any explanatory power for intersex. We tested size, Gosner stage, temperature (range and average), metals, water quality, organochlorines, and hydrocarbons, and none of these provided models with lower ("better") AIC than the base model, which included no predictive factors. This is an interesting result, but it may be that with only 163 frogs examined for intersex, we did not have enough statistical power to assess trends with environmental factors.

The controlled experiment yielded an interesting insight because 39 out of 44 frogs that survived to metamorphosis were male, and the remaining five were intersex. This condition was not associated with site sediment and water because it occurred in all treatments. The production of male frogs is probably the result of high rearing temperature, as has been demonstrated in earlier research (Witschi 1929), but the temperatures in our experiment ranged from 13.7 ºC to a maximum of 22.7 ºC, much lower than previously known to control sex determination in wood frogs (32 ±2 ºC; Witschi 1929).

DISCUSSION

Skeletal Abnormalities and Malformations

The goal of this study was to identify specific stressors associated with amphibian abnormalities and assess whether these were associated with human disturbance. We have identified dragonflies, some toxic metals, and higher temperatures for the skeletal abnormalities and malformations, and frog size and developmental stage as covariates.

The strong positive association between larval dragonfly abundance and skeletal abnormalities is satisfying because it provides a biological model for the ultimate cause of asymmetrical limb abnormalities, both deformities and malformations, in Kenai Refuge wood frogs. The available literature on limb regeneration after early injury suggests that injuries can cause developmental malformations, like micromelia, but timing is key. Tadpoles have the ability to regenerate limbs amputated early in development, but this ability is lost as tadpoles progress toward metamorphosis (Forsyth 1946; Fry 1966; Kurabuchi and Inoue 1982). The location of the amputation along the developing limb bud also determines the developmental outcome of the healed limb. Fry (1966) found that if leopard frog (*R. pipiens*) limbs were amputated at an early developmental stage (prior to patterning of the joints and toes, when the developing limb was just a tiny stump), then these limbs regenerated into a fully-patterned limb, reduced in size (Figure 19). Yet, if that amputation occurred later in development, when joints and toes had already developed a pattern, the limb would heal into a stump lacking joints, toes, or other limb-like features (Fry 1966). Wood frogs similarly amputated showed less regenerative ability than leopard frogs, but tadpoles in this study were injured after patterning had already occurred, even in the earliest-stage treatments (Forsyth 1946). Another study of four species of Japanese frogs produced similar results: regenerative ability was present in all species, but varied among species and decreased with increasing age at amputation (Kurabuchi and Inoue 1982). Adult frogs in this study lost all regenerative ability for amputated limbs (Kurabuchi and Inoue 1982). More recent research proposes that tadpoles lose regenerative ability as they age because limb regeneration is incompatible with the developing immune system (Mescher and Neff 2005). These authors and others propose that the development of cellular and humoral immunity and consequent fibrosis and scarring at the wound site, which happens only in later-stage tadpoles, inhibits blastema formation, which is essential for limb regeneration (Harty et al. 2003).

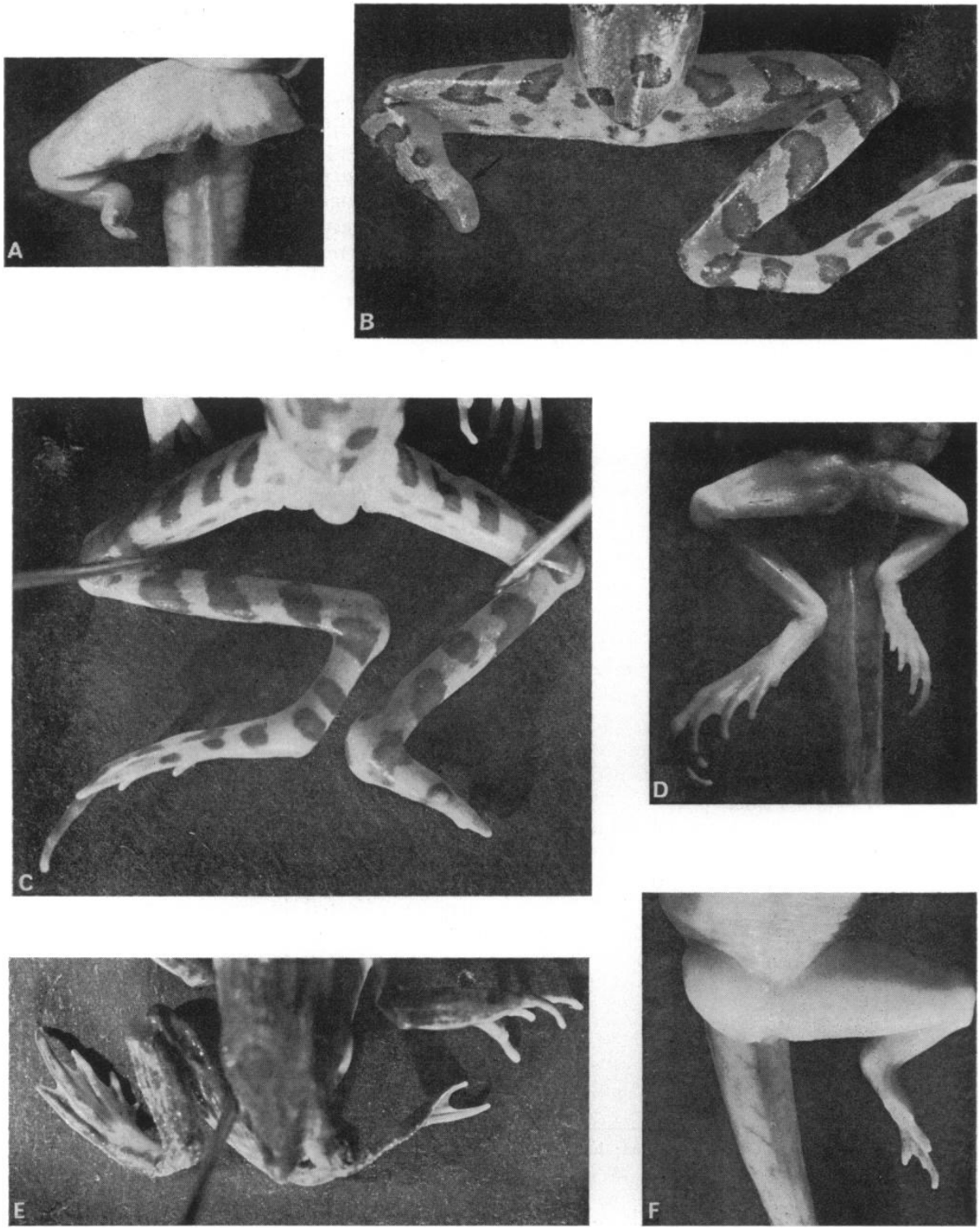


Figure 19. (Figure 1. from Fry 1966 with original caption) Regeneration of hind limb after amputation in Rana pipiens. A, Ventral view. At stage XI11 right hind leg amputated at shank. Here, stage XX, note abbreviated 4-toed limb. Left leg, amputated at thigh at stage XIII, is typical of nonregenerating limbs amputated at this level. B. Dorsal view. Left leg amputated at shank (arrow), stage XV. Accumulation blastema apparent 5 weeks later. No operation performed on right limb. C, Dorsal view. Amputation through foot was performed at stage XXII. Two months after operation, a regenerate with evidence of some toe formation is apparent. Left limb is unoperated control. D, Ventral view. Left leg amputated at thigh; stage VII. At stage XX well-developed, although somewhat abbreviated. 5-toed regenerate.

Taken together, these studies suggest that amputation injuries early in tadpole development can cause abnormalities like those seen in metamorphic Alaskan wood frogs. Yet, predation injury alone cannot explain the pattern in Reeves et al.'s (2008) data, where proximity to roads increases the risk of skeletal abnormalities, or the significance of metals and temperature in this study. The significant road effect implies that anthropogenic stressors influence predation risk: Below we explore how this might occur.

The significant effects of metals and temperature suggest something has been thrown out of balance in these ecosystems, and this mechanism could be ecological or physiological. The associations with metals in sediment (As, Cd, Cu, Se) and water (Fe, K, Pb, and Ba) are intriguing because many of these metals are above established toxic thresholds for aquatic organisms in Kenai study sites. Of the eight metals highlighted by statistical analyses as significant predictors of skeletal abnormalities, six of them (As, Cd, Cu, Ba, Fe, and Se) exceeded established criteria in at least one study site, and half of these (As, Ba, and Fe) are above threshold levels in most study sites. Evidence from controlled experiments with Kenai Refuge site sediment and water suggest toxicity to developing wood frogs from abiotic site media. It is the nature of that toxicity, and its effects on the predator-prey relationships in these systems, which deserve further attention.

Chemical contaminants or physicochemical habitat alterations (e.g. changes in dissolved oxygen or acidity) can affect both invertebrates and tadpoles, so the outcome of these stressors on predator-prey relations may be difficult to predict. For example, in a study examining low dissolved oxygen effects on salamander (*Ambystoma tigrinum*) predation on bullfrog (*R. catesbeiana*) tadpoles, both predator and prey behaved differently depending on the oxygen concentration of the water (McIntyre and McCollum 2000). Predation on tadpoles was less at low oxygen concentrations because salamanders stayed near the water surface and tadpoles stayed close to the bottom of the tanks, presumably because of physiological differences in oxygen requirement. At high oxygen concentrations, the two intermingled, causing greater predation on tadpole larvae. Another study examining predator-prey relationships in microcosms treated with nitrogen fertilizer and an insecticide had similarly complex results (Boone et al. 2007). The insecticide was more harmful to the salamander predator (*A. maculatum*) than it was to the anuran tadpole prey (*Bufo americanus* and *R. sphenocephala*), and therefore increased numbers of tadpoles by releasing them from predatory stress. Likewise, fertilizer treatment increased primary productivity (and thus food resources) for the tadpoles, increasing the survival and mass of all tadpoles in this treatment (Boone et al. 2007). In a compelling study on copper neurotoxicity to salmon, as little as 2 ppb copper in water inhibited the salmon's ability to detect predators, leading to higher predation on animals in treated water (Sandahl et al. 2007). Copper in this study inhibited salmon olfaction so that smolts in metal-enriched water did not detect conspecific alarm pheromones and altered their normal predator-avoidance behavior. Treated smolts were therefore attacked at significantly greater rates than controls. This final study provides one mechanism by which tadpoles in metal-rich Kenai sites may be worse at avoiding predators than tadpoles in other areas of Alaska (Reeves et al. 2008).

In other studies, contaminants have been shown to reduce tadpole fitness or size at metamorphosis, increasing tadpole vulnerability to predation (Boone and Semlitsch 2001, 2002; Boone and James 2003; Bridges 2000). In the Kenai study, smaller frogs were more likely to have skeletal abnormalities and malformations. It could be that toxicity from metals in Kenai site sediment and water slows their development and increases the amount of time during which they are vulnerable to gape-limited invertebrate predators, such as dragonflies (Brodie and Formanowicz 1983).

Nevertheless, size is a complicated variable because it interacts with both tadpole behavior and larval period. Larval amphibians experience tradeoffs between the length of larval period and size at metamorphosis: a shorter larval period can result in smaller size at metamorphosis. At least one study suggests predators control larval period length (Vonesh and Warkentin 2006). These authors found smaller tadpoles that metamorphosed earlier when exposed to chemical cues of an aquatic predator, and larger tadpoles that metamorphosed later when exposed to cues from a terrestrial predator. For tadpoles, increased foraging often leads to large size – a behavioral strategy that has a payoff of faster growth but carries greater risk of predation (Monello et al. 2006). In simple systems, foraging increases predation risk because of increased predator encounter, and hiding reduces it (Tejedo 1993; McIntyre and McCollum 2000). Yet, more foraging can also increase size in phenotypically plastic organisms like wood frog tadpoles (Relyea 2002), and large size decreases predation risk by gape-limited predators (Brodie and Formanowicz 1983). Further, although it is tempting to generalize that changes in foraging behavior alone cause changes in size, that would be an oversimplification. Abiotic factors such as chemical contaminants (Relyea 2005), pH (Rosenberg and Pierce 1995), and chemical cues from caged predators (Relyea 2001) can all reduce size in metamorphic amphibians. The nature of the interaction between abiotic (temperature, metals, and pH) and biotic factors (invertebrates, plant cover, and tadpoles) is a ripe area for future research.

Warmer temperatures can differentially increase the growth of either predator or prey, leading to a differential competitive advantage. For example, in one study higher temperatures led to faster growth of *Hyla (psuedacris) regilla* tadpoles, enabling them to escape size-specific predation by newt *(Notonecta kirbyi)* predators faster than tadpoles raised at lower temperature (Anderson et al. 2001). This benefit was differential, however, because warmer temperatures also favored the predator: for tadpoles of a given mass, the probability of capture was higher at warmer temperatures. By an unknown mechanism worthy of future consideration, the positive association between abnormalities and temperature in the Kenai Refuge study system suggests warmer temperature may somehow favor invertebrate predators. Warmer temperatures may increase the abundance of dragonfly larvae or change the timing of dragonfly presence or development relative to tadpole growth. The temperature effect found in this study may be particularly significant in the face of global climate change.

The increased prevalence of skeletal abnormalities at later developmental stages is probably sampling bias created by different capture techniques. Whereas dip-netting for earlier stage metamorphs (Gosner 42-44) samples abnormal and normal individuals with comparable efficiency, capturing later stage metamorphs on land may result in the disproportionate collection

of the less-mobile abnormal animals. Moreover, normal metamorphs leave the breeding area quickly, but frogs with skeletal abnormalities may stay closer to water, where they can dive from predators instead of relying on missing or misshapen limbs to escape.

Eye Abnormalities

It is difficult to say what is causing the eye abnormalities, or to determine whether the lack of pigmentation in one or both eyes is an abnormality at all. Histological examination of frogs with unpigmented irises suggested that the iris was present and only missing the normal golden pigmentation (Sutherland, D.R., personal communication). The best correlations with this condition in field situations were with some species of water beetles. A competing model, with slightly worse explanatory power, included only metals in water, pH, and organochlorine concentration in sediment. We did not find a significant association between distance to the road and eye abnormalities in the Alaska-wide data set (Reeves et al. 2008). The only significant predictor for this type of abnormalities in five Alaskan refuges was year sampled; the abnormalities were more prevalent during warmer years (Reeves et al. 2008). Other authors have described this condition as caused by a recessive genetic mutation (Nishioka 1977) or as associated with early season temperature extremes or urban contamination (Vershinin 2002). In the multi-refuge study, we observed this abnormality at all refuges except the Arctic, which was surveyed at a time when protocols did not specify the unpigmented iris as an abnormality. Evidence from controlled experiments suggests only that abiotic factors, especially those leading to high density or poor water quality, or genetic predisposition may drive the occurrence of unpigmented irises in wild populations. More study is needed to determine what causes this abnormality and whether this condition adversely affects the frog.

Disease

We found higher temperatures and more acid conditions to be positively associated with a newly-discovered disease of conservation concern. Warmer temperatures can lead to differential pathogen success relative to hosts, as has been shown with spruce bark beetles on the Kenai Refuge (Berg et al. 2006). In this study, warmer summers increased development rates of the pathogenic beetles from two years to one, leading to fast increases in beetle populations. This increase in pathogen population combined with drought stress in the spruce tree hosts (*Picea* spp.) led to decimation of mature spruce forests in the refuge in the 1990s (Berg et al. 2006). Warming can also change pathogen ranges or increase pathogen virulence within a location by creating more favorable conditions in areas previously inhospitable for temperature or moisture reasons. This has been found to be the case with the frog pathogen Bd in Central America, where climate shifts have led to conditions more favorable for disease spread into montane regions (Pounds et al. 2006). Another pertinent example of temperature-induced pathogen range expansions is the documented spread of *Perkinsus marinus* from Long Island to Maine in warmer conditions. *P. marinus* has been found to be associated with ENSO in the Gulf of Mexico. Incidence was low in cold, wet El Nino years and high during hotter and drier La Nina years. The *P. marinus* example is especially relevant because preliminary genetic analyses of the disease we documented in Kenai wood frogs showed this protozoan organism to be closely related to both *P. marinus* and the fungal pathogen of fish, *Ichthyophonus*, perhaps creating a genetic bridge between these two organisms (Green, D., Personal communication). The

interactions between temperature and this new amphibian disease deserve additional study, especially because of impending climate change.

Acid conditions may stress wood frog tadpoles physiologically, leading to lower disease resistance. Gosner and Black (1957) found lower hatch rates for wood frogs in more acid conditions, and Rosenberg and Pierce (1995) found frogs reared in acid conditions were smaller. Both studies suggest acid conditions are stressful to wood frog tadpoles. It may also be that acid conditions somehow favor this pathogen, assisting its reproduction and survival, but so little is known about this disease there is no research on its life cycle. We also found associations with TDS and metals in water. These associations should help target future study of this disease.

INTERSEX

We used histology to compare gonad structure in frogs from ponds near roads and development to those inhabiting remote ponds. Our field results, a male-biased sex ratio in metamorphs and a high prevalence of intersex frogs, suggest endocrine disruption in wood frogs from Kenai, in both apparently normal and visually abnormal animals from developed and remote sites. Nothing we measured was a significant predictor of intersex gonads, but we probably did not have a high enough sample number to assess environmental correlates with this abnormality type. Both normal and abnormal animals, as determined by visual examination of external characteristics, had intersex gonads.

Literature suggests wood frogs should have a 1:1 sex ratio at metamorphosis and should not have intersex gonads (Witschi 1929; Hayes 1998), yet high temperatures have been shown to cause wood frogs to turn into males (Witschi 1929). Witschi (1929) reared frogs near the limits of their physiological tolerance (32±2 ºC), which resulted in genetic females developing testes. The temperatures in the sediment toxicity study that produced only males and intersex frogs were set to mimic surface temperatures observed in the field, and did not exceed 23 ºC. It may be that Alaskan wood frogs are adjusted to lower water temperatures, and therefore are sensitive to temperature-induced masculinization, and this is an interesting area for future research. The most prevalent abnormality reported in the literature for PCBs and pesticides is feminization of male frogs (Hayes *et al*. 2002; Tavera-Mendoza *et al*. 2001; Qin *et al*. 2003) therefore PCBs are another potential cause of this abnormality. Because both high temperatures and PCBs have been shown to cause endocrine disruption in amphibians, these are two initial hypotheses for the intersex condition in Kenai wood frogs. More study is needed on the phenomenon of intersex wood frogs in Kenai.

HUMAN DISTURBANCE

It remains to be seen whether Kenai frog abnormalities are related to human disturbance. Toxic metals in study sites may be present because of geologic parent material, or elevated due to anthropogenic factors like deposition of global pollutants or local contamination from roads or resource extraction. An alternative hypothesis is that naturally present but normally immobile metals migrate into wetlands because of biogeochemical reactions following landscape disturbance. This study was not designed to evaluate the origin of earth elements in site sediment

and water; these may be naturally occurring or artificially elevated. The origin of metals in Kenai study sites deserves further consideration.

It is probably indisputable at this point that global temperatures are warming because of human input of greenhouse gasses to the atmosphere. This is so much the case that geologists have proposed the current epoch be named the "Anthropocene" (Zalasiewicz et al. 2008). If higher temperatures play a causative role in the frog abnormalities we have observed, by shifting predator-prey dynamics in these systems or by another mechanism, then we may also argue the abnormalities have been caused by human disturbance. Again, this study was not designed to make this kind of a connection.

We have identified a correlation between frog abnormalities and roads in a previous study (Reeves et al. 2008), which may or may not be spurious, based upon locations we chose to collect data. There was a significant association between proximity to roads and prevalence of abnormalities in the Kenai Refuge data set as well, but this also may have more to do with site selection, constrained necessarily by location of available habitat and logistics.

Dragonflies are a naturally-occurring biotic factor, and we propose that the relationship between dragonfly predators and their wood frog prey has been thrown out of balance by abiotic factors, such as elevated metal concentrations and changing temperatures. Additionally, our preliminary results on disease suggest that abiotic factors like temperature and acidity are correlated with problematic amphibian diseases we observed in this study.

Unfortunately, more research is needed before we completely understand the causative mechanisms for wood frog abnormalities in this study. When we do understand the mechanisms, we will know whether anthropogenic stressors are an important causative factor.

CONCLUSIONS

Skeletal abnormalities and malformations were positively related to temperature, metals, and the early season abundance of dragonfly larvae. High temperatures, some toxic metals in sediment (As, Cd, Cu, and Se) and water (Ba, Fe, K, and Pb), and dragonflies all increased the probability of skeletal abnormalities. Most metals identified by the statistical analysis were above water quality and sediment toxicity threshold concentrations, at least in some sites. In controlled experiments we found toxicity but not teratogenicity due to abiotic factors in site sediment and water. These resulted in significant differences in hatching success, size, and time to metamorphosis, yet we never found asymmetrical limb abnormalities in controlled experiments, implying another stressor is needed to produce this type of abnormality. The results of our analyses suggest this stressor is dragonfly larvae. The most plausible hypotheses is that abiotic factors (contaminants and higher than normal temperatures) have disrupted either the ecological system or the frogs' biological systems to either favor increased predation or alter limb regeneration after early injury.

The causes of the eye abnormalities remain elusive. Our study results show somewhat equivocal correlations between certain species of water beetle larvae or, alternatively, abiotic conditions such as pH and chemical contaminants. Experimental results, though scant for this abnormality type, suggest this condition may be related to stressful rearing conditions or genetics. More research is needed to determine whether an unpigmented iris is detrimental to the frog or not.

High temperatures and acid conditions were correlated with an increased risk of infection by the *Perkinsus*-like protozoan organism, first identified in this study. The best predictive models also included TDS, which exacerbated the condition, and some inorganic elements, which alleviated it. More effort should be spent to understand this new disease of conservation concern, especially as it relates to imminent changes in the Alaska climate.

We also found a spatial association between Bd, another disease of conservation concern, and a recreational access corridor, the Swanson River Canoe Route. More research is needed to understand the spread of this disease as it relates to recreational land use.

We found a high proportion of intersex frogs, but no significant correlations with factors measured in the field, probably because the relatively low sample size inhibited our ability to detect correlations where present. When tadpoles were reared experimentally at high and consistent temperatures, most of them were male, which suggests that higher than normal temperatures for this region during our study may be playing a role in gonadal abnormalities. Because PCB concentrations in Kenai study sites were high, and PCBs are an established cause of endocrine disruption, PCBs are also worthy of evaluation as cause for intersex gonads in Kenai Refuge wood frogs.

PCB levels were higher than expected and fairly consistent across Kenai Refuge sites. PCBs warrant further investigation because PCB concentrations were high and uniform and because of the history of environmental contamination with PCBs on the Refuge. Preliminary analysis suggests the congener profile of the PCBs we found in study sites matches the profile of PCBs spilled in the 1970s and 1980s, yet more analysis is needed in this area. The one site sampled during a forest fire had total PCB (and other OC) concentrations orders of magnitude greater than all other study sites. PCBs deposited into the Kenai NWR system in earlier years may be mobilized by fire, and may remain a problem.

MANAGEMENT RECOMMENDATIONS AND FUTURE DIRECTIONS

Determine the nature of correlations between metals, temperature, frog size, and predation leading to skeletal abnormalities – This requires a combination of a more detailed statistical analysis of this data set and controlled experimentation.

Determine the source of elevated metal concentrations in study site sediment and water – Is this driven by regional geology, or global transport of pollutants from Eastern Europe and China, or is it because of local inputs or driven by biogeochemical transformations of disturbed habitat?

Evaluate the effect of road disturbance on hydrology and metals solubility – The high metal concentrations may be a result of regional geology, or may result from landscape disturbances (like roads), which may mobilize contaminants (like iron) into water. This relationship warrants further research and consideration. Can land management change metal availability in Kenai, perhaps limiting the incidence of frog malformations in the future?

Further investigate the main eye abnormality, the unpigmented iris – Is this condition detrimental to frogs? Can they see? Is it even an abnormality? If so, what causes it?

Evaluate the role of temperature, pH, and contaminants on the Perkinsus-like protozoan disease organism found in this study – Are these correlations causative? If so, will projected higher temperatures increase the virulence or range of this disease?

Evaluate the role of recreational users in spreading Bd – Reeves (2008) noted Bd only in the Kenai Refuge and only along a recreational access corridor (i.e. The Swanson River Canoe Route), and the roads leading to it. We did not find the disease on the oil fields, or in remote areas of the Tetlin or Innoko Refuges. Are recreational users spreading Bd to Alaskan wood frogs? More study is needed.

Consider implementing protocols for recreational users to prevent introduction of diseases like Bd and other exotic or invasive species – If recreational users are spreading wildlife diseases, can we implement policies to reduce disease spread, such as cleaning gear and public education.

Conduct a formal analysis of PCB congeners to determine whether PCBs in site sediment are a result of earlier environmental contamination and remediation – The analysis of PCB congeners discussed in this study was not formal, rather it was the chemist looking at the data. A formal comparison of the congener-specific results would determine more conclusively whether the PCB signature matches the PCB Arochlors remediated by onsite incineration in the early 1980s. If contamination on the refuge is widespread, and there are higher trophic level effects, a source of the contamination can be tied to a responsible party.

Assess the effects of wildfire on PCB fate and transport – Evaluate whether fires are mobilizing PCBs previously released into the Kenai Refuge ecosystem. Is there is bioaccumulation in, or negative effects on, top predators in Kenai.

Determine the cause of intersex wood frogs – This will require controlled experimentation. We suggest that either high temperatures or PCBs may be disrupting wood frog gonadal morphology, which could negatively affect reproductive fitness in these populations.

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Appendix A - Invertebrate Study Reports

Predatory Invertebrate Sampling
In Kenai National Wildlife Refuge, 2005

By **Peter D. Jensen**
William E. Walton

Prepared for:

US Fish and Wildlife Service, Region 7
Environmental Contaminants Division

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Acknowledgements

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Executive Summary

A nationwide survey is underway to monitor wood frog populations in an effort to gauge ecosystem health. As part of this survey, higher (5.5-19%) than average rates (0-2%) of abnormal frogs were discovered in Alaska's Kenai National Wildlife Refuge (NWR). To determine the cause of the high abnormality rates, an investigation was undertaken to examine several factors that may contribute to the observed abnormalities. One of these factors is the occurrence and density of predatory invertebrates.

In this investigation, we surveyed predatory invertebrates with an aquatic sweep net at 21 pond sites and deployed predator exclusion cages in 3 of the pond sites where invertebrate predators were surveyed. For the survey, three aquatic net sweeps covering 10 meters each were performed for a total of 1.1 m^3 or approximately 2120 L of water sampled at each site during each sampling period. Sweeps were performed once in each pond between the dates of May 11 to May 24, 2005 as the tadpoles were hatching and again between the dates of June 28 to July 11, 2005 as the frogs were emerging from the pond as metamorphs. Invertebrate predators including zygopterans (damselfly nymphs), anisopterans (dragonfly nymphs), dytiscid (water beetle) larvae and adults, and a large species of *Dolomedes* (fishing spider) were collected and sorted from the sweeps in the field. The specimens were then identified, recorded, and preserved in 70% ethanol in the laboratory.

For the predator exclusion study, cylindrical commercial bait cages (76 cm diameter by 76 cm high) were modified with fiberglass window screening and fishing net floats. One hundred young tadpoles were introduced into each cage before the cages were closed and deployed near vegetation in the study ponds. The cages were monitored biweekly to record the development of the tadpoles.

The incidence of abnormalities in wood frog metamorphs was directly related to the number of dragonfly nymphs collected in early and late sweep samples and to the mean dragonfly nymph abundance for both sampling dates; although the abundance of predatory dragonflies explained < 55% of the variation in *Rana sylvatica* limb abnormalities. More than 3,000 predatory invertebrates were catalogued from 126 sweeps. The abundance of each invertebrate predator species varied significantly among the study site ponds and, with a few exceptions, (*Cordulia* nymph abundance in both surveys; *Acilius* abundance in the early survey),

no significant patterns of association with frog limb abnormalities and each invertebrate taxon were observed.

The invertebrate predator exclusion cage study was successful in allowing tadpoles to complete metamorphosis with limited foraging opportunities, but unsuccessful in excluding predators. The cages provided valuable information to help further modify the cage design and deployment. While the 2005 exclusion cage study provided some frog abnormality figures, caution is recommended in interpreting these data until further cage modifications can be performed.

The 2005 field season was successful for the invertebrate study despite forest fires that burned some study sites. However, it is recommended that at least another year of invertebrate sampling be performed. This would allow for an account of invertebrate population fluctuations and consequently a more robust statistical comparison between invertebrate species frequency and frog abnormalities. Another field season would also enable the deployment of modified exclusion cages, capable of providing a yes/no answer to the question of whether invertebrate predators are responsible for the elevated rates of frog abnormalities found in the Kenai NWR.

Introduction

High numbers of abnormal wood frogs (*Rana sylvatica*) have been found on the Kenai National Wildlife Refuge (NWR) during consecutive years (2000-2002) of monitoring for the U.S. Fish and Wildlife Service's (Service) National Abnormal Amphibian Program (NAAP). As it was beyond the scope of NAAP to investigate possible causes for the high number of abnormal frogs found on the Kenai NWR, this investigation was undertaken to investigate potential contributing factors to the phenomenon in this study. Specifically, this report addresses the potential of predatory invertebrates to cause limb abnormalities through unsuccessful but injury-causing predation events.

Abnormalities consistently observed on the Kenai NWR include the following: shrunken limbs, missing limbs or parts of limbs, and other anomalies of the skin, head or body. The most frequently observed abnormalities occur in the rear legs. For example, in 2002, of the 54 abnormal frogs collected, 55 percent of the abnormalities observed affected the hind limbs (Reeves and Trust, 2003). The Kenai NWR is host to many invertebrate predators that are capable of inflicting tissue damage to sensitive developmental tissue in tadpoles that may lead to deformities in adult frogs (Ouellet, 2000). These invertebrates include dragonfly nymphs, damselfly nymphs, semi-aquatic spiders, and dytiscid beetle larvae and adults.

The intent of the biomonitoring was to determine the extent of the correlation between invertebrate predator abundance with frog abnormalities. A high correlation between predator abundance and limb abnormalities in the wood frogs would be strong evidence that the high rate of amphibian abnormalities in Kenai NWR *R. sylvatica* populations are a natural phenomenon and not due to anthropogenic disturbance or contamination. A lack of correlation would indicate that the abnormalities detected in the amphibian population are not likely caused by the invertebrate predators.

Purpose

The objective of the invertebrate sampling and cage deployment is to determine whether or not unsuccessful but injurious attacks by invertebrate predators are responsible for higher than average abnormalities detected in the Kenai NWR NAAP.

The invertebrate sampling and caging program in the Kenai NWR addresses the following factors:

1. Invertebrate population density
2. Amphibian abnormality frequency in an environment excluding invertebrate predators

Location and schedule of sampling

Twenty-one ponds within the boundaries of the Kenai NWR and included in the NAAP were selected for invertebrate sampling in 2005 (Figure 1). Each site was sampled twice during the period that immature wood frogs were present. The first sweep samples were taken between May 10 and May 25th, and the second sweep samples were taken between June 27th and July 11th (Table 1). Two ponds could not be swept a second time because they had dried out before the second visit.

Three ponds included in the NAAP were selected for the predator exclusion cage study and included two roadside sites (1, 8) and one remote site (54). Cages were deployed on May 17th, May 17th, and May 26th, respectively, and monitored biweekly to check on development of frogs and on the effectiveness of predator exclusion.

***Figure 1*. Approximate location of the 21 ponds sampled for invertebrate density. Ponds with asterisks also included exclusion cages.**

***Table 1*. Schedule of pond visits for aquatic sweeps.**

Pond Site	Site characterization	Date of early season sample	Date of late season sample
1	Road	5/17/05	6/29/05
2	Road	5/17/05	6/29/05
3	Road	5/17/05	6/29/05
8	Road	5/19/05	6/30/05
12	Road	5/13/05	6/30/05
14	Road	5/13/05	6/30/05
21	Road	5/16/05	7/1/05
31	Remote	5/25/05	6/28/05
46	Remote	5/18/05	7/6/05
47	Remote	5/18/05	7/6/05
51	Remote	5/26/05	7/11/05
54	Remote	5/26/05	7/11/05
55	Remote	5/26/05	7/11/05
56	Remote	5/26/05	7/11/05
60	Remote	5/11/05	7/3/05
62	Remote	5/11/05	7/3/05
90	Road	5/20/05	6/28/05
95	Road	5/20/05	7/4/05
97	Road	5/20/05	7/4/05
111	Road	5/20/05	7/4/05 (dry)
141	Road	5/16/05	7/1/05 (dry)

Methods

Data analyses were performed using Microsoft Excel 2001 and Statview. A nonparametric MannWhitney U-test was used to compare road and remote site invertebrate density in addition to differences between invertebrate density in early (May) and late (July) season sampling. A correlation Z-test and multiple regression were used to determine correlation between amphibian abnormality incidence and direct, transformed, or ranked invertebrate species density values. The invertebrate density values were normalized with the following transformations: *Aehsna tuberculifera* - square root, *Cordulia shurtleffii* – inverse, *Lestes disjunctus* – log, *Dytiscus* – normal, *Acilius* – inverse, *Rhantus* – inverse. A ranking system was also used to compensate for the extreme variability in the species density values. A value of 1 was assigned if no individual of a particular species was found in a pond; 2 if only one individual was found; 3 if two to ten individuals were found; 4 if ≥ 11 individuals were found. Abnormal frog data were sorted into

two categories in an attempt to distinguish between the effects of early vs. late season invertebrate populations. The first category included only historical injuries (missing or shortened limbs, notches in tails) while the second included only fresh injuries (blood, bruising, protruding bone). Significant differences required an $\alpha = 0.05$.

Invertebrate Sampling

Rationale

The density of different predatory invertebrate species will be correlated with the frequency of amphibian abnormalities. A statistically significant correlation will indicate an association between a particular invertebrate species and injuries to wood frogs. The lack of a statistically significant correlation between predatory invertebrate density and wood frog abnormalities will indicate that it is unlikely that predatory invertebrates are causing the amphibian abnormalities.

Sample Collection and Analysis

Sweeps were performed with an aquatic D-net with a 30-cm diameter. On each sample date, three 10-meter transects were swept parallel to the shore of each pond to bring the total volume sampled at each pond to 2120 liters, or just over 2 cubic liters. Each pond was sampled twice during the season. Two ponds were not swept a second time because they had dried out before the second visit. The protocol at each pond consisted of delineating three distinct areas of the pond that could be sampled safely, but that might provide access to the most diverse habitats.

Sweep samples were sorted in two stages. The first sort was performed in modified plastic tubs containing a panel of fiberglass window screen in the bottom. The plastic tubs were nested to allow water to flush upwards through the screen panel to disturb vegetation and sediment obtained during the sweep samples. All predatory invertebrates larger than 5 mm were collected from the samples and stored in 100% ethanol in redundant labeled 100-ml whirl-paks. We focused on invertebrates larger than 5 mm because larger macro-invertebrates are adequately sampled using dip-nets (McCormick and Polis 1982). The field sort was performed due to the difficulty of adequately preserving and transporting delicate invertebrates in addition to vegetation and or sediment from the sweep samples. While some invertebrates were undoubtedly missed due to the field sorting, great care was taken in the field to maximize specimen recovery.

Laboratory sorting involved identification to family and preservation of labeled specimens grouped by site, date, and family in 100% ethanol in 30-ml glass vials. Representative specimens were transported to the Department of Entomology at the University of California, Riverside and identified under 5-25X magnification to genus and species. All remaining samples were left in custody of the project director at the USFWS Anchorage field office.

Invertebrate exclusion cage study

Rationale

The predator exclusion cages were used to allow tadpoles to develop in their natural environment without predation pressure. An absence of abnormalities in the tadpoles and recent metamorphs reared in cages excluding predators compared to a higher level of abnormalities in individuals living in the same pond or leaving that pond would indicate that the invertebrate predators likely cause the abnormalities through unsuccessful but injurious predation attempts. No statistically significant difference in the frequency of abnormalities between individuals reared in predator-free cages and individuals collected from the pond holding the cages would indicate that the predatory invertebrates are not likely responsible for the observed abnormalities.

Cage Deployment and Monitoring

Bait cages were purchased from Team NuMark Inc. (Victoria, TX), with measurements of 76.2 cm (30 in.) diameter and 76.2 cm (30 in.) deep (Figure 2). These cages would contain approximately 300 L of water and according to previous studies (Berven et al. 1983, Berven et al. 1985) should provide enough volume for 100 tadpoles to complete development. The cages were modified by sewing fiberglass window screen to the outside of the cages on the sides and bottom with 10-lb fishing line to reduce the size of the mesh and restrict invertebrate access to the cage. The cages included floats to keep the top of the cage above the surface of the water, which were augmented with four fishing net buoys on each cage. The tops of the cages were above water and were not covered with additional screen in order to allow unrestricted access through the drawstring opening for tadpole development monitoring. The small hole at the center of the drawstring closing was blocked with an inverted plastic funnel zip-tied to a 13 by

13 cm square of foam. The foam pad also provided a dry site for matured metamorphs to rest upon.

Tadpole eggs were collected from pond sites 1 and 8 in early May and were allowed to develop without risk of predation in site-specific aquaria inside the field station until cage deployment. Due to the difficulty of accessing pond 54, and the unlikelihood that the tadpoles would survive transportation, tadpoles were collected from egg masses in pond 54 and introduced into the cages when the cages were deployed. The cages were carefully deployed close to vegetation in water at least 75 cm deep whenever possible. Three cages were deployed at each of three ponds. The ponds chosen included two roadside sites (1, 8) and one remote site (54). One hundred tadpoles were introduced into each cage and were monitored approximately biweekly to monitor development of the tadpoles and the effectiveness of predator exclusion. The tadpole metamorphs were catalogued and released when they reached Gosner stage 42 or later.

Results and Discussion

Predatory Invertebrates

We focused on aquatic invertebrates in our sampling, however one semi-aquatic spider species was captured and determined to consume tadpoles. The scientific names of the terrestrial spider and the predatory aquatic invertebrates are included in Table 2.

The largest of the predatory invertebrates was the 5th instar aeshnid dragonfly nymphs. These nymphs would likely require more than one season to develop (Higgins and Brigham 1982). Fifth instar nymphs could likely attack fairly late gosner stage tadpoles or even small adults (Henrikson 1990), and smaller nymphs (3rd and 4th instars) can readily attack early gosner stage tadpoles (up to ~25). Formanowicz (1986) determined that larger (i.e. 5th instar) aeshnids are more successful at capturing larger prey than are smaller nymphs and consume many more prey. There were three distinct cohorts of *Aeshna* although the largest nymphs were by far the fewest in number. It has been shown that most predation pressure on tadpoles occurs when tadpoles are small, because more abundant, smaller predators, cannot handle large prey (Brodie and Formanowicz 1983). We included Zygopterans (damselflies) in our collection for this reason, and because of their ability to strike and potentially inflict injury in tadpoles.

Coleoptera was the second most abundant predacious taxon in all samples. *Dytiscus* (Dytiscidae) included the largest-bodied individuals, and are known to be voracious and opportunistic predators of frog tadpoles (Formanowicz 1986, Henrikson 1990). However, large *Dytiscus* were not captured in great quantity. Other members of the family Dytiscidae that we collected (*Rhantus* spp.) are also known to prey on invertebrates (including other Dytiscidae), as well as frog larvae (Henrikson 1990) and fish, though life history information is available almost exclusively for the *Dytiscus* spp. (Young 1967, Formanowicz 1982, LeClair et al. 1986).

The spider species that we captured belongs to a family of fishing spiders in the genus *Dolomedes*. These are robust cursorial spiders generally located near permanent bodies of fresh water. They can run and sail on the water, are able to dive and swim under water, and have been documented feeding on *Rana sylvatica*, on each of the predator taxa we collected and on other arthropods in the pond community (Zimmermann and Spence 1989). While we were able to collect specimens for identification purposes, these predators were able to run quickly out of our nets making consistent collection and density determination impossible.

***Table 2*. Predatory Invertebrate Taxa Collected and Identified from the KNWR study sites.**

Order	**Suborder**	**Family**	**Subfamily**	**Genera**	**species**
Odonata	Anisoptera	Aeshnidae		*Aeshna*	*tuberculifera*
		Libelllulidae	Corduliinae	*Cordulia*	*shurtleffii*
	Zygoptera	Lestidae		*Lestes*	*disjunctus*
Coleoptera		Dytiscidae		*Dytiscus*	
		Dytiscidae		*Acilius*	
		Dytiscidae		*Rhantus*	
Aranae		Pisauridae		*Dolomedes*	*triton*

The taxa that we recorded at the study ponds were consistent with those reported in a previous study in the KNWR by Prussian et al. (2001). While the total number of taxa that we recorded was far less that reported by Prussian et al., our collecting was not intended to take an inventory of every invertebrate species that is considered a predator.

We did not make an effort to collect hemipterans (Corixidae), predatory trichopterans, or leeches in this study. The hemipterans use a piercing-sucking mode of feeding that is unlikely to cause the loss of limbs or traumatic injuries. The predatory trichopterans are not very motile and unlikely to attack tadpoles. The leeches were difficult to capture consistently as they were able to squeeze through the mesh on our nets.

In an effort to gain as complete of a picture of the taxa likely to inflict injury on the tadpoles we will make modifications to our collection technique to consistently collect corixids, leeches and *Dolomedes* spiders. A review of the literature indicated that corixids feed on both eggs and tadpoles (Henrikson 1990), and could potentially cause damage to developmental tissue and not death if scared off the amphibian prey. We will likely perform a time-limited search for the spiders at the pond's edge at each sampling site to get an estimate of spider density. To prevent the loss of the leeches we will contain the bottom of the collection net in a plastic container after each sweep to prevent their escape.

Invertebrate density

We catalogued 3745 invertebrate specimens from 126 sweep samples. The density of the invertebrate predators was variable between ponds (Figs. 2 and 3) but in general did not vary significantly. In fact, the only significant difference in species density that occurred between the early and late season sampling periods occurred in the *Acilius* beetles. It is likely that this significant increase ($p = 0.046$) was due to an increase in the size of the beetle larvae over the season and the higher likelihood of their capture or detection in the pond samples.

We also compared the density of each species at ponds categorized as road or remote (see Table 1). Abundance of dragonfly nymphs of the genus *Aeshna* in the late season samples in remote ponds was significantly ($p = 0.017$) higher than in road ponds but no significant differences between the other taxa in either sampling period. The higher abundance of *Aeshna tuberculifera* at the remote ponds could indicate a preference of the adults for remote sites, a higher survival rate of the nymphs, or a different assemblage of species due to competition or predation. Nilsson (1986) and Larson (1985) established that the less abundant and diverse dytiscids fauna of stable habitats may be due to competition from other invertebrate predators. The higher abundance in our late season samples at the remote ponds corresponded with a positive significant correlation between *Aeshna tuberculifera* and *Cordulia shurtleeffii* ($r = 0.853$, $p = 0.002$) in addition to between *Aeshna tuberculifera* and *Lestes disjunctus* ($r = 0.683$, $p = 0.041$). However, this significant correlation was not unique to the remote sites (Table 3), and no significant negative correlations were detected at any site during early or late season, indicating that the correlations are likely due to a habitat variable that we did not measure.

We performed a literature search to compare the invertebrate density values that we observed with those of studies in other regions. Most invertebrate surveys report species diversity as opposed to density; the comparisons that we were able to make are limited. In addition, those studies that reported invertebrate density did not use similar sampling techniques or reported their results in unconvertible format like dry weight (Shalles 1989). For example the densities of aquatic beetles and odonate nymphs were reported (Downie 1998) based upon trap catches. These are not at all comparable to our results as the traps are present in the field for much longer period of time and have much different catch efficacy for different taxa (Downie 1998).

Armitage et al. (2003) performed 15-second sampling sweeps with a comparable D-net (900-mm mesh, 230 x 255 mm frame, 275 mm bag depth) and provided abundance at the family level. The sampling was performed at Rushton Ditch, located on the floodplain of the lower River Frome at East Stoke in southern England, consisting of pasture with a small proportion of arable land on the northern edge and occasional patches of riparian woodland. They found an average of 57 odonates per sweep with a range of 0 to 300, and an average of 22 coleoptera, with

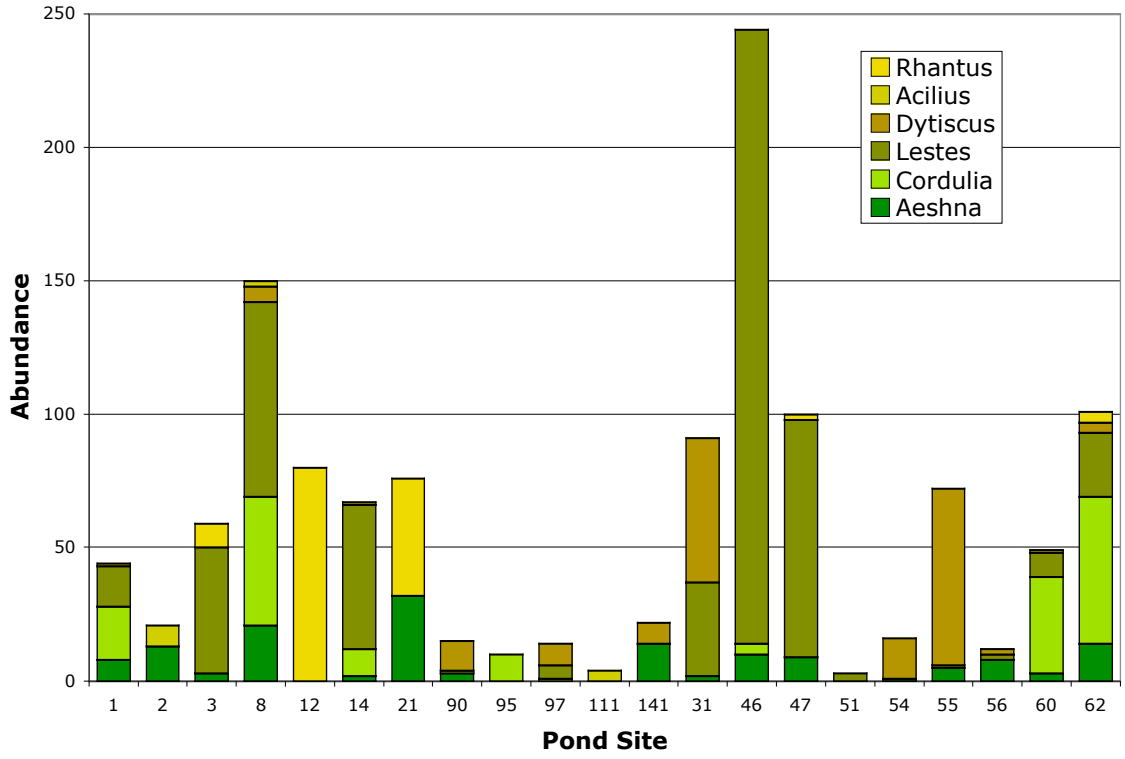

***Figure 2*. Abundance of invertebrate predators per $2m^3$ collected during early season 2005 sweeps.**

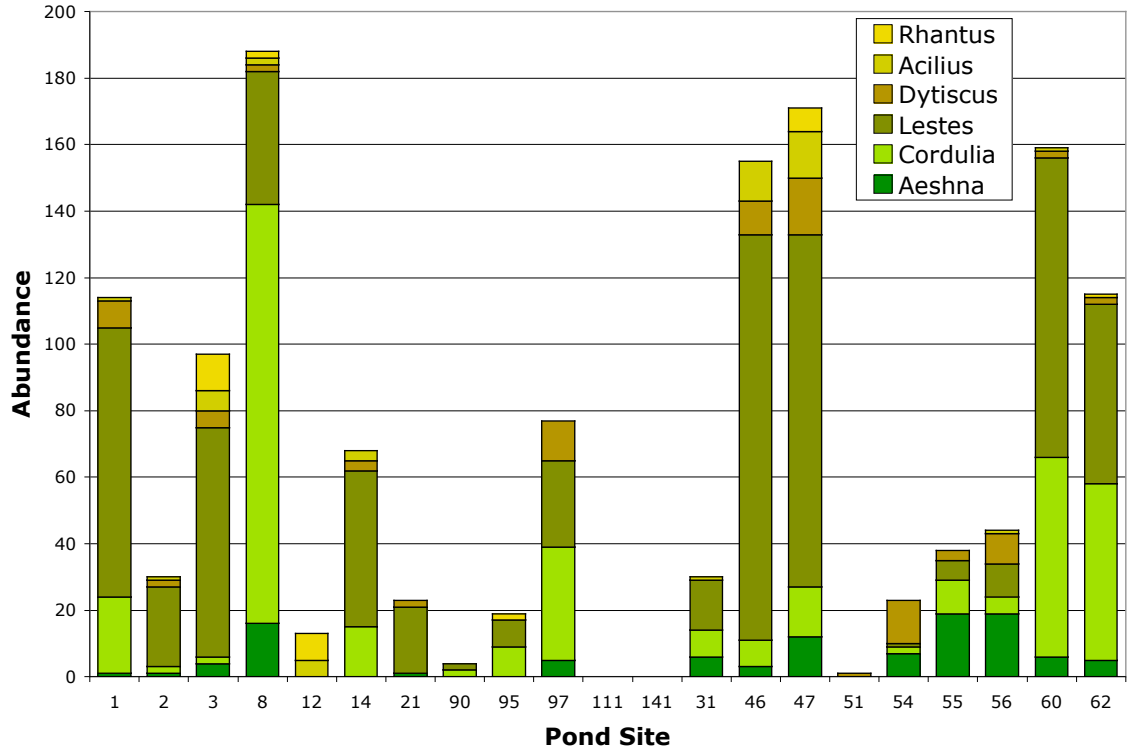

***Figure 3*. Abundance of invertebrate predators per 2m^3 collected during late season 2005 sweeps**

a range from 0 to 100. Upon conversion to similar area swept, our sweeps provided an average of 25 odonates and 2.7 coleoptera, with an odonate range from 0 to 98, and a coleopteran average of 0-33 per sweep.

Bosi (2001) studied shallow marshes (200 000 m^2) in Malalbergo, Bologna. The two marshes showed a maximum depth of less than 1 m and were planted with poplars, willows, and around their shorelines. The sampling points were represented by nine littoral areas with a mean depth of 0.308 ± 0.075 m with abundant vegetal detritus. Sampling consisted of 10 alternate sweeps with a semicircular net (diameter: 0.40 m). The average number of coleopterans was 102 in samples from April to August, with a range of 0 to 400. More specifically, Bosi found 0 to 2 of *Dytiscus* spp, 0 *Acilius* spp., and 27 to 360 *Rhantus* sp. Our samples contained an average of 8 coleopterans from May to August with a range of 0-150. We found 0-66 *Dytiscus* spp., 0-14 *Acilius* spp., and 0-80 *Rhantus* spp. From these numbers it appears that our sample ponds had greater species richness, as they were not dominated by beetles from the *Rhantus* genus. However, our overall numbers were still slightly lower. The species dominance could be related to many other factors including water depth, nutrients, water temperature, dissolved oxygen, species distribution, size of habitat, substrate type, water chemistry, toxic secondary chemicals

from nearby vegetation, habitat isolation, and the duration of dry and aquatic cycles (Williams 1997).

Johansson (1991) studied interactions between odonate nymphs that included taking 20 cylinder samples (diameter = 30 cm) at depths ranging from 20 to 40 cm. The cylinder was swept three times with a handnet to collect some of the captured odonates. Most importantly, Johansson did not find any negative correlations between the abundances of the different dragonfly nymphs found in his samples. We converted the densities that he recorded to match ours by volume by simply multiplying by four. Thus, Johansson found approximately 120 leucorrhinid dragonfly nymphs, with a range of 0 to 360, 24 ceonagrionid nymphs, ranging from 0 to 64, and 20 aeshnid nymphs, ranging from 0 to 48 per square meter. This adds up to an average of 164 odonate nymphs per square meter with a range of 0 to 436 per square meter. Our samples provided approximately 31 per square meter, ranging from 0 to 105. Our aeshnid densities were lower, although only by half, as we found an average of 6 per square meter, with a range of 0 to 24.

Standen (1999) sampled invertebrates in mires in the Flow Country of Caithness and Sutherlandin north-east Scotland. She used a long handled D-frame sweep net fitted with 1 mm mesh size bag, between 24 April and 19 May, 1994. She swept in a 2 m figure eight pattern 20 times in each pool (1.3 times as much volume as we sampled), sorted the samples from debris in a pan in the field and then preserved the samples in alcohol. Three odonate species and three Dytiscid beetle species were among the taxa reported. The average number of specimens for each species were as follows: *Enallagma* - 32.1, *Sympetrum* - 30.2, *Aeshna* - 35.8, *Acilius* - 17.5, *Rhantus* - 7.5, *Agabus* (dytiscid) - 12.5. The average number of odonates per sample was 98, and coleopterans was 38. Our samples (multiplied by 1.3) contained an average of 33 odonates per sample, and 3.5 coleopterans, including an average of 8 aeshnids, 2 *Acilius*, and 5 *Rhantus* specimens.

Duffy and Douglas (1994) sampled aquatic invertebrates using a 1.5-m long 7.5 cm diameter core sampler in Noxubee National Wildlife Refuge in Mississippi from December 1987 to April 1988. The sampler collected aquatic invertebrates from both the sediment and overlying water column. Understandably, this sampling technique provided relatively low numbers of highly motile predatory invertebrates. The highest mean density values (number/m^2) from 5 sample locations were 3 for a species of odonate, and 13 for a species of dytiscid.

Overall, those studies with comparable techniques or reporting methods had much higher invertebrate densities than our ponds. This is not surprising given the oligotrophic nature, cold

temperature, relatively small size, and short summer season characteristic of bogs and found at our field sites (Spitzer and Danks 2006)

Abnormal Amphibian Correlation

The incidence of abnormalities in wood frog metamorphs was directly related to the number of dragonfly nymphs (*Aeshna* + *Cordulia*) collected in early and late sweep samples and to the mean dragonfly nymph abundance for both sampling dates (Fig. 4); although the abundance of predatory dragonflies explained < 55% of the variation in *Rana sylvatica* limb abnormalities. The multiple regression of transformed or ranked invertebrate density did not result in any significant relationship between amphibian abnormality incidence and any combination of individual invertebrate species density.

The correlation values from the Z-test analysis (Table 3) show that the incidence of abnormalities in amphibians correlated only with the density of early season *Acilius* at remote sites, early season *Cordulia* at road sites, and late season *Cordulia* at remote sites. The lack of a more prominent pattern, or even some consistency in the correlation results highlights the need for additional data collection. As we are planning to collect more predatory invertebrate species in the 2006 field season, and with an additional season of data to use in the Z-test correlation analysis, it is possible that additional relationships may still emerge. A further consideration is the grading of amphibian abnormalities. Tadpoles are graded for abnormalities at the end of the season but these tadpoles may not accurately represent predation pressure and injury rate. While evidence exists that suggests that tadpoles can survive with substantial tail damage (Figiel and Semlitsch, 1991), there is a substantial cost associated with tail loss. Tadpoles with tail damage tend to develop more slowly than tadpoles with intact tails (Morin, 1985; Wilbur and Semlitsch, 1990; Parichy and Kaplan, 1992). Retarded growth can then lead to increased predation in tadpoles because size plays a role in successfully escaping predators. Small tadpoles are more vulnerable than large tadpoles to predation by insect larvae (e.g., Travis et al., 1985; Formanowicz, 1986; Caldwell, 1994). In staged experiments, Brodie and Formanowicz (1983) found that smaller tadpoles exhibited less injury than larger ones simply because small ones were more often completely consumed when attacked. Therefore, the very tadpoles that we wish to document are also those most likely to be consumed by predators. An absence of tail damage or other abnormalities detected in our samples may indicate that those

tadpoles are consumed completely once captured, rather than indicating that the tadpoles are good at avoiding or escaping capture. Therefore, we may not have found any correlations because the detected abnormalities are representative only of a small percentage of tadpoles that managed to survive with an injury. If possible any additional injury information collected during the gosner stage monitoring of the tadpoles that is performed throughout the season may provide a more accurate incidence of tadpole injury.

***Table 3*. Correlation values for significant interactions between amphibian abnormalities and invertebrate density across 21 pond sites.**

	Aeshna	*Cordulia*	*Lestes*	*Dytiscus*	*Acilius*	*Rhantus*	Abnormals
Transformed							
Early season							
Aeshna	--						
Cordulia		--	0.705				
Lestes		0.705	--				
Dytiscus				--	0.612		
Acilius				0.612	--		0.753*
Rhantus						--	
Late season							
Aeshna	--						
Cordulia		--					0.698*
Lestes			--				
Dytiscus				--			
Acilius					--		
Rhantus						--	
Ranked							
Early season							
Aeshna	--						
Cordulia		--					0.658
Lestes			--				
Dytiscus				--			
Acilius					--		
Rhantus						--	
Late season							
Aeshna	--	0.617†	0.737†				
Cordulia	0.617†	--	0.736†	0.613			
Lestes	0.737†	0.736†	--	0.837			
Dytiscus		0.613	0.837	--			
Acilius					--		
Rhantus						--	

* indicates remote sites, † indicates road and remote sites

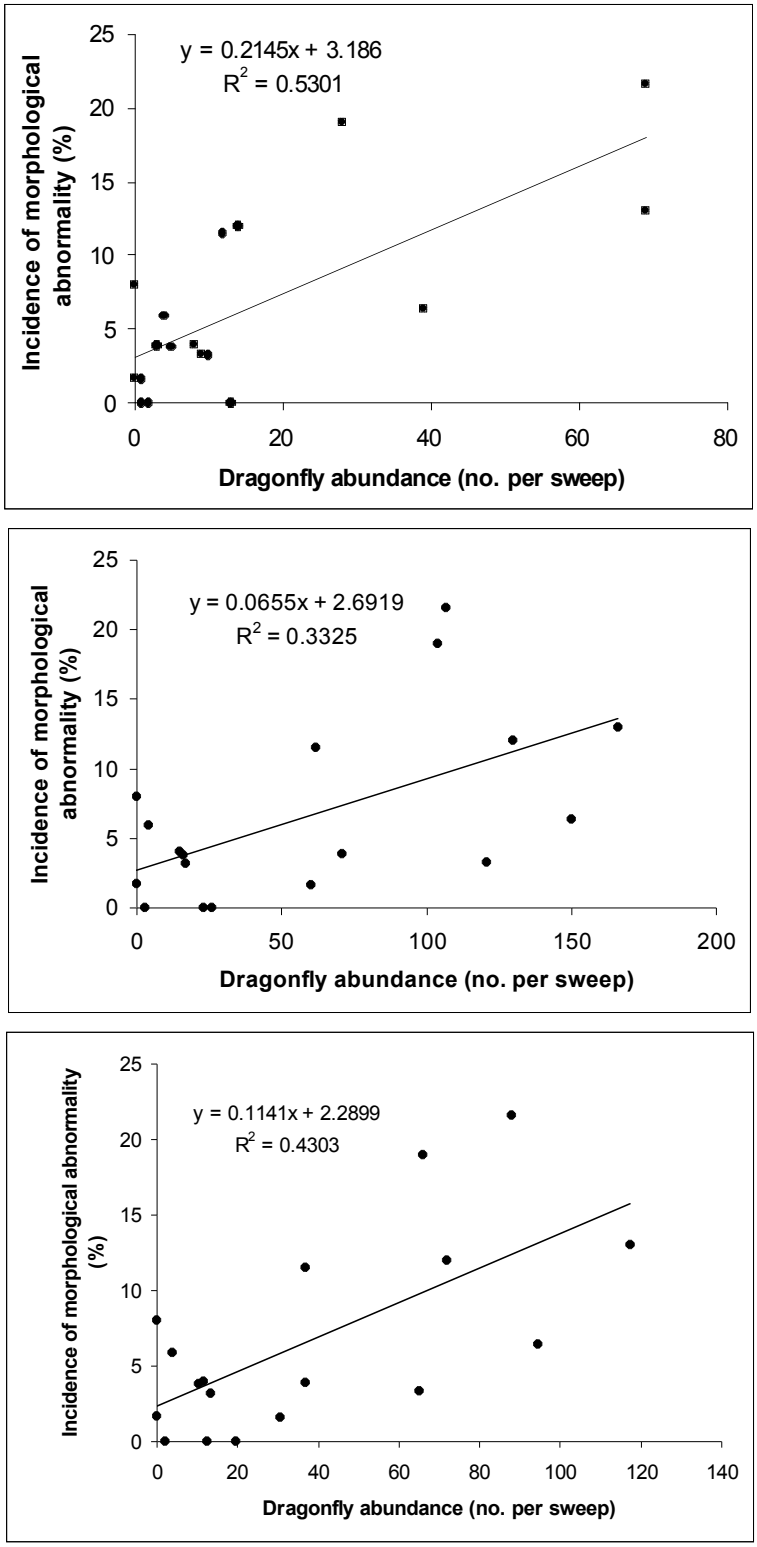

Figure 4. **Relationship of wood frog morphological abnormalities vs. dragonfly nymph abundance in early samples (top), late samples (middle) and the mean abundance in both samples from each pond (bottom).**

Invertebrate Exclusion Cage Study

The cage study was designed to complement the correlation survey component of our study by providing an experimental result to answer to the question of whether or not the invertebrate predators were causing abnormalities in the amphibians. If predators were excluded and abnormalities persisted, the predators would not likely be responsible. If no abnormalities were detected in the amphibians in the cages and abnormalities were observed in metamorphs that developed in the ponds outside the cages, we could conclude that the predators might be responsible for the abnormalities. Other factors modified by the cages included UV light and cage injury. Since UV light exposure is another potential factor in the rate of abnormalities and the cages modify the light levels to which the tadpoles are exposed, we cannot be absolutely sure that the predators are the causal agent. In addition, we found tadpoles mid-season that appeared trapped between the two layers of mesh in the cages. If these tadpoles are injured because of being trapped and potentially pinched between layers of the cage as a result of our monitoring efforts, our results will be confounded and misleading.

Unfortunately, we did not obtain any useable results from the exclusion cage experiments as both the tadpoles and the predators were much more difficult to contain than anticipated. Initially the cages did not float as we expected, some tadpoles escaped and some predators gained access to the cages. We modified the cages and re-stocked the cages with new tadpoles but still found predators in all but two of the cages at the end of the season. In addition, we found a low rate of abnormalities in two of the cages, although none were limb abnormalities. Accordingly, we planned to further modify the cages for the 2006 field season to prevent predator access. A larger problem, however, was the low rate of tadpole growth and survival observed in the cages. We cannot know the effects of the predators that gained entry to the cages, but we do know that the cages produced very small frogs that had completed metamorphosis. The stunted growth likely indicates a low level of nutrient availability in the cages, which presents a much more difficult problem. How will we add nutrients? How much should we add, and how often? Many of the ponds that we sample are oligotrophic and the introduction of nutrient pellets to the cages could potentially alter the pond habitat.

Low nutrient levels, potential tadpole injury, along with the physical difficulty of transporting, deploying, and monitoring the cages are all factors which may discontinue the use of the exclusion cages in the 2006 field season. The relationship between dragonfly abundance

and *R. sylvatica* limb abnormalities, the initial cost and labor invested in the cages, along with the lessons learned from the 2005 season may promote the continued use of the cages in the 2006 field season. Since some of the monitoring and all of the recovery of the cages was performed by frog crews that were not primarily responsible for the invertebrate component of the study, continued use of the cages will depend upon the results of discussions with crew leaders before the 2006 field season.

Conclusions

The abundance of dragonfly nymphs (*Aeshna* + *Cordulia*) was significantly related to the incidence of abnormalities in *Rana sylvatica* metamorphs. Although < 55% of the variation in the incidence of wood frog abnormalities among habitats was explained by dragonfly abundance in sweep samples, the relationship between the two variables was statistically significant for early and late season samples, as well as for the average dragonfly nymph abundance across both samples. Except *Cordulia* nymph abundance on both sampling dates and *Acilius* abundance in the early samples, individual insect predator taxa were generally not strongly associated with amphibian abnormality incidence across the 21 pond sites sampled.

The density of predatory invertebrates in our samples was lower than those reported in studies at other locations around the world, but were characteristic of the oligotrophic bog habitats that were primarily sampled on the Kenai NWR.

The invertebrate collection techniques provided an abundance of specimens and elucidated a relationship between dragonfly nymph abundance and tadpole limb abnormalities. However, we will modify the collection protocol slightly to more effectively assess several additional predatory species next field season. These additional taxa will provide an opportunity to test for more potential associations between both individual invertebrate species densities and amphibian abnormalities.

The invertebrate predator exclusion cage study was successful in allowing tadpoles to complete metamorphosis, albeit with limited foraging opportunities, but was unsuccessful at excluding predators. However, the exclusion study provided preliminary findings that will be useful for cage modifications and new ideas for approaching the next field season. While the 2005 exclusion cage study provided estimates of frog abnormality incidence, caution is recommended in interpreting these data until further cage modifications are made and the study is repeated. The final decision on cage deployment in 2006 will be made after further consultation with the project leaders.

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Appendix 1. Invertebrate Sweep Sample Data

Numbers of amphibian abnormalities and corresponding invertebrate densities from early season sweep samples.

Pond site	Abnormal Incidence	Dragonfly *Aeshna*	Dragonfly *Cordulia*	Damselfly *Lestes*	Beetle large *Dytiscus*	Beetle med *Acilius*	Beetle small *Rhantus*
1	19.0	8	20	15	1	0	0
2	0.0	13	0	0	0	8	0
3	3.9	3	0	47	0	0	9
8	13.0	21	48	73	6	2	0
12	1.7	0	0	0	0	0	80
14	11.5	2	10	54	1	0	0
21		32	0	0	0	0	44
90	5.9	3	1	0	11	0	0
95	3.2	0	10	0	0	0	0
97	1.6	0	1	5	8	0	0
111	8.0	0	0	0	0	4	0
141		14	0	0	8	0	0
31	0.0	2	0	35	54	0	0
46	12.0	10	4	230	0	0	0
47	3.3	9	0	89	0	0	2
51		0	0	3	0	0	0
54	0.0	1	0	0	15	0	0
55	3.8	5	0	1	66	0	0
56	4.0	8	0	2	2	0	0
60	6.4	3	36	9	1	0	0
62	21.6	14	55	24	4	0	4

Numbers of amphibian abnormalities and corresponding invertebrate densities from late season sweep samples.

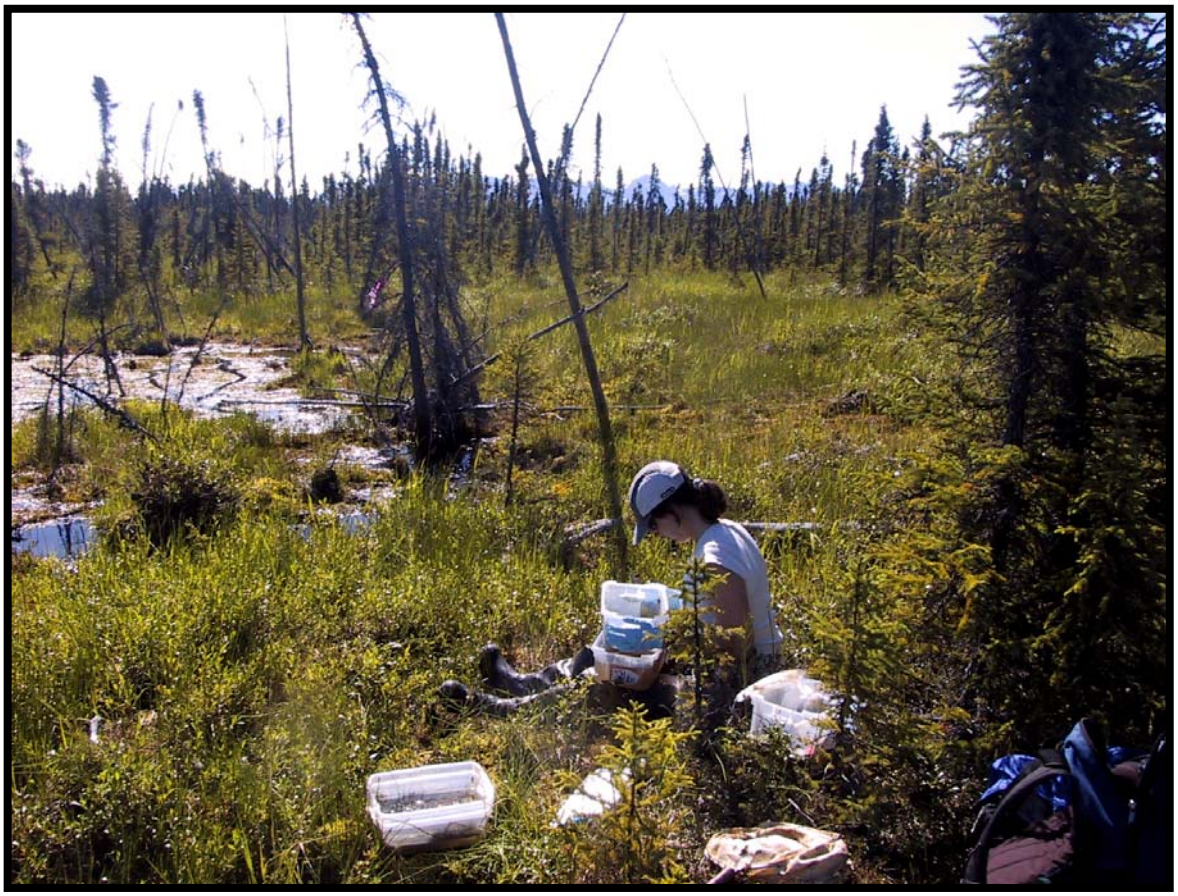
Pond site	Abnormal Incidence	Dragonfly *Aeshna*	Dragonfly *Cordulia*	Damselfly *Lestes*	Beetle large *Dytiscus*	Beetle med *Acilius*	Beetle small *Rhantus*
1	5.6	1	23	81	8	1	0
2	18.0	1	2	24	2	1	0
3	2.0	4	2	69	5	6	11
8	9.1	16	126	40	2	2	2
12	6.6	0	0	0	0	5	8
14	16.4	0	15	47	3	3	0
21		1	0	20	2	0	0
90	5.9	0	2	2	0	0	0
95	10.4	0	9	8	0	0	2
97	8.7	5	34	26	12	0	0
111	14.8	0	0	0	0	0	0
141		0	0	0	0	0	0
31	6.8	6	8	15	0	1	0
46	6.4	3	8	122	10	12	0
47	10.3	12	15	106	17	14	7
51		0	0	0	1	0	0
54	0.0	7	2	1	13	0	0
55	12.3	19	10	6	3	0	0
56	2.0	19	5	10	9	1	0
60	4.3	6	60	90	2	1	0
62	16.7	5	53	54	2	0	1

Appendix 2. Invertebrate Exclusion Cage Data

Pond	Cage	Data	Abnormal	Normal	Total
KNA01	1	Avg. Snout to Vent Length (mm)		15	15
		Count of Normal/Abnormal		23	23
	2	Avg. Snout to Vent Length (mm)		13	13
		Count of Normal/Abnormal		12	12
	3	Avg. Snout to Vent Length (mm)		14	14
		Count of Normal/Abnormal		14	14
KNA01 Avg. Snout to Vent Length (mm)				14	14
KNA01 Count of Normal/Abnormal				49	49
KNA08	1	Avg. Snout to Vent Length (mm)		14	14
		Count of Normal/Abnormal		24	24
	2	Avg. Snout to Vent Length (mm)	11	12	12
		Count of Normal/Abnormal	2	25	27
	3	Avg. Snout to Vent Length (mm)	13	14	14
		Count of Normal/Abnormal	3	15	18
KNA08 Avg. Snout to Vent Length (mm)			12.2	13	13
KNA08 Count of Normal/Abnormal			5	64	69
KNA54	1	Avg. Snout to Vent Length (mm)		14	14
		Count of Normal/Abnormal		2	2
	2	Avg. Snout to Vent Length (mm)		16	16
		Count of Normal/Abnormal		6	6
KNA54 Avg. Snout to Vent Length (mm)				16	16
KNA54 Count of Normal/Abnormal				8	8
Total Avg. Snout to Vent Length (mm)			12.2	14	14
Total Count of Normal/Abnormal			5	121	126

Predatory Invertebrate Sampling
In Kenai National Wildlife Refuge, 2006

By Peter D. Jensen
William E. Walton



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Acknowledgements

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Executive Summary

A nationwide survey is underway to monitor wood frog populations in an effort to gauge ecosystem health. As part of this survey abnormal frogs were discovered in Alaska's Kenai National Wildlife Refuge (NWR) at rates higher than the national average. To determine the cause of the high abnormality rates, an investigation was undertaken to examine several factors that may contribute to the observed abnormalities. One of these factors is the occurrence and density of predatory invertebrates.

In this investigation, we surveyed predatory invertebrates with an aquatic sweep net at 27 pond sites and deployed predator exclusion cages in 3 of the pond sites where invertebrate predators were surveyed. For the survey, three aquatic net sweeps covering 10 meters each were performed for a total of 2.1 m^3 or approximately 2120 L of water sampled at each site during each sampling period. Sweeps were performed once in each pond in the Kenai NWR between the dates of May 4 to May 19, 2006 as the tadpoles were hatching and again between the dates of July 10 to July 18, 2006 as the frogs were emerging from the pond as metamorphs. Sweeps were performed once in the Yukon Delta NWR between the dates of July 2 to July 4, 2006. Invertebrate predators including zygopterans (damselfly nymphs), anisopterans (dragonfly nymphs), dytiscid (water beetle) larvae and adults, and a large species of *Dolomedes* (fishing spider) were collected and sorted from the sweeps in the field. The specimens were then identified, recorded, and preserved in 70% ethanol in the laboratory.

For the predator exclusion study, cylindrical commercial bait cages (76 cm diameter by 76 cm high) were modified with fiberglass window screening and fishing net floats. Fifty young tadpoles were introduced into each cage before the cages were closed and deployed near vegetation in the study ponds. The cages were monitored biweekly to record the development of the tadpoles.

More than 3,000 predatory invertebrates were catalogued from 138 sweeps. Diversity indices showed increasing predatory invertebrate diversity and richness over the season, as well as higher diversity and richness in shallow sites compared to deep sites. Direct relationships between individual invertebrate taxa and specific injury types were detected using linear regression and ANOVA, indicating at least a correlation between five of the observed invertebrate predators and observed injury rates across the study sites. Furthermore, a strong time component and further predator-injury correlations were detected using a multivariate

redundancy analysis. High positive correlations were found to occur between Skeletal Malformation rates and five invertebrate predatory taxa abundances in early season sampling, when tadpoles would likely be smallest and most vulnerable. These predatory taxa included two species of dragonfly nymphs (*Aeshna sitchensis, Leucorrhinia proxima*), and three beetle genera (*Dytiscus*, *Acilius*, and *Ilybius*). We also detected high negative correlations between Skeletal Injury rates late in the season and two large predatory dragonflyspecies (*Aeshna sitchensis*, *Leucorrhinia glacialis*), along with high positive correlations to two smaller predatory genera. These, along with other RDA analyses suggest that while invertebrate predators could likely be responsible for injuries early in the season, there appears to be another cause for the injuries that occur late in the season, which is also causing the absence of the larger invertebrate predator taxa.

The invertebrate predator exclusion cage study was successful in allowing tadpoles to complete metamorphosis, and relatively successful at excluding predators. While not conclusive, the cage study results suggest that predatory invertebrates could be the cause of metamorph injury.

Introduction

High numbers of abnormal wood frogs (*Rana sylvatica*) have been found on the Kenai National Wildlife Refuge (NWR) during consecutive years (2000-2002) of monitoring for the U.S. Fish and Wildlife Service's (Service) National Abnormal Amphibian Program (NAAP). As it was beyond the scope of NAAP to investigate possible causes for the high number of abnormal frogs found on the Kenai NWR, this investigation was undertaken to investigate potential contributing factors to the phenomenon in this study. Specifically, this report addresses the potential of predatory invertebrates to cause limb abnormalities through unsuccessful but injury-causing predation events.

Abnormalities consistently observed on the Kenai NWR include the following: shrunken limbs, missing limbs or parts of limbs, and other anomalies of the skin, head or body. The most frequently observed abnormalities occur in the rear legs. For example, in 2002, of the 54 abnormal frogs collected, 55 percent of the abnormalities observed affected the hind limbs (Reeves and Trust, 2003). The Kenai NWR is host to many invertebrate predators that are capable of inflicting tissue damage to sensitive developmental tissue in tadpoles that may lead to deformities in adult frogs (Ouellet, 2000). These invertebrates include dragonfly nymphs, damselfly nymphs, and dytiscid beetle larvae and adults.

The intent of the biomonitoring was to determine the extent of correlation between invertebrate predator abundance with frog abnormalities. A high correlation between predator abundance and limb abnormalities in the wood frogs would be strong suggestive evidence that the high rate of amphibian abnormalities in Kenai NWR *R. sylvatica* populations are a natural phenomenon and not due to anthropogenic disturbance or contamination. A lack of correlation would indicate that the abnormalities detected in the amphibian population are not likely caused by the invertebrate predators.

In this second year of biomonitoring, we increased the number of predatory invertebrate categories to include water boatmen (Family Corixidae), leeches (Hirudinae), and spiders (Genera Dolomedes) as suggested in the 2005 report. In addition, following a second suggestion from our 2005 report we made further modifications to the cages used in the second component of the study to further exclude predatory invertebrates.

Purpose

The objective of the invertebrate sampling and cage deployment is to determine whether or not unsuccessful but injurious attacks by invertebrate predators are responsible for higher than average abnormalities detected in the Kenai NWR NAAP.

The invertebrate sampling and caging program in the Kenai NWR addresses the following factors:

1. Invertebrate population density
2. Amphibian abnormality frequency in an environment excluding invertebrate predators.

Location and schedule of sampling in 2006

Twenty-one ponds within the boundaries of the Kenai NWR and included in the NAAP were selected for invertebrate sampling 2006 (Figure 1). Each site was sampled twice during the period that immature wood frogs were present. The first sweep samples were taken between May 4 to May 19, 2006 as the tadpoles were hatching, and again between the dates of July 10 to July 18, 2006 (Table 1). The high altitude sites (50, 51, 54, 55) were not swept for the first time until June 21, 2006 because of late de-icing. The sweeps were performed by Stacie Jensen.

In a repeat of the 2005 season, two ponds (111, 141) could not be swept a second time because they had dried out before the second visit in July.

Seven ponds within the boundaries of the Yukon Delta NWR were also sampled in 2006. Each site was sampled once between the dates of July 2 and July 4, 2006 (Table 1).

Three ponds included in the NAAP were again selected for the predator exclusion cage study. The sites selected for use in 2006 included all roadside sites (1, 3, 8) to allow for easier monitoring, deployment, and recovery, but also because deicing of the high altitude ponds occurred much later in 2006 than in 2005. Cages were deployed on May 17th and monitored biweekly to check on development of frogs and on the effectiveness of predator exclusion.

***Figure 1*. Approximate location of the 21 ponds sampled for invertebrate density. Ponds with asterisks also included exclusion cages.**

***Table 1*. Schedule of pond visits for aquatic sweeps.**

Pond Site (KNA)	Site characterization	Date of early season sample	Date of late season sample
1	Road	5/4/06	7/11/06
2	Road	5/4/06	7/11/06
3	Road	5/4/06	7/11/06
8	Road	5/15/06	7/10/06
12	Road	5/10/06	7/10/06
14	Road	5/10/06	7/11/06
21	Road	5/15/06	7/18/06
31	Remote	5/19/06	7/31/06
46	Remote	5/12/06	7/13/06
47	Remote	5/12/06	7/13/06
51	Remote	6/21/06	7/17/06
54	Remote	6/21/06	7/17/06
55	Remote	6/21/06	7/17/06
56	Remote	6/21/06	7/17/06
60	Remote	5/11/06	7/14/06
62	Remote	5/11/06	7/14/06
90	Road	5/15/06	7/11/06
95	Road	5/16/06	7/10/06
97	Road	5/16/06	7/10/06
111	Road	5/11/06	(dry)
141	Road	5/16/06	(dry)

Methods

Data analyses were performed using Microsoft Excel 2001 and Conoco. A paired two sample t-test for means was used to compare mean abundances of invertebrate taxa across both early (May) vs. late (July) season sampling and 2005 vs. 2006. A z-test for means was used to compare diversity indices between deep and shallow sites in 2006. A one-way ANOVA was used to test for significance in the linear regression of individual invertebrate predator taxa on injury types.

Detrended Correspondence Analysis (DCA) was used to identify the gradient lengths to determine whether linear or unimodal models should be used in the multivariate analysis. Redundancy Analysis (RDA) was used to examine the correlation between tadpole injury types and each of the predatory species, taking into account sampling date and pond characteristics including depth, area, and distance from the road.

Abnormal frog data were categorized using the decision rules set by the project leader Mari Reeves (Appendix 1). We used only four categories of injury in our analyses: Fleshwounds, Skeletal Injuries, Skeletal Malformations, and Skeletal Abnormalites. Significant differences required an $\alpha = 0.05$.

Invertebrate Sampling

Rationale

The density of different predatory invertebrate species will be correlated with the frequency of amphibian abnormalities. A statistically significant correlation will indicate an association between a particular invertebrate species and injuries to wood frogs. The lack of a statistically significant correlation between predatory invertebrate density and wood frog abnormalities will indicate that it is unlikely that predatory invertebrates are causing the amphibian abnormalities.

Sample Collection and Analysis

Sweeps were performed with an aquatic D-net with a 30-cm diameter. On each sample date, three 10-meter transects were swept parallel to the shore of each pond to bring the total volume sampled at each pond to 2120 liters. Each pond was sampled twice during the season. Two ponds were not swept a second time because they had dried out before the second visit. The protocol at each pond consisted of delineating three distinct areas of the pond that could be sampled safely, but that might provide access to the most diverse habitats.

Sweep samples were sorted in two stages. The first sort was performed in modified plastic tubs containing a panel of fiberglass window screen in the bottom. The plastic tubs were nested to allow water to flush upwards through the screen panel to disturb vegetation and sediment obtained during the sweep samples. All predatory invertebrates larger than 5 mm were collected from the samples and stored in 100% ethanol in redundant labeled 100-ml whirl-paks. We focused on invertebrates larger than 5 mm because larger macro-invertebrates are adequately sampled using dip-nets (McCormick and Polis 1982). The field sort was performed due to the difficulty of adequately preserving and transporting delicate invertebrates in addition to vegetation and or sediment from the sweep samples. While some invertebrates were undoubtedly missed due to the field sorting, great care was taken in the field to maximize specimen recovery.

In an effort to gain as complete of a picture of the taxa likely to inflict injury on the tadpoles we made modifications to our 2005 collection protocol to consistently collect corixids, leeches and *Dolomedes* spiders. A review of the literature indicated that corixids feed on both eggs and tadpoles (Henrikson 1990), and could potentially cause damage to developmental tissue or death. We performed a five-minute visual survey for the spiders at the pond's edge at each sampling site to get an estimate of spider density. To prevent the loss of the leeches we contained the bottom of the collection net in a plastic container after each sweep to prevent their escape. Corixids were collected from the sweep samples and enumerated, instead of being discarded in the fields sorting in 2005.

Laboratory sorting in 2006 involved identification to family or genus using a digital microscope, and preservation of labeled specimens grouped by site, date, and family in 30-ml glass vials with 100% ethanol. Digital photographs were taken of representatives from each sample in the Soldatna field laboratory. Representative specimens were transported to the Department of Entomology at the University of Maryland, College Park and identified under 5-25X magnification to genus. All remaining samples were left in custody of the project director at the USFWS Anchorage field office.

Invertebrate exclusion cage study

Rationale

The predator exclusion cages were used to allow tadpoles to develop in their natural environment without predation pressure. An absence of abnormalities in the tadpoles and recent metamorphs reared in cages excluding predators compared to a higher level of abnormalities in individuals living in the same pond or leaving that pond would indicate that the invertebrate predators likely cause the abnormalities through unsuccessful but injurious predation attempts. No statistically significant difference in the frequency of abnormalities between individuals reared in predator-free cages and individuals collected from the pond holding the cages would indicate that the predatory invertebrates are not likely responsible for the observed abnormalities.

Cage Deployment and Monitoring

Cages from 2005 were re-used. They were originally purchased from Team NuMark Inc. (Victoria, TX), with measurements of 76.2 cm (30 in.) diameter and 76.2 cm (30 in.) deep.

These cages would contain approximately 300 L of water and according to previous studies (Berven et al. 1983, Berven et al. 1985) should provide enough volume for 100 tadpoles to complete development. The cages were modified by sewing fiberglass window screen to the outside of the cages on the sides and bottom with 10-lb fishing line to reduce the size of the mesh and restrict invertebrate access to the cage. The cages included floats to keep the top of the cage above the surface of the water, which were augmented with four fishing net buoys on each cage. In May of 2006 the cages were inspected for damage, repaired, and then modified with a 1.5 m screen sleeve over the top of the cage opening to improve invertebrate exclusion. In addition,

Tadpole eggs were located in pond sites 1, 3, and 8 on May 17th, 2006 and were immediately sorted, counted, and added to the cages at those sites (Figure 2). The cages were carefully deployed close to vegetation in water at least 75 cm deep whenever possible. Three cages were deployed at each of the three ponds. Only fifty eggs were introduced into each cage in 2006 (100 were added in 2005) due to the small size of the metamorphs observed in the cages in 2005. The cages were monitored approximately biweekly to monitor development of the tadpoles and the effectiveness of predator exclusion. The tadpole metamorphs were catalogued and released when they reached Gosner stage 42 or later.

***Figure 2*. Separation and counting of *Rana sylvatica* egg masses for exclusion cages**

Results and Discussion

Predatory Invertebrates

We focused on aquatic insects, one semi-aquatic spider species, and leeches in our sampling. The scientific names of the terrestrial spider and the predatory aquatic invertebrates are included in Table 2.

The largest of the predatory invertebrates were the 5th instar aeshnid dragonfly nymphs from the species *Aeshna sitchensis* (Figure 3). These nymphs would likely require more than one season to develop (Higgins and Brigham 1982). Fifth instar nymphs could likely attack

Figure 3*. Fifth instar nymph of the dragonfly *Aeshna sitchensis.

fairly late gosner stage tadpoles or even small adults (Henrikson 1990), and smaller nymphs (3rd and 4th instars) can readily attack early gosner stage tadpoles. Formanowicz (1986) determined that larger aeshnids are more successful at capturing larger prey than are smaller nymphs and consume many more prey. However Bates and Beebee (1988) recorded feeding by small and large Aeshnid larvae on toad nymphs (*Bufo calamita*) greater than 12 mm in length in aquarium studies. Bates and Beebee (1988) also found feeding by Sympetrum and Libellulid nymphs, both of which we collected in our samples. It has been shown that most predation pressure on tadpoles occurs when tadpoles are small, because more abundant, smaller predators, cannot handle large prey (Brodie and Formanowicz 1983). Although Zygopterans (damselflies) are likely too small to kill a tadpole (Figure 4), we included them in our collection because of their ability to strike and potentially inflict injury in tadpoles.

***Figure 4*. A damselfly nymph from the genus *Lestes*.**

Coleoptera was the second most abundant predacious taxon in all samples. *Dytiscus* (Dytiscidae) included the largest-bodied individuals (Figure 5), and are known to be voracious and opportunistic predators of frog tadpoles (Formanowicz 1986, Henrikson 1990). However, large *Dytiscus* were not captured in great quantity. Other members of the family Dytiscidae that we collected (*Rhantus* spp.) are also known to prey on invertebrates (including other Dytiscidae), as well as frog larvae (Henrikson 1990) and fish, though life history information is available

***Figure 5*. Head and thorax of a large water beetle larva from the genus *Dytiscus*.**

almost exclusively for the *Dytiscus* spp. (Young 1967, Formanowicz 1982, LeClair et al. 1986). Bates and Beebee (1988) did, however, record low rates of feeding by beetles in the genera Colymbetes, Acilius, Ilybius, Agabus, Rhantus, and Graphoderus on Natterjack toads (*Bufo*

calamita) shorter than 12 mm in length in aquarium studies. We collected representatives of each of these genera in our sampling.

The spider species that we captured belongs to a family of fishing spiders in the genus *Dolomedes*. These are robust cursorial spiders generally located near permanent bodies of fresh water. They can run and sail on the water, are able to dive and swim under water, and have been documented feeding on *Rana sylvatica*, on each of the predator taxa we collected and on other arthropods in the pond community (Zimmermann and Spence 1989). As we encountered difficulty in containing these spiders after capture in 2005, we performed a five-minute walking survey of the ponds edge at each site to enumerate these large spiders. This sampling technique provided very low numbers of Dolomedes, although it also seemed that fewer spiders were captured in the sweep nets in 2006 than in 2005.

We also collected corixids in 2006 as suggested after the 2005 season report. The hemipterans use a piercing-sucking mode of feeding that could cause injury early in the tadpole's life that might cause malformations observed in the metamorphs later in the season. Bates and Beebee (1988) tested corixids, but found no feeding on small (<12 mm) or large (>12 mm) *Bufo calamita* tadpoles. We found large numbers of corixids at many sites (Figures 6 and 7).

We also made modifications to our collection technique to capture leeches (Hirudinea) in the 2006 sampling as suggested after the 2005 report. After sweeping with the D-net, we carried the net over a bucket before sorting in order to catch any escaping leeches. Unfortunately we did not capture many Hirudinea in 2006 (Figure 7).

The taxa that we recorded at the study ponds were consistent with those reported in a previous study in the KNWR by Prussian et al. (2001). While the total number of taxa that we recorded was less that reported by Prussian et al., our collecting was not intended to take an inventory of every invertebrate species that is considered a predator, but only those large enough at some stage in their lives to potentially cause injury to the tadpoles. For example, we did not make an effort to collect predatory trichopterans in this study as predatory trichopterans are not very motile and also unlikely/unable to attack tadpoles.

Invertebrate abundance and density

We catalogued 3375 invertebrate specimens from 138 sweep samples. The abundance of the invertebrate predators was variable between ponds and over time (Figures 6 and 7). We

compared several diversity indices between early (May) and late (July) samples, as well as between shallow and deep site samples using a paired two-sample t-test for means, and a z-test for means, respectively, for all sites that we were able to sample twice. Table 4 shows the means for each index. It is not at all surprising that many of the measures are higher for the July samples, since our May sampling occurred very soon after de-icing, and because many species

***Table 2*. Predatory Invertebrate Taxa Collected and Identified from the KNWR study sites.**

Order	Suborder	Family	Genera	Species
Odonata	Anisoptera	Aeshnidae	*Aeshna*	*sitchensis*
		Libelllulidae	*Leucorrhinia*	*proxima*
			Leucorrhinia	*glacialis*
			Libellula	
	Zygoptera	Lestidae	*Lestes*	*disjunctus*
		Coenagrionidae	*Coenagrion*	
Coleoptera		Dytiscidae	*Dytiscus*	
			Acilius	
			Agabus	
			Colymbetes	
			Copelatus	
			Graphoderus	
			Ilybius	
			Neoscutopterus	
			Rhantus	
Hemiptera		Corixidae		
Aranae		Pisauridae	*Dolomedes*	*triton*

***Table 3.* Predatory Invertebrate Taxa Collected and Identified from the YDNWR sites.**

Order	Suborder	Family	Genera	species
Odonata	Anisoptera	Libelllulidae	*Leucorrhinia*	*proxima*
	Zygoptera	Lestidae	*Lestes*	*disjunctus*
		Coenagrionidae	*Coenagrion*	
Coleoptera		Dytiscidae	*Dytiscus*	
			Acilius	
			Agabus	
			Graphoderus	
			Rhantus	
Hemiptera		Corixidae		

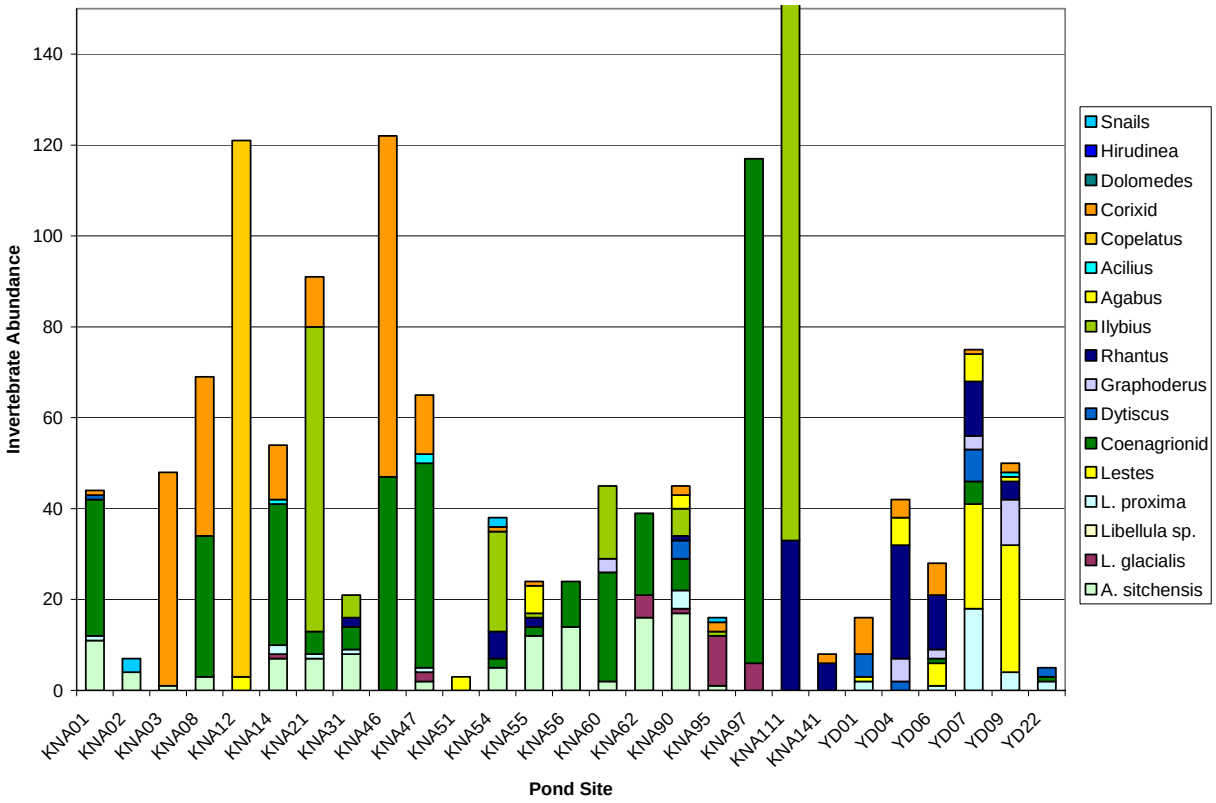

***Figure 6*. Invertebrate predator abundance in early season 2006 sweeps.**

that require only one year to mature may have been still in eggs or such small instars that we did not catch or identify them. It also makes sense that the shallow ponds had higher indices as the deeper ponds were often oligotrophic kettle ponds with potentially less diverse vegetation. These differences underscore the importance of using these groupings as covariables in our multivariate analysis, as well as for blocking using these groupings for random permutation Monte Carlo significance testing.

The abundances of the invertebrate predators were comparable between the two years of sampling. A paired two sample t-test for means showed that *Leucorrhinia glacialis*, *Dytiscus* sp., *Libellula* sp., *Graphoderus* sp., and *Neoscutopterus* sp. (5 of the 16 taxa) were significantly more abundant ($p \geq 0.04$) in 2005 than in 2006. Of these taxa, *Neoscutopterus* sp. was not collected at all in 2006, but was found in the 2005 samples upon reexamination with the digital microscope on site. Of the remaining four taxa, *L. glacialis* was not significantly different between 2005 and 2006 May or July sweeps, but *Libellula* sp. and *Graphoderus* sp. were significantly more

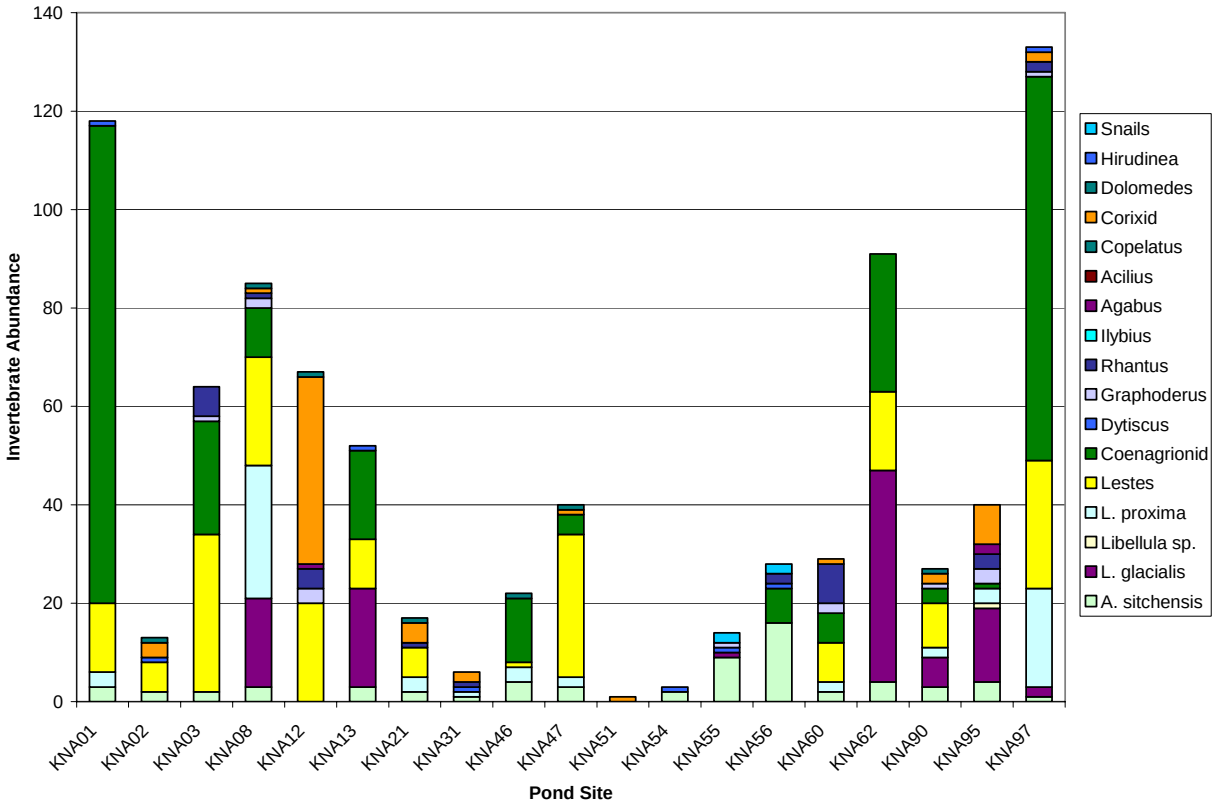

***Figure 7*. Invertebrate predator abundance in late season 2006 sweeps.**

abundant in May of 2005 than May 2006, while *Dytiscus* sp. and *Graphoderus* sp. were significantly more abundant in July 2005 than July 2006. Our sampling began much earlier in the season in 2006 due to colder weather in April and May, which could explain the drop in abundances of *Libellula* sp. and *Graphoderus* sp. in May of 2006. However, no explanation for the decrease in *Dytiscus* sp. and *Graphoderus* sp in July of 2006 compared with July 2005 is readily apparent. With the majority of the remaining predatory species not changing significantly in abundance between the two years, it is likely that the decrease in *Dytiscus* sp. and *Graphoderus* sp. is simply an example of individual population fluctuation.

Overall, those studies with comparable techniques or reporting methods had higher invertebrate densities than our ponds (Armitage 2003, Bosi 2001, Duffy and Douglas 1994, Johansson 1991, Standen 1999). This is not surprising given the oligotrophic nature, cold temperature, relatively small size, and short summer season characteristic of bogs and found at our field sites (Spitzer and Danks 2006).

Table 4. **Mean Diversity Indices (± SE) for May vs. July Samples, and Shallow vs. Deep Sites.**

	Early (May)	Late (July)	Shallow	Deep
Diversity	2.51 (± 0.34)	3.22 (± 0.27)*	3.34 (± 0.39)*	2.48 (± 0.23)
Richness	3.00 (± 0.41)	3.97 (± 0.31)*	4.07 (± 0.46)*	3.03 (± 0.27)
Evenness	0.85 (± 0.02)	0.81 (± 0.02)	0.83 (± 0.02)	0.83 (± 0.02)
Shannon's Index	0.97 (± 0.12)	1.32 (± 0.08)*	1.29 (± 0.13)*	1.03 (± 0.09)

* indicates mean is significantly greater ($p \geq 0.05$)

Abnormal Amphibian Correlation

Much of the collected invertebrate abundance data from 2006 was not useable due to a lack of frog injury data. Insect abundance from sites KNA03, KNA21, KNA51, KNA54, KNA55, KNA56, KNA62, KNA96, KNA111, KNA141, YD01, YD04, YD06, YD07, YD09, and YD22 were not used in any of the following correlation analyses.

The incidence of abnormalities in wood frog metamorphs was directly related to the number of several invertebrate taxa collected in early and late sweep samples. Table 5 shows linear regression results for single invertebrate taxa and each injury type.

To better analyze interactions between all of the predatory species, each of the injury types of interest, and some of the pond characteristics that were recorded, we performed a multivariate analysis using the multivariate statistical software package Canoco (ter Braak and Smilauer 2006). As a first step, we determined which type of multivariate analysis should be performed. We checked the gradient length of the predatory invertebrate species abundances

Table 5. **Adjusted R-square Values for Significant Linear Regression Results ($p > 0.05$) Between Individual Invertebrate Taxa and Injury Rates.**

	Flesh Wound		Skeletal Injury		Skeletal Malformation		Skeletal Abnormality	
	May	**July**	**May**	**July**	**May**	**July**	**May**	**July**
A. sitchensis		0.57		0.31	0.43			
L. proxima	0.33				0.59			
Corixids				0.48				
***Dytiscus* sp.**					0.67			
***Graphoderus* sp.**				0.31				

using an indirect Detrended Correspondence Analysis (DCA) with no transformation of the species data and the gradients detrended by segment. The DCA showed a gradient of 7.9, well above the threshold of 4.0 required to trigger a unimodal model based analysis (Leps and Smilauer 2003). However, inspection of the DCA biplot revealed that only two predatory invertebrate genera were responsible for the high gradient value (Figure 8) and could be justifiably be removed from the analysis as outliers. Larvae in the genera *Copelatus* were found at only site 12 in May 2006 at high density. *Agabus* larvae were the only other genera also found at site 12 in May 2006.

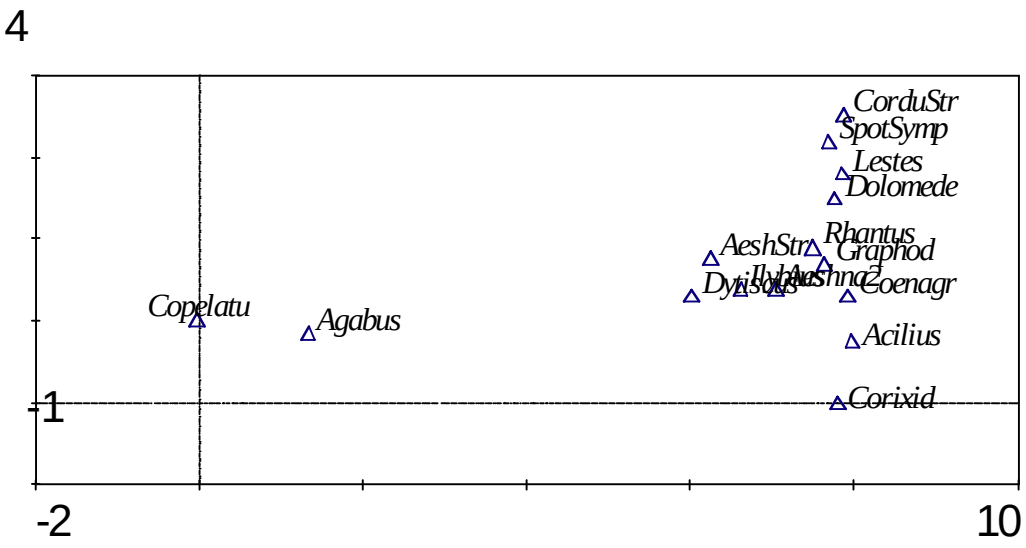

***Figure 8*. DCA plot of predatory invertebrate groups. This plot illustrates the justification for removal of the genera *Copelatus* and *Agabus* from the subsequent RDA analysis.**

A subsequent DCA of the predatory invertebrate groups using the same analysis conditions, but with the genera *Copelatus* and *Agabus* removed, and determined that the gradient was 3.24 – well within the range of linear model analysis.

An RDA of the predatory invertebrate groups (minus *Agabus* and *Copelatus*), using the percent injury rates for fleshwounds, skeletal abnormalities, skeletal malformations, and skeletal injury as the environmental variables was performed with the following conditions: Biplot scaling was focused on inter-species correlations and centered by species, species scores were post-transformed (divided by standard deviation) to provide a more uniform biplot, the species scores were log-transformed before analysis to normalize the data, with A=10 and B=1 in the

equation Y'=log (A*Y + B). We used pond characteristics as covariables to account for differences in injury rates or invertebrate abundance due to sampling time (May vs. July), pond depth (deep vs. shallow), pond distance to nearest road, and pond area. A Monte Carlo significance test was then performed to test the significance of the first ordination axis, and the significance of all of the canonical axes together with 999 reduced model permutations, and with permutation blocks defined by time (May vs. July) and pond depth (deep vs. shallow).

The RDA indicated that the covariables accounted for 38% of the variance seen in the invertebrate abundances. An additional 11% of the variance was explained by the first multivariate axis, 4% by the second, and 3% by the third. The species environment correlations for the first two axes were 89% and 65% respectively. Both the first axis (p = 0.0030) and all canonical axes together (p = 0.0290) were significant. The RDA results are summarized in the Invertebrate-Injury rate biplot (Figure 9). **Interpretation rules for linear model biplots suggest that vectors with less than a 90 angle between are positively correlated, a 90 angle indicates no correlation at all, while an angle greater than 90 indicates a negative correlation.** It is apparent from the biplot that most of the invertebrate groups are negatively correlated with Fleshwound and Skeletal Injury rates. It appears that most of the larger predators (dragonfly nymphs, *Dytiscus* larvae) are most highly correlated with skeletal malformation or

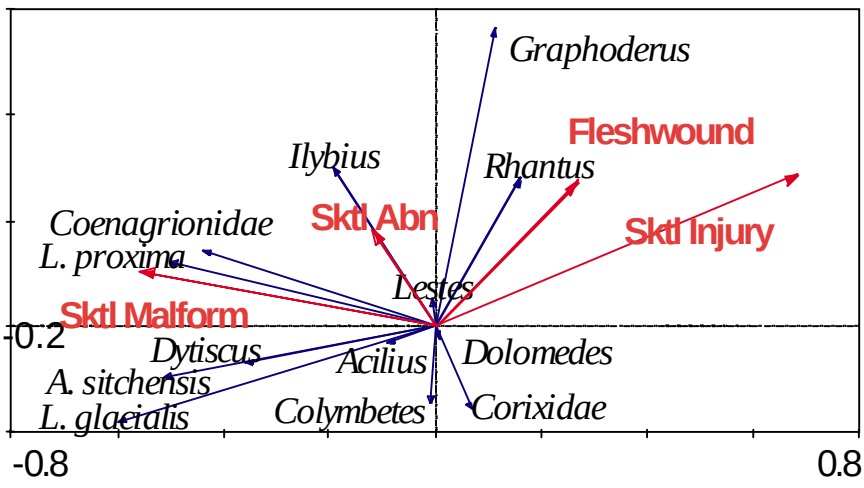

***Figure 9*. RDA biplot of correlation between predatory invertebrate abundance and tadpole injury rate, correcting for sampling time and pond characteristics.**

skeletal abnormality rates. Of the smaller invertebrates, *Graphoderus* and *Rhantus* are the only taxa closely correlated with Fleshwound rates and with Skeletal Injury rates.

To make these results more readily interpretable, and to determine which species were most highly correlated with each type of injury, we performed an RDA on each individual injury type, with invertebrate inclusion in the biplot limited by the amount of variance explained by the first axis in each analysis. Using the same settings as in the previous RDA, but deleting all but one injury type we found that there was no significant first axis for Fleshwound or Skeletal Abnormality rates. The first axis was significant, however, for Skeletal Malformation rates ($p = 0.0090$) accounting for 8% of the variance (Figure 10), and for Skeletal Injury rates ($p = 0.0070$) accounting for 8% of the variance (Figure 11). These biplots are more easily interpretable since only those species that are well fitted by the first ordination axis (the injury rate of interest). In each biplot (Figure 10 and 11) since the RDA explains 8% of the variability in the species data, a species with an average 'explainability' by the first ordination axis will have at least 8% of the variability in its values explained by that axis. **In short, this provides a filter that excludes all but the species with the highest correlation with the injury rate of interest.**

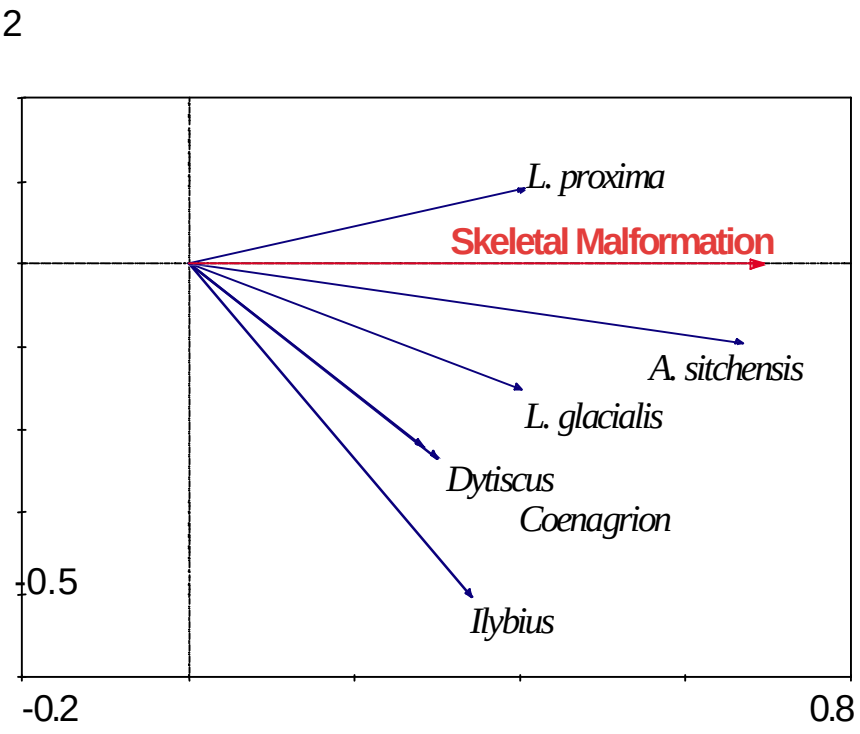

***Figure 10*. RDA biplot showing only species significantly correlated with Skeletal Malformation rates.**

We can see in Figure 10 that the species most highly correlated with Skeletal Malformation rates is the dragonfly nymph *Aedes sitchensis*, the largest of the dragonflies that we collected in the study sites. This interpretation is based on the smallest angle between the *Aedes sitchensis* species vector and the Injury rate vector. Using this rule, *Leucorrhinia proxima* (dragonfly nymph) has the next highest correlation, followed by *Leucorrhinia glacialis* (dragonfly nymph), *Coenagrion* (damselfly), *Dytiscus* (beetle), and finally *Ilybius* (beetle). In addition, a projection of the species vector tip onto the Injury rate vector (greater value with greater distance from the origin) provides evidence for the value of environmental value for that species. Using this rule provides a similar order of the species, with *Ilybius* having greater value than *Coenagrion* and *Dytiscus*. Overall we see that the dragonfly nymphs as a group are the most highly correlated with Skeletal Malformation, followed by two beetles.

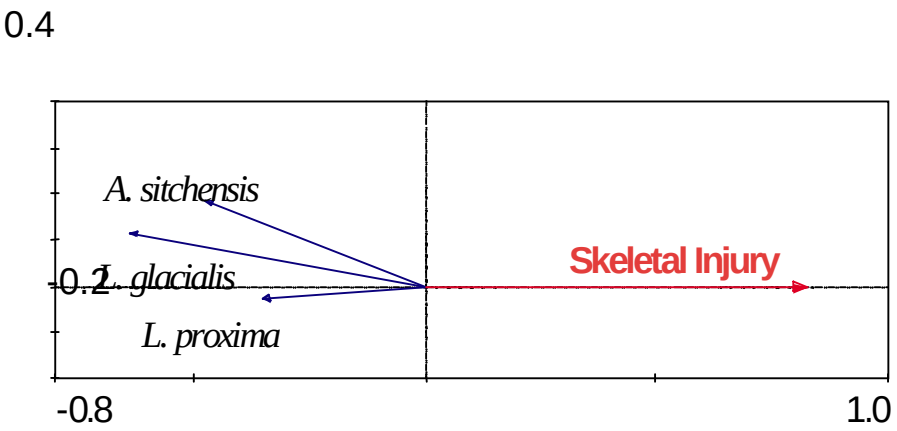

***Figure 11*. RDA biplot showing only species significantly correlated with Skeletal Injury rates.**

Figure 11 shows the opposite type of correlations to Figure 10. In this case, all of the species are negatively correlated with Skeletal injury. Once again it is the dragonfly species demonstrating the highest correlation, albeit negative. It appears in this biplot, however, that another environmental factor is causing the absence of dragonfly larvae, and the presence of Skeletal Injury. We will discuss this in further detail below.

Since the injury types correspond roughly to historical (Skeletal abnormality, Skeletal malformation) and recent (Fleshwound, Skeletal Injury) categories of injury, we performed an RDA to look at the influence of early and late invertebrate abundances on these injury types.

The biplot for invertebrate samples collected in May 2006 and historical type injuries (Skeletal abnormality, Skeletal malformation) is shown in Figure 12. The first canonical axis was significant (p = 0.0020) in a Monte Carlo reduced permutation significance test, the covariables accounted for 34% of the total variance, while the first axis accounted for 25% of the remaining variance, with a 97% species-environment correlation. It is interesting to note how different

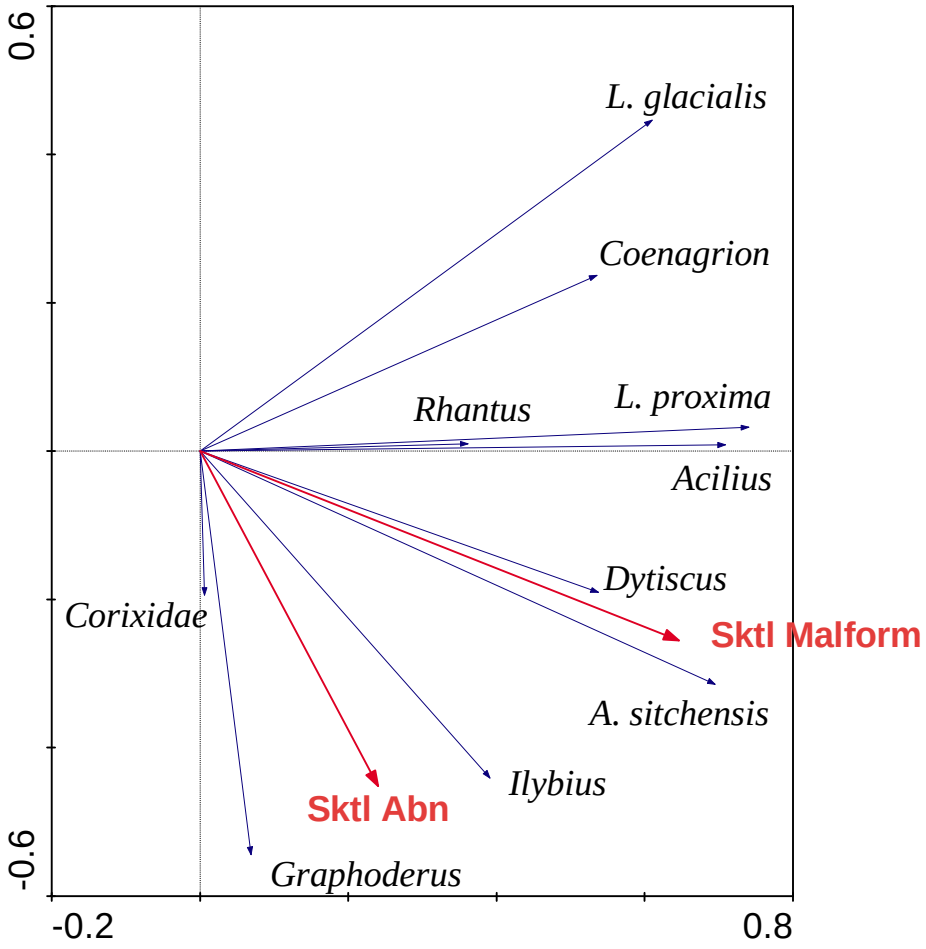

***Figure 12*. May 2006 Invertebrate Abundance with Historical Injury Types.**

invertebrate groups changed in correlation with the injury types, although the largest predator *Aeshna sitchensis* is still the most highly correlated with Skeletal Malformation rates. In this biplot we see an increase in the correlation and importance of *Dytiscus*, *Acilius*, and *Ilybius* (beetles), and a decrease in the correlation and importance of the two *Leucorrhinia* species (dragonflies). Once again, we singled out each of the injury types to increase the interpretability

of the biplots. The first axis for Skeletal Abnormality rates was not significant in a Monte Carlo permutation test. The first axis for Skeletal Malformation was significant in a Monte Carlo permutation test (p = 0.0140), the covariables accounted for 34% of the total variance, while the first axis accounted for 22% of the remaining variance, with a 94% species-environment correlation. The corresponding biplot is shown in Figure 13. This biplot reveals that the high

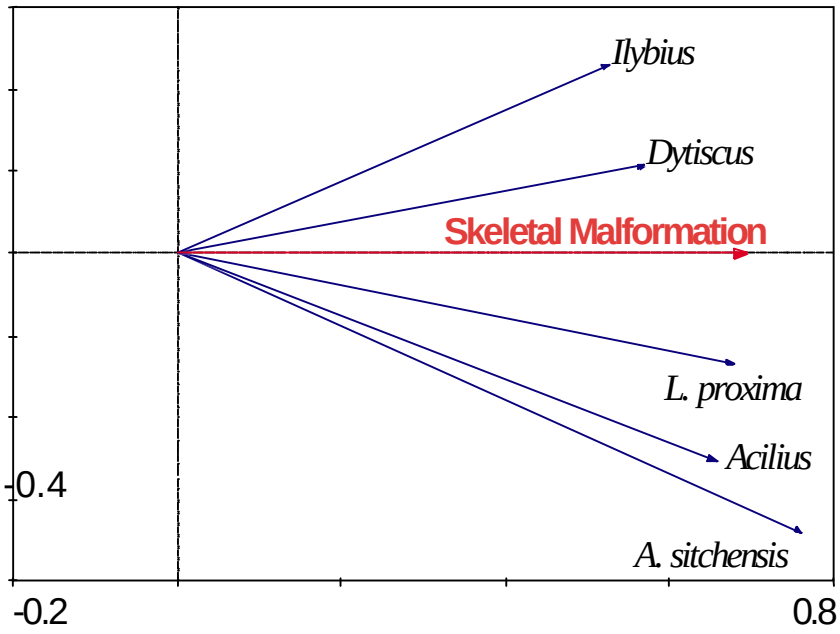

***Figure 13*. RDA Biplot of Skeletal Malformation Rate With May 2006 Invertebrate Predator Abundance.**

rate of correlation between *A. sitchensis* and Skeletal Malformation rates was a product of the correlation of *A. sitchensis* with both of the injury types in Figure 12. We see in Figure 13 that the highest correlation in the May 2006 samples actually occurred between *Dytiscus*, *L. proxima*, and the Skeletal Malformation rates, although recall that any species included in the single injury biplots was included only if it met or exceeded the correlation value of the first axis. In this biplot the inclusion boundary was set at 22%, therefore all of the species in Figure 13 are highly correlated with Skeletal Malformation rates. This high and significant correlation suggests that two dragonfly species, and three beetle species may be responsible for historical type injuries.

When we examined July 2006 invertebrate predator abundances we found very different results. The first axis was not significant in a Monte Carlo permutation test for Fleshwound

rates, but it was significant (p = 0.0260) for Skeletal Injury rates. The covariables accounted for 46% of the total variance, while the first axis accounted for 15% of the remaining variance, with a 88% species-environment correlation. The corresponding biplot is shown in Figure 14. In this

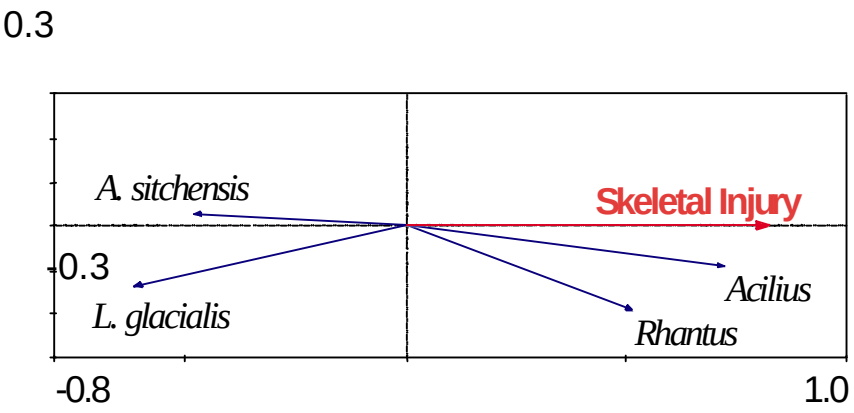

***Figure 14*. RDA Biplot of Skeletal Injury with July 2006 Predator Abundance.**

analysis we see that the larger predators (dragonfly larvae *A. sitchensis* and *L. glacialis*) are again negatively correlated with recent Skeletal Injury, just as we saw in Figure 11. The high correlation of *Acilius* and *Rhantus* (beetles) with Skeletal Injury is interesting because it is unlikely that these medium sized beetles could remove a limb from a metamorph. It may be more likely that this correlation is evidence once again of another environmental factor that causes the absence of larger dragonfly predators, but allows the presence of smaller beetle larvae, and also causes skeletal injury. While this combination of abundances may be due to several different environmental factors, it could also be plausible that one factor, such as a larger vertebrate predator could also be the cause. Great effort put forth by the field sampling crew to avoid large predatory vertebrates precluded the potential observation of a causal factor in the incidence of metamorph recent skeletal injury.

An additional consideration is the grading of amphibian abnormalities. Tadpoles are graded for abnormalities at the end of the season but these tadpoles may not accurately represent predation pressure and injury rate. While evidence exists that suggests that tadpoles can survive with substantial tail damage (Figiel and Semlitsch, 1991), there is a substantial cost associated with tail loss. Tadpoles with tail damage tend to develop more slowly than tadpoles with intact tails (Morin, 1985; Wilbur and Semlitsch, 1990; Parichy and Kaplan, 1992). Retarded growth

can then lead to increased predation in tadpoles because size plays a role in successfully escaping predators. Small tadpoles are more vulnerable than large tadpoles to predation by insect larvae (e.g., Travis et al., 1985; Formanowicz, 1986; Caldwell, 1994). In staged experiments, Brodie and Formanowicz (1983) found that smaller tadpoles exhibited less injury than larger ones simply because small ones were more often completely consumed when attacked. Therefore, the very tadpoles that we wish to document are also those most likely to be consumed by predators. An absence of tail damage or other abnormalities detected in our samples may indicate that those tadpoles are consumed completely once captured, rather than indicating that the tadpoles are good at avoiding or escaping capture. Therefore, the correlations that we did find might have been even higher if they were not representative of only of a small percentage of tadpoles that managed to survive with an injury.

We performed these same analyses incorporating the abundance and injury data from 2005. While some of the combined 2005/06 relationships were significant, we believe it was only due to the weight of the 2006 data, as no 2005 correlations alone were significant. We could conclude that the 2006 data was anomalous, however, greater experience and confidence in our second year of sampling, identification, and data analysis lends much more weight to the 2006 correlations than those from 2005.

Invertebrate Exclusion Cage Study

The cage study was designed to complement the correlation survey component of our study by providing an experimental result to answer to the question of whether or not the invertebrate predators were causing abnormalities in the amphibians. If predators were excluded and abnormalities persisted, the predators would not likely be responsible. If no abnormalities were detected in the amphibians in the cages and abnormalities were observed in metamorphs that developed in the ponds outside the cages, we could conclude that the predators might be responsible for the abnormalities. Other factors modified by the cages included UV light, cage injury, and elimination of vertebrate predators. Since UV light exposure was another potential factor in the rate of abnormalities and the cages modified the light levels to which the tadpoles were exposed, we cannot be absolutely sure that the predators were the causal agent. Damage caused to the developing tadpoles by the cages themselves (during monitoring and cage removal) would also confound the results, and therefore cages were handled carefully at every

opportunity. The exclusion of vertebrate predators by the cages was another confounding factor that could not be avoided. The cages did, however, allow the tadpoles to develop in a pond environment with no (or very few) invertebrate predators.

In 2005, we did not obtain any useable results from the exclusion cage experiments as both the tadpoles and the predators were much more difficult to contain than anticipated. In 2006 the cages were further modified and were more successful at excluding predators. In addition only 50 eggs were added to each cage (as opposed to 100 in 2005) to reduce food competition between the developing tadpoles.

The results from the 2006 study were mixed once again. Several cages contained no tadpoles very early in the study, indicating that the eggs had not survived the handling and new environment. In the cages that did produce tadpoles, however, very few predators gained access, and we observed a very low injury rate. The cages from pond KNA01 produced only 12 metamorphs, but with no injuries categorized as abnormal. Two cages from KNA03 fared better, with 33 metamorphs, and no injuries despite the presence of 3 small predators. Interestingly, the third cage from KNA03 produced no tadpoles, although 1 large dragonfly larvae was found in the cage. The cages from KNA08 produced 41 metamorphs. One metamorph had two bleeding limbs, although this may have been caused by the cage extraction. Only three small predators were found in the cage with the injured metamorph. Interestingly, this same cage also produced two metamorphs with eye abnormalities (one hollow, one missing). Eye abnormalities were not analyzed as part of the injury types associated with insect predators.

Overall it appears that very few, if any injuries in the cages could be attributed to the few small insect predators found in the cages. While certainly not indicative of causality, we can tentatively conclude that the exclusion of (most) invertebrate predators eliminated the skeletal type injuries found in metamorphs outside of the cages.

Conclusions

More than 3,000 predatory invertebrates were catalogued from 138 sweeps. Diversity indices showed increasing predatory invertebrate diversity and richness over the season, as well as higher diversity and richness in shallow sites compared to deep sites.

Direct relationships between individual invertebrate taxa and specific injury types were detected using linear regression and ANOVA, indicating at least a correlation between five of the observed invertebrate predators and observed injury rates across the study sites. Furthermore, a strong time component and further predator-injury correlations were detected using a multivariate redundancy analysis. High positive correlations were found to occur between Skeletal Malformation rates and five invertebrate predatory taxa abundances in early season sampling, when tadpoles would likely be smallest and most vulnerable. These predatory taxa included two species of dragonfly nymphs (*Aeshna sitchensis*, *Leucorrhinia proxima*), and three beetle genera (*Dytiscus*, *Acilius*, and *Ilybius*). We also detected high negative correlations between Skeletal Injury rates late in the season and two large predatory dragonfly species (*Aeshna sitchensis*, *Leucorrhinia glacialis*), along with high positive correlations to two smaller predatory genera. These, along with other RDA analyses suggest that while invertebrate predators could likely be responsible for injuries early in the season, there appears to be another cause for the injuries that occur late in the season, which is also causing the absence of the larger invertebrate predatory taxa.

The invertebrate predator exclusion cage study was successful in allowing tadpoles to complete metamorphosis, and relatively successful at excluding predators. While not conclusive, the cage study results suggest that predatory invertebrates could be the cause of metamorph injury.

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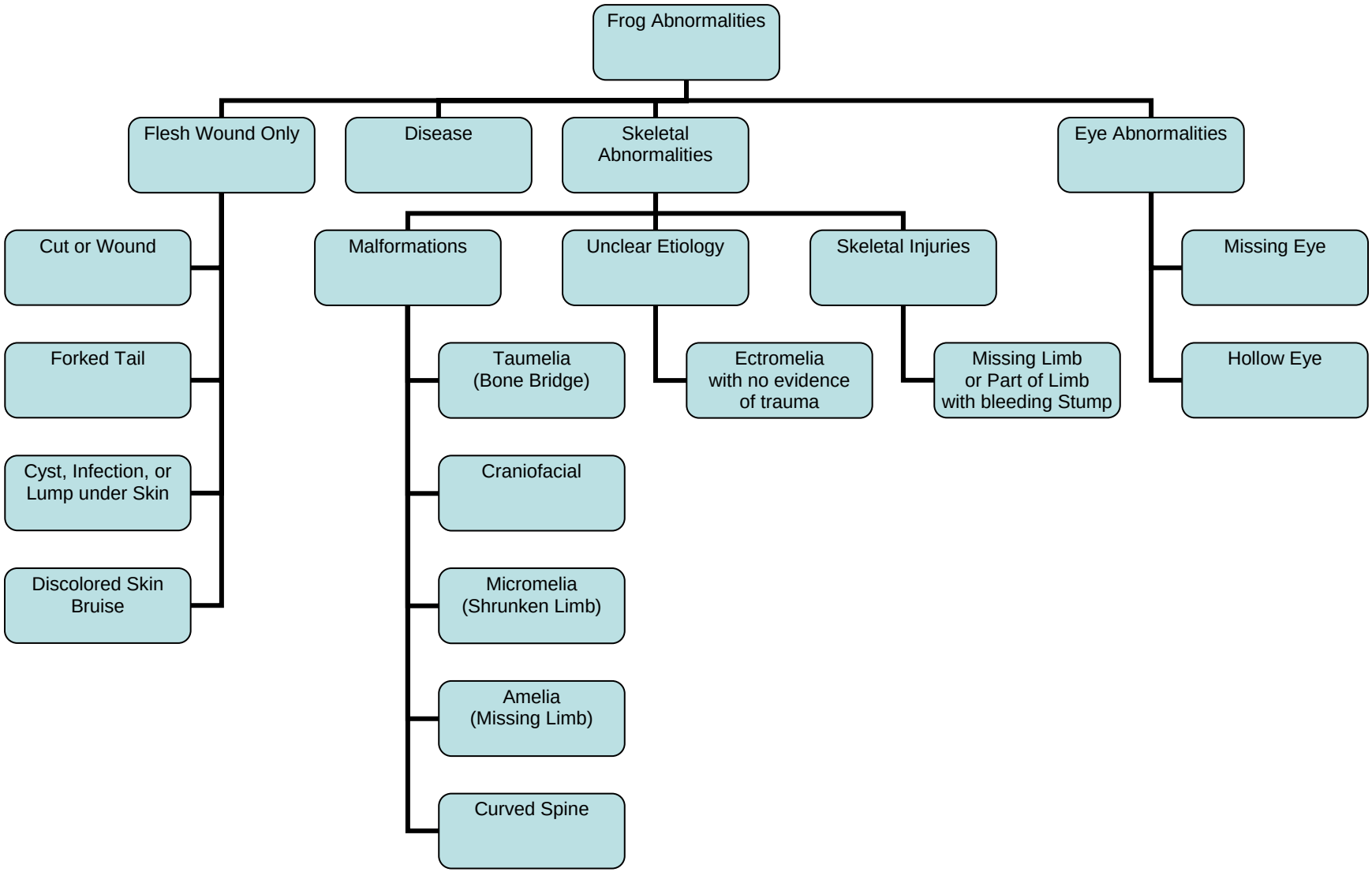
Appendix 1. Injury Decision Rules (courtesy of Mari Reeves)



Appendix 2. Invertebrate Sweep Sample Data

Invertebrate counts from May 2006 (early season) sweep samples.

	L.glacialis	*Libellula*	*L.proxima*	*Lestes*	*Coenag.*	*Dytiscus*	*Graph.*	*Rhantus*	*Ilybius*	*Agabus*	*Acilius*	*Copelatus*	Corixidae	*Dolomedes*	Hirudinea
KNA01	0	0	1	0	30	1	0	0	0	0	0	0	1	0	0
KNA02	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
KNA03	0	0	0	0	0	0	0	0	0	0	0	0	47	0	0
KNA08	0	0	0	0	31	0	0	0	0	0	0	0	35	0	0
KNA111	0	0	0	0	0	0	0	33	258	0	1	0	8	0	0
KNA12	0	0	0	0	0	0	0	0	0	3	0	118	0	0	0
KNA14	1	0	2	0	31	0	0	0	0	0	1	0	12	0	0
KNA141	0	0	0	0	0	0	0	6	0	0	0	0	2	0	0
KNA21	0	0	1	0	5	0	0	0	67	0	0	0	11	0	0
KNA31	0	0	1	0	5	0	0	2	5	0	0	0	0	0	0
KNA46	0	0	0	0	47	0	0	0	0	0	0	0	75	0	0
KNA47	2	0	1	0	45	0	0	0	0	0	2	0	13	0	0
KNA51	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0
KNA54	0	0	0	0	2	0	0	6	22	0	0	0	1	0	0
KNA55	0	0	0	0	2	0	0	2	1	6	0	0	1	0	0
KNA56	0	0	0	0	10	0	0	0	0	0	0	0	0	0	0
KNA60	0	0	0	0	24	0	3	0	16	0	0	0	0	0	0
KNA62	5	0	0	0	18	0	0	0	0	0	0	0	0	0	0
KNA90	1	0	4	0	7	4	0	1	6	3	0	0	2	0	0
KNA95	11	0	0	0	0	0	0	0	1	0	0	0	2	0	0
KNA97	6	0	0	0	111	0	0	0	0	0	0	0	0	0	0
YD01	0	0	2	1	0	5	0	0	0	0	0	0	8	0	0
YD04	0	0	0	0	0	2	5	25	0	6	0	0	4	0	0
YD06	0	0	1	5	1	0	2	12	0	0	0	0	7	0	0
YD07	0	0	18	23	5	7	3	12	0	6	0	0	1	0	0
YD09	0	0	4	28	0	0	10	4	0	1	1	0	2	0	0
YD22	0	0	2	0	1	2	0	0	0	0	0	0	0	0	0

Invertebrate counts from July 2006 (late season) sweep samples.

	L.glacialis	*Libellula*	*L.proxima*	*Lestes*	*Coenag.*	*Dytiscus*	*Graph.*	*Rhantus*	*Ilybius*	*Agabus*	*Acilius*	*Copelatus*	Corixidae	*Dolomedes*	Hirudinea
KNA01	0	0	3	14	97	1	0	0	0	0	0	0	0	0	0
KNA02	0	0	0	6	0	1	0	0	0	0	0	0	3	1	0
KNA03	0	0	0	32	23	0	1	6	0	0	0	0	0	0	0
KNA08	18	0	27	22	10	0	2	1	0	0	0	0	1	1	0
KNA12	0	0	0	20	0	0	3	4	0	1	0	0	38	1	0
KNA13	20	0	0	10	18	1	0	0	0	0	0	0	0	0	0
KNA21	0	0	3	6	0	0	0	1	0	0	0	0	4	1	0
KNA31	0	0	1	0	0	1	0	1	0	0	0	0	2	0	0
KNA46	0	0	3	1	13	0	0	0	0	0	0	0	0	1	0
KNA47	0	0	2	29	4	0	0	0	0	0	0	0	1	1	0
KNA51	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
KNA54	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
KNA55	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0
KNA56	0	0	0	0	7	1	0	2	0	0	0	0	0	0	0
KNA60	0	0	2	8	6	0	2	8	0	0	0	0	1	0	0
KNA62	43	0	0	16	28	0	0	0	0	0	0	0	0	0	0
KNA90	6	0	2	9	3	0	1	0	0	0	0	0	2	1	0
KNA95	15	1	3	0	1	0	3	3	0	2	0	0	8	0	0
KNA97	2	0	20	26	78	0	1	2	0	0	0	0	2	0	1
KNA111	Dry														
KNA141	Dry														

Appendix 3. Injury Rates Used in Correlation Analysis for 2005/2006

Year	Site	Fleshwound	Skeletal Injury	Skeletal Malformation	Skeletal Abnormality
2006	KNA01	0.119266	0.018349	0.0180	0.037000
2006	KNA03	0.103448	0.017241	0.0000	0.052000
2006	KNA08	0.142857	0.010989	0.0110	0.033000
2006	KNA12	0.25	0.0625	0.0000	0.062500
2006	KNA14	0.044944	0	0.0110	0.011236
2006	KNA31	0.196078	0.009804	0.0000	0.009804
2006	KNA46	0.06	0.02	0.0100	0.040000
2006	KNA47	0.010417	0	0.0100	0.010417
2006	KNA60	0.09	0.02	0.0000	0.020000
2006	KNA90	0.013333	0.013333	0.1200	0.160000
2006	KNA97	0.321429	0	0.0000	0.000000
2005	KNA01-3	0.031746	0.031746	0.111111	0.158730
2005	KNA03-3	0.019608	0.000000	0.019608	0.039216
2005	KNA08-3	0.044118	0.029412	0.029412	0.088235
2005	KNA12-3	0.068966	0.000000	0.017241	0.017241
2005	KNA14-3	0.054545	0.018182	0.072727	0.072727
2005	KNA31-3	0.068627	0.000000	0.000000	0.000000
2005	KNA46-3	0.000000	0.040000	0.080000	0.120000
2005	KNA47-3	0.084211	0.000000	0.021053	0.031579
2005	KNA60-3	0.020000	0.040000	0.020000	0.060000
2005	KNA90-3	0.037037	0.000000	0.018519	0.037037
2005	KNA97-3	0.078125	0.015625	0.000000	0.015625

Appendix 4. Invertebrate Exclusion Cage Data for 2006.

Site	Cage#	Date	SVL	TAL	Gosner	CAGE COMMENTS	FROG COMMENTS
KNA01	1	6/23/2006	21	27	43	SAW SMALL LEBILLULID IN CAGE #1	
KNA01	1	8/4/2006	10	12	43	SAW SMALL LEBILLULID IN CAGE #1	
KNA01	1	8/4/2006	9	21	42	SAW SMALL LEBILLULID IN CAGE #1	
KNA01	2	8/4/2006	12	1	45	PULLED CAGES	
KNA01	2	8/9/2006			41	PULLED CAGES	
KNA01	2	8/9/2006			41	PULLED CAGES	NOTCHED TAIL
KNA01	2	8/9/2006			41	PULLED CAGES	
KNA01	2	8/9/2006			38	PULLED CAGES	
KNA01	2	8/9/2006			37	PULLED CAGES	
KNA01	2	8/9/2006			38	PULLED CAGES	END OF TAIL CUT OFF
KNA01	2	8/9/2006	16	18	42	PULLED CAGES	
KNA01	2	8/9/2006	16	26	42	PULLED CAGES	
KNA03	1	7/25/2006	12	21	42	DAMSELFLY INSIDE ON FLOAT	
KNA03	1	7/25/2006	14	16	43	DAMSELFLY INSIDE ON FLOAT	
KNA03	1	7/25/2006	14	19	42	DAMSELFLY INSIDE ON FLOAT	
KNA03	1	7/25/2006	15	18	42	DAMSELFLY INSIDE ON FLOAT	
KNA03	2	7/25/2006	13	0	46		
KNA03	2	7/25/2006	14	1	45		
KNA03	2	7/25/2006	14	7	43		
KNA03	2	7/25/2006	15	0	46		
KNA03	2	7/25/2006	14	0	46		
KNA03	2	7/25/2006	16	0	46		
KNA03	3	7/25/2006	14	24	42		
KNA03	3	7/25/2006	15	19	42		
KNA03	3	7/25/2006	14	22	42		
KNA03	3	7/25/2006	14	23	42		
KNA03	1	8/4/2006	13	0	46	SAW SMALL LEBILLULID IN CAGE #1	
KNA03	1	8/4/2006	14	1	45	SAW SMALL LEBILLULID IN CAGE #1	
KNA03	1	8/4/2006	13	2	45	SAW SMALL LEBILLULID IN CAGE #1	
KNA03	1	8/4/2006	13	1	45	SAW SMALL LEBILLULID IN CAGE #1	
KNA03	1	8/4/2006	12	12	42	SAW SMALL LEBILLULID IN CAGE #1	
KNA03	1	8/4/2006	11	1	45	SAW SMALL LEBILLULID IN CAGE #1	

Site	Cage#	Date	SVL	TAL	Gosner	CAGE COMMENTS	FROG COMMENTS
KNA03	1	8/4/2006	10	0	46	SAW SMALL LEBILLULID IN CAGE #1	
KNA03	2	8/4/2006	15	2	45		
KNA03	2	8/4/2006	12	0	46		
KNA03	2	8/4/2006	12	0	46		
KNA03	3	8/4/2006	12	0	46		
KNA03	3	8/4/2006	12	21	43		
KNA03	1	8/9/2006			37	2 CM DAMSEL FLY LARVA AND 5 CM LEECH IN CAGE	
KNA03	1	8/9/2006			41	2 CM DAMSEL FLY LARVA AND 5 CM LEECH IN CAGE	
KNA03	1	8/9/2006	16	27	42	2 CM DAMSEL FLY LARVA AND 5 CM LEECH IN CAGE	
KNA03	1	8/9/2006	15	11	45	2 CM DAMSEL FLY LARVA AND 5 CM LEECH IN CAGE	
KNA03	1	8/9/2006			34	2 CM DAMSEL FLY LARVA AND 5 CM LEECH IN CAGE	
KNA03	2	8/9/2006			38	1.5 CM LEBELLULID	
KNA03	3	8/9/2006				3 CM DRAGONFLY LARVA IN CAGE. NO FROGS. PULLED CAGE	
KNA08	1	7/25/2006				CAGE EMPTY AND OUT OF THE WATER	
KNA08	2	7/25/2006	14	0	46		
KNA08	2	7/25/2006	13	15	44		
KNA08	2	7/25/2006	15	5	45		
KNA08	2	7/25/2006	15	1	45		
KNA08	2	7/25/2006	14	24	44		
KNA08	2	7/25/2006	15	25	42		
KNA08	2	7/25/2006	15	22	42		
KNA08	2	7/25/2006	16	25	42		
KNA08	2	7/25/2006	14	23	43		
KNA08	2	7/25/2006	13	18	43		
KNA08	2	7/25/2006	13	17	43		
KNA08	3	7/25/2006	15	1	45		
KNA08	3	7/25/2006	10	0	46		
KNA08	3	7/25/2006	17	22	43		HOLLOW RIGHT EYE
KNA08	3	7/25/2006	16	1	45		
KNA08	3	7/25/2006	16	3	45		
KNA08	3	7/25/2006	18	21	44		MISSING RIGHT EYE
KNA08	3	7/25/2006	17	15	44		
KNA08	3	7/25/2006	15	27	42		
KNA08	1	8/4/2006	15	28	42		

Site	Cage#	Date	SVL	TAL	Gosner	CAGE COMMENTS	FROG COMMENTS
KNA08	1	8/4/2006	14	0	46		
KNA08	1	8/4/2006	14	0	46		
KNA08	1	8/4/2006	12	0	46		
KNA08	1	8/4/2006	13	1	45		
KNA08	1	8/4/2006	13	26	43		
KNA08	1	8/4/2006	14	0	46		
KNA08	1	8/4/2006	14	0	46		
KNA08	3	8/4/2006	13	1	45		
KNA08	2	8/9/2006	18	29	42	SMALL SPIDER IN CAGE 2	
KNA08	2	8/9/2006	17	20	43	SMALL SPIDER IN CAGE 2	
KNA08	2	8/9/2006	17	17	44	SMALL SPIDER IN CAGE 2	
KNA08	2	8/9/2006	15	5	45	SMALL SPIDER IN CAGE 2	
KNA08	2	8/9/2006	16	25	42	SMALL SPIDER IN CAGE 2	Tumor or growth in chest, inside skin. Released
KNA08	2	8/9/2006	16	22	42	SMALL SPIDER IN CAGE 2	
KNA08	2	8/9/2006	16	5	45	SMALL SPIDER IN CAGE 2	
KNA08	3	8/9/2006	16	0	46	Damselfly larva 2.5 cm long, 7 mm and 1.5 cm long dragonfly larvae	
KNA08	3	8/9/2006	15	10	44	Damselfly larva 2.5 cm long, 7 mm and 1.5 cm long dragonfly larvae	BLOODY RFL AND LHL. RELEASED
KNA08	3	8/9/2006	17	29	43	Damselfly larva 2.5 cm long, 7 mm and 1.5 cm long dragonfly larvae	
KNA08	3	8/9/2006	16	0	46	Damselfly larva 2.5 cm long, 7 mm and 1.5 cm long dragonfly larvae	
KNA08	3	8/9/2006	16	0	46	Damselfly larva 2.5 cm long, 7 mm and 1.5 cm long dragonfly larvae	
KNA08	3	8/9/2006			39	Damselfly larva 2.5 cm long, 7 mm and 1.5 cm long dragonfly larvae	

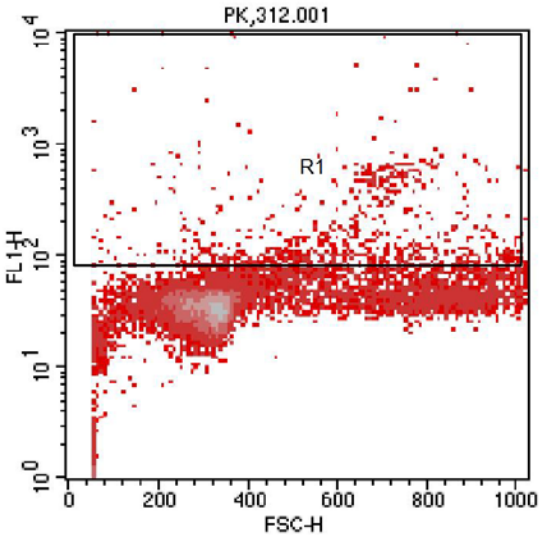
Appendix B - DNA and Biomarker Report

USGS
science for a changing world

National Wetlands Research Center, USGS, Lafayette

A Biomarker Approach for Measuring Cytogenetic Impacts to Anurans: Wood Frogs (*Rana sylvatica)* from Kenai National Wildlife Refuge, Alaska

by Jill A. Jenkins



This report is preliminary and has not been reviewed for conformity with U.S. Geological Survey editorial standards or with the North American Stratigraphic Code.

Outside front cover photographs:

Flow cytometric dot plot showing nuclei from peripheral blood from wood frogs from the Kenai National Wildlife Refuge, Alaska. Nuclei are labeled with a fluorescent marker showing levels of DNA repair protein.

One pond sampling site in winter 2005 from the Kenai National Wildlife Refuge, Alaska.

Submitted by: Jill A. Jenkins *signed*
Research Microbiologist, Wetlands Ecology Branch

Supervisor: Jack Waide *signed*
Branch Chief, Wetlands Ecology Branch

To: Kim Trust, Branch Chief, Environmental Contaminants Program, USFWS Region 7, Alaska Fish and Wildlife Field Office, Anchorage, Alaska 99501-2249

Subject: Final Report USFWS 70181-4-N205

Kenai Investigations group, February 2004.

Contents

Preface and Acknowledgements

The Kenai National Wildlife Refuge (KNWR) located on the Kenai Peninsula in south central Alaska, is the most frequently visited refuge in Alaska, and is one of two Alaskan refuges on the road system (Trust and Reeves 2004). High numbers (Ouellet 2000) of physical abnormalities in wood frogs (*Rana sylvatica*) have been documented in consecutive years on the KNWR. Known sources of contaminants within the refuge have also been documented. In an attempt to identify and evaluate factors that contribute to the causality of these physical abnormalities in wood frogs sampled from KNWR, the USFWS contributed financial resources to support a research project at the USGS National Wetlands Research Center (NWRC). This report describes and documents the main results of this project, in particular the development and use of three biomarkers optimized for use in detecting cytogenetic impacts using anuran blood from sampling that occurred during 2004 – 2006. Herein, each biomarker is explained and described in relation to anuran scientific literature reviewed. The report presents each dataset with associated results and summarizes statistical analyses obtained to date. Validated protocols for the individual biomarkers, that will be applicable with other anurans, are presented for the first time in print. Future statistical analyses applied to these dependent variables, along with others collected by USFWS and their collaborators, will yield more meaningful and environmentally relevant results with regard to the cytogenetic biomarkers and how they may or may not relate to wood frog abnormalities. These analyses and their interpretations will be documented in future joint publications.

Site designation and animal collections were made in accordance with the overall field sampling plan authored by the USFWS. Animals were processed either in the field by USFWS and contracted personnel, or by Dr. D. Sutherland of the Department of Biology at the University of Wisconsin. A battery of tests was conducted by the Kenai research group working during this time period. Biomarker development and blood analyses were carried out independently at NWRC.

The author thanks Kim Trust for serving as a mentor dedicated to preserving our nation's trust resources and for exhibiting excellent writing skills and attention to detail, and to Mari Reeves for her energetic quest to unravel the factors leading to high wood frog abnormality rates documented at KNWR. The author respectfully acknowledges the pioneering work of Dr. Sutherland, now deceased, in collaborative development of the blood collection and shipping

protocol from animals as small as one's thumbnail. Thanks are also extended to Don Lawson for diligent field efforts in 2006, and to Dr. Rassa Dale for statistical analyses of USGS data. Finally, thanks are extended to the entire assemblage of scientists (including Connie, Roxanna, Tina, Ed, and Fred) involved with these intellectually exciting efforts in attempting to understand the parameters involved with this abnormality phenomenon.

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The use of trade names in this report is solely for identification purposes and does not constitute endorsement by the U.S. Department of the Interior.

Introduction

Monitoring phenotypic traits of animals can provide an effective means of identifying biological impacts of environmental stressors, including environmental contaminants and climate change (Hoffmann and Daborn 2007). However, studying biomarkers that are functional measures of exposure to environmental stressors, typically expressed at the suborganismal level of biological organization (Adams 2002), provides an objective assessment of environmental changes. Biomarkers are early-warning indicators of pollution, since their responses occur before irreversible damage to organisms or to entire ecosystems (Adams 2002; Barni et al. 2007). Using a comparative animal approach in environmental studies whereby lines of inquiry are applied across taxonomic lines, conclusions are strengthened by virtue of crossing evolutionary barriers. One crucial measurable physiological biomarker of exposure is DNA. By virtue of the universality of DNA molecular structure and the physiological processes involved in maintaining its replicative fidelity for future generations, DNA biomarker studies provide an approach that is comparable across animal species, whether invertebrate or vertebrate. Because of the evolutionarily conserved nature of the modes of action related to repair and structure maintenance, multiple species comparisons are valid.

The purpose of this particular research study was to contribute information regarding potential impacts to DNA from wood frogs, *Rana sylavatica*, sampled from selected sites within Kenai National Wildlife Refuge (KNWR). These analyses were completed in accordance with an On-Refuge Investigations Sub-Activity by USFWS for Region 7, whereby the KNWR was the site of an investigation into potential impacts from contaminants to wood frogs, many of which exhibit specific physical abnormalities.

The use of complementary biomarkers is a sound method for investigations into impacts of environmental stressors, yielding a robust assessment of animal condition and likely causes of identified abnormalities. In this regard, this report provides a synthesis of background information on each of the three biomarkers responses measured: DNA integrity, apoptosis, and DNA repair protein. This report describes how the three evolutionarily conserved biomarkers are associated mechanistically. For each biomarker, previously obtained research results applicable to this study as well as with anurans (or aquatic animals) in general are summarized. Protocols validated and data generated for each biomarker endpoint from samples sent from the

field are provided and interpreted. Results of statistical analyses, although preliminary in nature, are also presented.

Background

Amphibian populations around the world are declining at alarming rates (Hopkins et al. 2006; Skerratt et al. 2007), and are also showing increasing frequencies of physical or developmental abnormalities (Ouellet 2000). By virtue of the North American Amphibian Monitoring Program, relatively high numbers (Ouellet 2000) of abnormal wood frogs (*Rana sylvatica*) were documented on the KNWR in Alaska during consecutive years of monitoring since 2000 (Trust and Tangermann 2002; Trust and Reeves 2004). Complex interactions likely contribute to malformations in wild frog populations, including such factors as parasitic trematodes (*Ribeiroia ondatrae*) (Johnson and Sutherland 2003; Skelly et al. 2007), amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) (Skerratt et al. 2007), ultraviolet B radiation (Blaustein et al. 1997), and exposure to xenobiotic pesticides or chemicals (Wake 1991; Carey and Bryant 1995; Alford and Richards 1999; Blaustein and Kiesecker 2002). Understanding such difficult scientific problems is advanced through an interdisciplinary research approach that integrates biological processes, from the molecular level to that of organisms and ecological communities (Wilcox and Colwell 2005).

Amphibians are valuable indicators of environmental stress in that they have semi-permeable skin (Lee and Stuebing 1990) and live in close contact with water as larvae, with most species having contact with land as adults (Blaustein and Kiesecker 2002). They may be considered sentinel species in aquatic habitats because crucial phases of their development occurs in water, generally not venturing far from where they were hatched (Blaustein and Johnson 2003). A field-based approach for studying amphibian malformations that addresses chemical agents associated with wetlands deserves particular attention (Skelly et al. 2007). Wood frogs reproduce in early spring as the snow melts, in shallow waters ranging from roadside ditches to open ponds, some of which are ephemeral. Episodic exposures of young animals to contaminants may occur during sensitive periods of embryonic development, when even low doses can impact health or development (Birnbaum 1995). The KNWR has experienced various types of contaminant stressors, including those linked with oil and gas development, pesticide use, mining, and polychlorinated biphenyls (Parson 2001; Trust and Reeves 2004).

Noxious chemical compounds can have effects on DNA and on cellular division, potentially leading to the induction of mutagenesis and carcinogenesis (Fimognari et al. 1999). Many waterborne pollutants have cytogenetic properties which cause enhanced frequencies of chromosomal aberrations or the alteration of the structure of DNA. Because of the universality of the DNA molecule, agents that are genotoxic for one group of living organisms are typically genotoxic for other groups (Al-Sabti 1985). Thus, molecular genotoxicity biomarkers can be effective early warning tools (Maria et al. 2002).

The objective of this study was to develop and employ three specific cytogenetic biomarkers for use with wood frogs: DNA integrity, DNA repair protein presence, and apoptosis. All of these processes are mechanistically linked, occurring somewhat sequentially kinetically; therefore, their measurement provides an integrated and comprehensive assessment of the cytogenetic process. Investigations into relationships among these three response variables along with other dependent and independent variables will yield important information about possible cause and effect mechanisms associated with wood frog abnormalities at the KNWR. Relevant variables include such parameters as distance to road, site type (remote or developed), elevation, contaminant levels and types, as well as morphology, histologic sex, and Gosner developmental stage. In subsequent analyses, multiple logistic regression models will be developed, similar to the robust approach taken by Taylor (2005), facilitating the use of multivariate statistical techniques in modeling the relative effects of different factors possibly linked to the occurrence of wood frog abnormalities at KNWR. Correlation matrix analysis is another way to approach the teasing out of important relationships among contaminants and biomarkers.

DNA Integrity

Genotoxic agents increase DNA damage and rates of mutation which can be heritable through mutations in germ line cells and teratogenic effects (Mitchelmore and Chipman 1998). DNA lesions are the first event in the carcinogenetic process induced by a genotoxic agent (Maria et al. 2002). Exposure to xenobiotics produces reactive oxygen species (ROS) that can result in cellular and nucleic acid damage. Oxidative DNA damage – functional or structural alterations resulting from the insults of ROS (Shen and Ong 2000) – can result during contaminant biotransformation processes that generate ROS, as well as by transition metals (Qu et al. 2001), metabolism, and inflammation (Martinez et al. 2003; Sorensen et al. 2003).

Oxidative DNA damage may also arise from a suppressed antioxidant defense system (Ciereszko et al. 1999; Dabrowski and Ciereszko 2001; Qu et al. 2001).

Ionizing radiation can cause strand breakage directly, whereas UV light or genotoxic chemicals can cause alterations to the DNA molecules that are candidates for repair and thus for strand breaks (Shugart and Theodorakis 1994). Factors interacting to assault DNA, such as ultraviolet B together with octylphenol, were found to alter the expression of hypothalamic genes and affect hindlimb emergence in *Rana pipiens* tadpoles (Crump et al. 2002). However, UV exposures have often produced bilateral and symmetrical abnormalities, not reflective of those found in the wild (see Taylor 2005).

Genomic DNA alterations and fragmentations are widely used in physiological, genetic and toxicological studies, and several biotechnical assays are available for measuring DNA strand breaks (e.g., DNA alkaline unwinding assay, sperm chromatin structure assay, single cell gel electrophoresis, other electrophoretic techniques, and flow cytometry) (Berwick and Vineis 2000). The advantage of flow cytometry for measuring DNA integrity (as well as other characteristics of cells or nuclei) is that thousands of cells can be analyzed in a few seconds with objective precision, giving a statistically precise evaluation via a reproducible technique, and multiple parameters can be measured from one cell simultaneously. Fresh or fixed cells can be used (Clemo et al. 1993).

Field studies documenting altered blood DNA content or profiles upon exposure have included radionuclotides with turtles and ducks (George et al. 1991), mercury with largemouth bass (Sugg et al. 1995), petrochemicals with wild rodents (McBee and Bickham 1988), aromatic hydrocarbons with English sole (Jenner et al. 1990), radioisotopes with slider turtles (Lamb et al. 1991), contaminants with great blue herons (Custer et al. 1997), and pesticides with green frogs (Lowcock et al. 1997). Levels of double stranded (ds) DNA breaks were found to be negatively correlated with mosquitofish fecundity (Theodorakis et al. 1997). DNA fragmentation and other apoptotic features were induced in tropical clawed frogs *Silurana tropicalis* by 3,3’5-triiodothyronine (Goto et al. 2006). DNA strand breaks were induced by cadmium (II), a known human and animal carcinogen, in cells from *Pleurodeles* brain (Calevro et al. 1998). The pancreas of *Bufo bufo* was shown to undergo programmed cell death or apoptosis during metamorphosis (Accordi and Chimenti 2001). Flow cytometry results did not demonstrate genotoxicity in anuran larvae in a controlled study (Freeman and Rayburn 2004a).

Apoptosis

In addition to DNA repair, the apoptotic process should be taken into account for a comprehensive understanding of the consequences of a genotoxic insult (Fimognari at al. 1999). Apoptosis is programmed cell death mediated through multiple steps of enzyme and substrate interactions along a timeline whereby early and late apoptotic steps can be distinguished, and the three phases of induction, execution, and degradation are distinguishable. Cell death is characterized by a series of morphological and biochemical changes resulting in their efficient elimination from tissues without eliciting inflammation. Apoptosis is critical in pathological and homeostatic processes, including development. Cells experiencing genotoxic damage may be removed by apoptosis. In this study, as described below (see methods), peripheral blood was the tissue selected for study as it was not experiencing apoptosis due to metamorphosis.

Some functional changes occurring during apoptosis include dissipation of the mitochondrial transmembrane potential by virtue of leaking of cytochrome C (which activates caspase 9 [Martin et al. 2007] that activates caspase 3) (Li and Darzynkiewicz 1999), increase in ROS, and finally DNA strand breaks, chromatin condensation, and cell shrinkage. During the process, proteins are stripped off the DNA, whereby it becomes more vulnerable to nicking; low molecular weight DNA will leak out of the nucleus. These lower molecular weight DNA peaks and apoptotic bodies are detectable by flow cytometry. A relatively late event is the transition of the inner membrane proteins (phosphatidyl serine, PS) from the inside of the cell to the outside, where they are easily stainable, but have been shown to have such damaged DNA that at this stage cells were not able to be measured by the comet assay (Basco and Eliason 2001). At the heart of the apoptosis pathway are the caspases (cysteine proteases with aspartate specificity), of which there are over 10, the overall machinery having been found to be evolutionarily conserved (Slee et al. 1999) between mammals and amphibians (Sakamaki et al 2004). Activation of the effector caspase 3 induces cell surface changes (e.g., PS switching) and nuclear events (Martin et al. 2007). Of all caspases, caspase-3 is among the most important because DNA strand breaks are generated (Paasch et al. 2004).

Loss of telomeres (end portion of chromosomes) function can lead to genetic instability and cancer progression, and it also induces apoptosis (Hemann et al. 2001). Normal cells maintain chromosome stability, while cancer cells are characterized by frequent chromosomal changes. Reproductive cancer in laboratory rodents and humans and endocrine disruption in wildlife has

been linked with atrazine, the most common pesticide contaminant of ground and surface water (Fan et al. 2007). Atrazine has also been linked to altered sexual development in amphibians (Hayes et al. 2002).

DNA Repair

Double stranded DNA lesions are the most serious type of damage (Bradbury and Jackson 2003), whereby these unrepaired ds breaks are the principle lesions leading to cell death or the formation of mutations and/or chromosomal aberrations (see Belloni et al. 2005). The measurement of DNA damage is a result of the interaction between the damaged DNA and the repair process. One endpoint of repair is sometimes the measure of the DNA damage, such as that measured with flow cytometry, micronucleus assay, or the comet assay. Other endpoints can be rates of repair or polymorphisms in the repair genes (Berwick and Vineis 2000). A direct reflection of the repair capacity is the measure of DNA-dependent protein kinase (DNA-PK) (Smith and Jackson 1999), one of the first kinases involved in signal transduction (Bradbury and Jackson 2003). This protein is activated by ds breaks in DNA and is a key in the predominant mechanism of repair termed nonhomologous end-joining (Smith and Jackson 1999). It is comprised of two subunits, with functions (Gu et al. 1998) and homologs for the catalytic subunit termed DNA-PKcs having been identified in *Xenopus* (Labhart 1997). The genes for the DNA-targeting subunits termed Ku70 and Ku 80 (Ku86) have been found in organisms ranging from yeast to human (Dynan and Yoo 1998) as the process for signaling and repair is highly conserved (Bradbury and Jackson 2003). The DNA-PKcs is recruited to the break and stabilizes the bridge and activates other factors needed for the DNA repair, and it may also be directly involved (Bradbury and Jackson 2003).

When the burden of genomic insult is too large to be effectively repaired, cells initiate apoptosis, thereby eliminating them from the population (Belloni et al. 2005). During apoptosis, the DNA-PKcs is specifically cleaved by caspase-3 with subsequent loss of activity. Cadmium was shown to interfere with the repair of oxidative DNA damage in brain cell cultures from larvae of Spanish ribbed newt *Pleurodeles waltl* (Calevro et al. 1998).

Materials and Methods

Study Area and Sampling Design

Pond selection was made by FWS personnel, and sampling performed similarly to previous years (Trust and Reeves 2004; Trust and Tangermann 2002). Pond locations (Fig. 1) were identified with a handheld GPS unit. Samples were collected and sent during or immediately after the field seasons of 2004, 2005, and 2006. In 2004, DNA integrity was measured, and the two other biomarker protocols were developed and optimized. In 2005 and 2006, data on all three biomarkers were obtained. In 2006, a controlled study was performed with water and sediment from selected sites, and those animals were tested for DNA integrity (Appendix 1).

Tissue Choice

Amphibian hematological parameters have been used to study natural environmental changes and to detect the presence of pollutants (see Barni et al. 2007; Stansley and Roscoe 1996). Micronuclei (cytoplasmic inclusions of chromosomal fragments or whole chromosomes not incorporated into the main nucleus during cell division as a result of DNA fragmentation or alteration of the mitotic apparatus [see Barni et al. 2007]) have been investigated with controlled studies of amphibians (e.g., Krauter 1993; Mouchet et al. 2005). Their incidence is low and much labor is involved with detecting them by microscopy. In comparison, large numbers of cells are preferable for detection by flow cytometry. Nucleated blood cells complicate this detection. In this study of wood frogs, blood cell numbers were limiting. DNA damage in amphibians has been detected by some controlled studies using the comet assay (e.g., Clements et al. 1997; Ralph and Petras 1998; Mouchet et al. 2005) Again, much labor is needed for assaying a relatively small number of nuclei by comet assay for ds DNA breaks. Because susceptibility of DNA to damage and its repair can differ between cell types and between quiescent and proliferating cells of a single cell type (Potter et al. 2002), blood cells of metamorphs of about the same size and age were obtained. Animals no younger than Gosner stage 42 (Gosner 1960) were used. This was beyond the metamorphic climax because larval mouthparts had been resorbed. Peripheral blood was a good tissue choice because of its fluid nature. Flow cytometry measures attributes of individual cells or nuclei flowing in a fluid stream. DNA fragmentation in

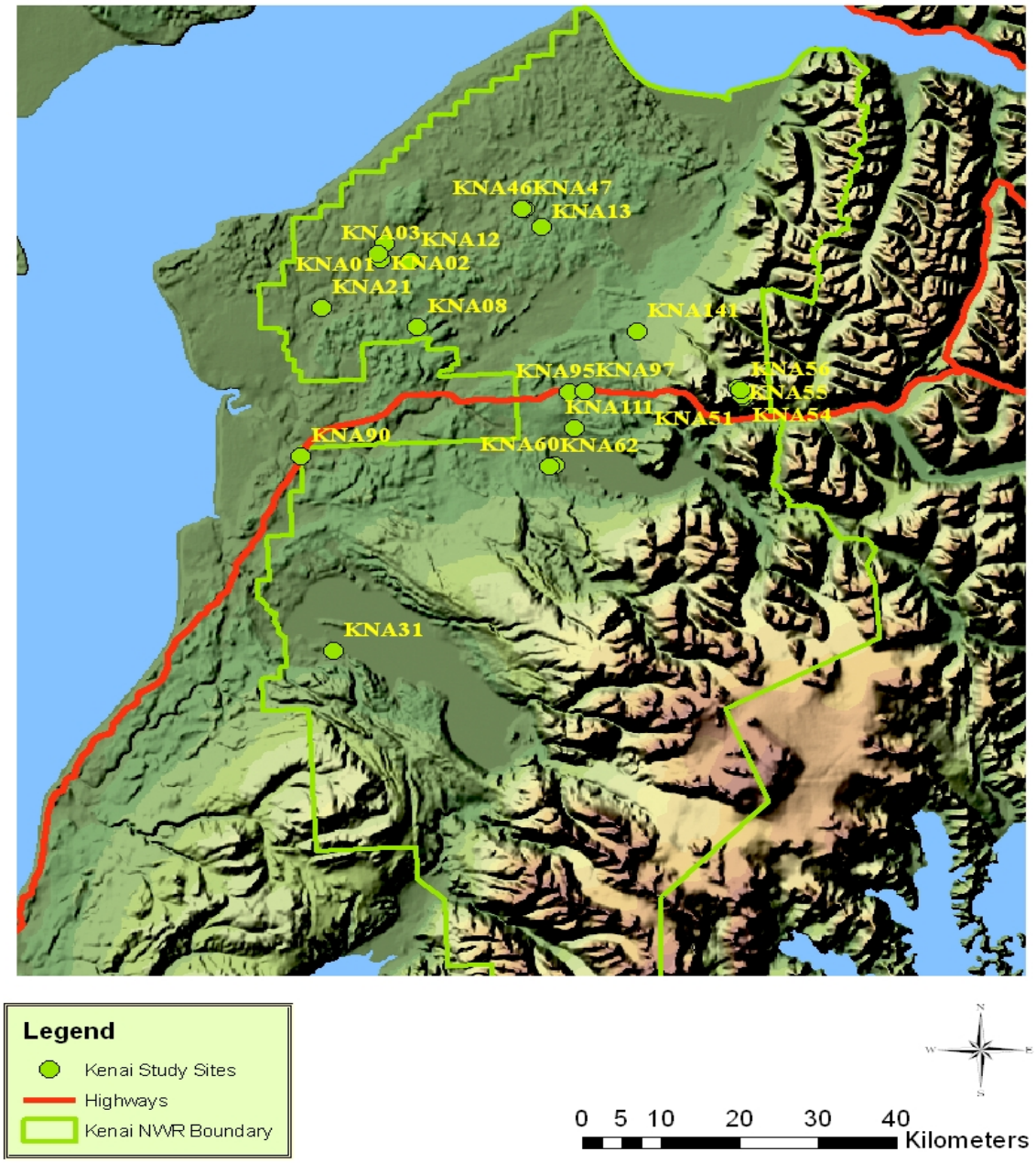


Figure. 1. Sampling sites for wood frogs from the Kenai National Wildlife Refuge.

post-larval bullfrog (*Rana catesbeiana*) was highest at the metamorphic climax (Hasebe et al. 1996), and postmetamorphic blood with non-larval hemoglobin remained in circulation a long time (Hasebe et al. 1999).

Animal Collection

Frogs were coded by field personnel at the KNWR so that other personnel would be unaware of their origin or whether the frogs were considered "abnormal" or from developed or remote sites. "Abnormality" was defined as anything physically anomalous with an animal based upon gross, visual observations in the field. Individual frogs were measured (snout-urostyle length), weighed (wet mass), and checked for abnormalities (Meteyer 2000). During 2004 – 2005, frogs were sent to Dr. Dan Sutherland at the University of Wisconsin- LaCrosse for assessment of parasite loads, animals were bled, and then euthanized. In 2006, animals were collected and processed at KNWR. Protocols for animal handling and experimentation were reviewed and approved by the Louisiana State University Department of Veterinary Sciences Institutional Animal Care and Use Committee.

Preliminary Experiments

Conditions were optimized for collection, shipping, and storage of blood. Frogs were anesthetized in an overdose of tricaine methane sulfonate (MS-222), and removed to petri dishes as soon as they were not responsive to prodding. Frogs were examined for malformations, snout-to-vent lengths (cm) were recorded, Gosner Stage (Gosner 1960) was recorded, tail remnant presence was recorded, and other metrics taken. Abnormalities were scored. Blood was collected with acid citrate dextrose (ACD) and placed into different media for storage: 0.5%, 1%, and 2% paraformaldehyde, 100% methanol, or 70% ethanol. Results of this preliminary experiment showed that 2% paraformaldehyde (Sigma Aldrich, St. Louis, MO; P6148) maintained the highest percentage of intact cells with the lowest level of DNA damage as compared with storage in the other media.

Blood Collection

In order to collect blood from each individual, microhematocrit tubes were filled 1/3 with acid citrate dextrose anticoagulant (ACD). A frog was anesthetized with an overdose of MS-222, and it was removed to a petri plate when no longer responsive to prodding. Each frog was examined for malformations and data recorded. Using a modification of ARMI SOP 101 (USGS NWHC 2007), a ventral incision was made near the inguinal area, then forward through the clavicle into the neck. The heart was punctured, and the atrium torn using clean microforceps and dissecting needles. The ACD from the microhematocrit tube was expelled on the heart

puncture and the tube filled with as much blood as possible, with repeated filling attempts and addition of ACD as needed. This whole blood suspension was then placed in 2% paraformaldehyde in phosphate buffered saline in labeled microfuge tubes, and tubes were mailed overnight with ice packs. Labels typically were tough tags (Diversified Biotech, Boston, MA). Pertinent information was recorded for each animal, including when and where the sample was taken from the field, when it was bled and fixed, and the frog ID number. Tubes were sent to NWRC via overnight express to arrive as early the next day as possible, stored in a cooler with ice packs to keep blood cool but not frozen.

Analyses of Blood for DNA Integrity

Nuclei were stained with equal volumes of 0.112% sodium citrate with 50 µg/mL propidium iodide (PI) (Sigma-Aldrich), RNAse A at 1 µg/mL (Sigma-Aldrich), and 0.1% (v/v) Triton X-100 (Sigma-Aldrich) for 30 min at 24°C. The distribution of nuclei in the G_0/G_1 phase of the cell cycle was analyzed with a flow cytometer (FACScalibur®; Becton Dickinson Immunocytometry Systems, San Jose, Ca [BDIS]) following instrument calibration using FACSComp (BDIS). Duplicate samples of nuclei were analyzed at 1 x 10^6 per mL at a rate of < 300 per second, and 5-10 K events were collected by using a 1024-channel FL2 parameter at 340 linear, with linear size and scatter parameters, and doublet discrimination mode. Histograms (Fig. 2), dot plots, and density plots were generated using CellQuest software (BDIS), and each replicate was displayed four ways: FSC versus SSC, FL2A histogram, FL2A versus FSC, and FL2W versus FL2A. These analyses allowed degraded samples to be distinguished from intact samples (Alanen et al 1989; Zbieranowski et al. 1993). The coefficient of variation (CV) was calculated at the full width of the peak at half the maximum height (Shapiro 1993). Cell Quest software (BDIS) was employed for analyses of flow cytometry data. The DNA integrities by CV width were categorized 1 to 4, with 4 being the least intact nuclear DNA (Fig. 2).

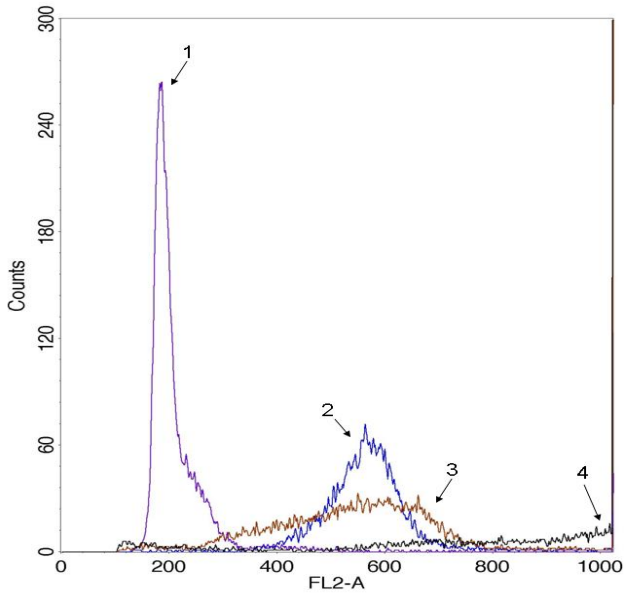


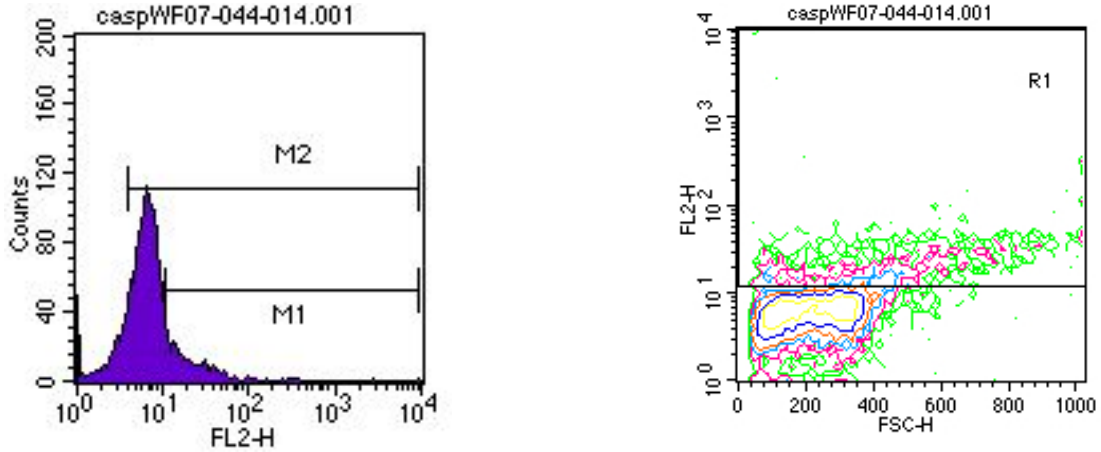
Fig. 2. Overlay of typical flow cytometric histograms showing fluorescently stained DNA (FL-2A) and the count of nuclei analyzed. DNA integrity categories are labeled "1" to "4" as best to worst, indicative of increasing peak width.

Analyses of Blood for Caspase 3

Part A. For detecting apoptosis using blood cells fixed as above, this procedure employed an antibody for the intracytoplasmic detection of the enzyme by using an antibody. The density of cells fixed in 2% paraformaldehyde in Dulbecco's Phosphate-Buffered Saline (PBS) (Gibco; Invitrogen, Carlsbad, CA; Cat. No. 14190-136) was adjusted to a concentration of 1×10^6 cells/mL using a hemocytometer. Cells were pelleted at 2500 rpm for 5 min and the supernatant discarded. Cells were washed twice by resuspension with 2% fetal bovine serum (FBS) (Sigma-Aldrich; Cat. No. F6178; lot 054K0365) in PBS, then resuspended in 500 µL lysing buffer (2% FBS in PBS with 0.5% Nonident-40 (NP-40; nonionic detergent) (Sigma-Aldrich; Cat. No. 21-3277) for 30 min at 4°C for membrane permeabilization to allow access of the antibodies for labeling the target protein (cytoplasmic caspase-3). Cells were centrifuged at 2005 rpm for 5 min and lysing buffer discarded. Cells were washed once with 2% FBS. Control cells included unstained and stained koi carp (*Cyprinus carpio carpio*) blood fixed and treated similarly. Each

of these steps in Part A are identical to those in the DNA repair protein detection assay below; hence, Part B is where the two protocols diverge. No more than 12 samples were processed at one time.

Part B. Cells were resuspended in 200 µl of 2% FBS and 40 µL of antibody [Phycoerythrin (PE)-conjugated, polyclonal rabbit anti-active Caspase-3 (Cat No. 557091), BD Biosciences, San Jose, CA] was added. Cells were incubated in the dark at 4°C for 2 hr. Cells were centrifuged at 2500 rpm for 5 min and the supernatant discarded, then resuspended in 500 µL of PBS. Stained cells were analyzed by flow cytometry as above. Samples were run in duplicate, and 5K to 10K cells per replicate were acquired using Cell Quest software (BDIS). The instrument settings were linear size (FSC) and scatter (SSC), and log fluorescence 2 (FL2H) at 585 nm (42 nm bandwidth). Cytograms were analyzed using histograms of FL2H, where markers were set for fluorescence of the main and high populations (Fig. 3A) and dotplots or contour plots where FSC was the x-axis parameter and fluorescence was the y-axis parameter, whereby those two regions were gated as above (Fig. 3B). Control cells were used for instrument adjustment baseline levels for similar gating among runs.



A) B)

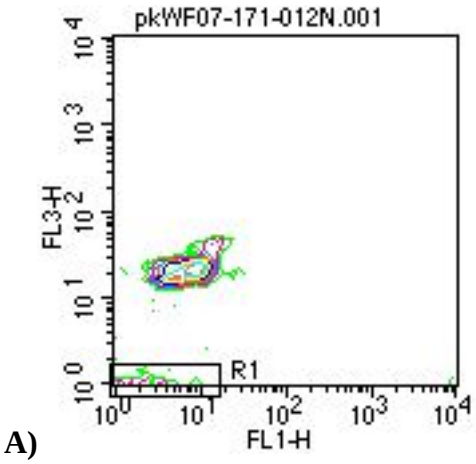
Fig. 3. A) Flow cytometric histogram showing fluorescence level of 10K blood cells from wood frog WF07-044-014, replicate 1, labeled with an antibody against caspase-3, reflective of apoptotic condition. Marker 1 (M1) indicates cells with higher levels of the enzyme. B) Flow cytometric contour plot of the same cells as (A), whereby Region 1 (R1) shows higher levels of the caspase-3 protein outside of the main population. Size is on the x-axis and red fluorescence due to anti-Caspase 3 binding is on the y-axis.

Analyses of Blood for DNA Repair Protein

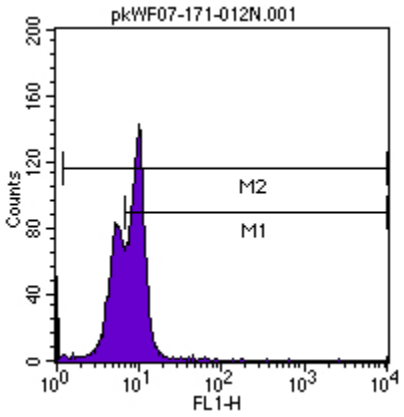
Part A. For detecting DNA repair protein using blood cells fixed as above, this procedure employed an antibody for the intranuclear detection of the catalytic subunit of DNA PK using a specific antibody. The steps for cell processing are the same as those for Part A in the Apoptosis section.

Part B. Cells were resuspended in 200 µl of 2% FBS and primary antibody at 1:100 dilution (vol µL : vol µL). The primary polyclonal antibody was anti-DNA dependent protein kinase produced in a rabbit, IgG isotype whereby the immunogen had been a recombinant fusion protein consisting of amino acids 2015-2134 human DNA-PK catalytic subunit with species reactivity proven including *Xenopus* (Calbiochem, EMD Biosciences, San Diego, CA; Cat. No. PC127). Cells were incubated at 4°C for 1 hr.

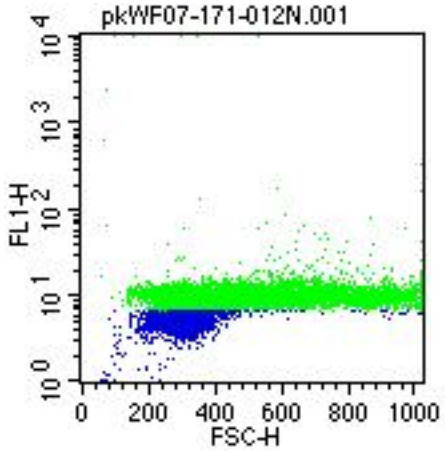
Cells were centrifuged at 2500 rpm for 5 min, supernatant discarded, and then washed with 500 µL 2% FBS-PBS. Cells were resuspended in 400 µL 2% FBS-PBS. The secondary antibody goat anti-rabbit IgG H and L chain specific fluorescein conjugate (Calbiochem, EMD Biosciences, San Diego, CA; Cat. No. 40139) was added at 1:1000 µL (vol µL : vol µL) and the mixture was incubated in the dark at 4°C for 1 hr. After incubation, cells were centrifuged at 2500 rpm for 5 min and the supernatant discarded. Cells were washed with 2% FBS-PBS, centrifuged as before and supernatant discarded. Cells were resuspended in 400 µL PBS and 1 µL of PI at 1 mg/mL added. Cells were incubated in the dark at 24°C for 30 min, and then analyzed by flow cytometry as above. The instrument settings were linear size (FSC) and scatter (SSC), and log fluorescence FL1H (emission wavelength 530nm with 30 nm bandwidth) for fluorescein (for antibody) and log fluorescence FL3H (emission wavelength > 670 nm) for PI. Cytograms were analyzed using FL1H vs. FL3H contour plots whereby debris at the intersection of those axes was removed prior to further analysis (Fig. 4A). Data analysis included histograms of FL1H where markers were set for fluorescence of the main and high populations (Fig. 4B), and dotplots where FSC was on the x-axis and fluorescence (FL1) was on the y-axis whereby regions were gated as above (Fig. 4C). Control cells were used as baseline levels for similar gating among runs.



A)



B)



C)

Fig. 4. A) Flow cytometric contour plot showing fluorescence level of 10K blood cells from wood frog WF07-171-012N replicate 1 incubated with an antibody against the catalytic subunit of DNA PK and secondary fluorescent antibody label to reflect the amount of the DNA repair protein. Fluorescence on the x-axis shows amount of green fluorescence or DNA repair protein, and that on the y-axis shows the cellular nucleic acid amount. Debris is gated out for percentage determinations of PK levels. B) Flow cytometric histogram showing two populations of cells stained for the PK repair protein. Marker 1 (M1) indicates cells with higher levels of the PK protein. C) Flow cytometric dot plot of the same cells, whereby the green dots indicate cells (M1 in 4B) with higher levels of the repair protein.

Statistical Analyses

Statistics were performed by using SAS (2003), and the alpha level was 0.05. For data from all sites and years (2004, 2005, 2006), the Logistic procedure was run with class variables:

- site type (developed [D] versus remote [R])
- site
- distance to road (km)
- elevation (low at < 500 ft and high $\geq$ 500 ft)
- length (mm): snout to vent body length (SVL)
- Gosner life stages, with early as 42-44 and late 45-46
- Abnormalites as No = 0, and Yes = 1 for all, eye, skeletal abnormalities, skeletal malformation, disease
- Caspase: as percent protein measured by gating (G) by dot plots and markers (M) by histograms
- PK: as percent protein kinase measuring DNA repair protein by gating
- DNA integrity: 4 classes, with 1 being most intact

For the controlled lab experiment, the Frequency Procedure was used. For the field data, class models were run using PROC GENMOD, PROC CATMOD and PROC FREQ. Length was included in the class variable model. The polynomial of road distance*elevation was included in the continuous variable. When site type was included as an effect, it contained redundant information of its characteristics of road distance (or D/R) and elevation. Thus, either site or distance and elevation were removed from the analysis. The hypothesis being tested was that there were abnormalities or biomarker differences among the site types, road distance, elevation, and Gosner life stages, and size. Data were analyzed by year.

Results

Abnormality Analyses

Using the definition of an Abnormality as being any gross deviation from the normal range in morphological variation, be it traumatic or developmental and that of malformation as a permanent structural defect resulting from abnormal development (Meteyer 2000), each "abnormality" type was analyzed separately.

Eye Abnormalities:

For 2004, there were no significant explanatory variables, such as site, road distance, D/R, elevation, Gosner stage, or snout-to-vent length.

For 2005, by CATMOD, there were no significant explanatory variables. By GENMOD, D/R, but not site, was a significant explanatory variable.

For 2006, again by GENMOD, D/R, but not site, was a significant explanatory variable. Length was a significant explanatory variable by continuous GENMOD.

Skeletal Abnormalities:

For 2004, there were no significant explanatory variables.

For 2005, Gosner stage was a significant explanatory variable by both CATMOD and GENMOD.

For 2006, by GENMOD, site and Gosner stage were significant explanatory variables.

Skeletal Malformations:

For 2004, elevation by CATMOD was a significant explanatory variable.

For 2005, Gosner was a significant explanatory variable by both CATMOD and GENMOD.

For 2006, Gosner and length were significant explanatory variables by GENMOD.

Disease:

For 2004, there were no significant explanatory variables.

For 2005, D/R and Gosner stage were significant by CATMOD and Gosner was significant by GENMOD.

For 2006, Gosner and length were significant by GENMOD.

Overall, there were no consistent significant explanatory variables throughout the years for each abnormality type, except that Gosner stage occurred more often than the others. When site was entered into the model, it contained redundant information with road distance and elevation and rendered the analyses inconclusive. If contamination status is entered into the site characterization, this will likely add considerable power to the statistical analyses and to the final results. Further collapsing of data (e.g., across all years) may yield another approach for more interpretable statistical analyses.

Analysis DNA Integrity in Controlled Study

For the 2006 controlled study with the biological endpoint of DNA integrity (Appendix 1), such that water and sediment in a random block design (Appendix 1) addressed the null hypothesis that wood frog metamorphs exposed to site water and sediment show the same DNA grade as metamorphs reared in control water and clean sand, there was a lack of frog survival and empty sample tubes resulting in many combinations of variables (96 total possible) not being filled. As a consequence, the experimental design was rendered unbalanced. Variables included 2 controls, and 6 experimental treatments (KNA01, KNA02, KNA03, KNA08, KNA12, and KNA90), shallow and deep depths, 3 parentages, and two replicates. Therefore, collapsing of data was needed, including DNA grades whereby high integrity was graded 1 and grades 2, 3, and 4 were pooled as low. Each metamorph was treated as one observation regardless of parentage, control sites were pooled, KNA12 analysis by site was eliminated because of only one observation, one model combined experimental sites, and for the one parentage with 8 metamorphs, dissimilarites in DNA integrity were examined for depths, independent of block. Categorical analysis was applied for the DNA integrity dependent variable, and sites needed to be pooled. Chi-square tests were not valid as cell counts were too low, however the analysis revealed no significant differences in DNA integrity by blocks, for depths across all sites, and for depths for pooled sites. Using pooled data (DNA grade and experimental or control sites) with categorical analysis, there were no significant differences in DNA integrity for pooled sites, depths, the interaction of site type with depth, or block. Among the replicate offspring pairs for the 8 parentages, 3 of 8 showed changes in DNA integrity, however that was not significant. Data are displayed in Appendix 1.

Analysis of Three Biomarkers from Field Samples

For the field data collected from 2004, 2005, and 2006, there were 12 sites from which samples were consistently obtained. Data from each year were, in general, analyzed separately. Two models were analyzed for each biomarker:

Continuous variable: = distance to road, elevation, Gosner, AVL, abnormalities

Continuous variable with polynomial: = distance to road * elevation

Or Class variable: = developed/remote, lo or hi (elevation), early or late (Gosner).

If SVL was significant, it was included in some class variable models.

For these analyses PROC GENMOD was applied, and PROC GLM was used for caspase and PK.

In general, road distance, elevation, Gosner, and length were typically not significant, and were inconsistent across the three biomarker endpoints. No abnormality effect was significant across all biomarker endpoints and years. Adding more site information, especially such as contaminants information, would be useful and add power in strengthening the models. If data are available, histological sex would be useful. Again, data may be pooled across years in future analyses. Data are presented in Table 1. Specifically, the following results were obtained for each biomarker considered separately:

DNA Integrity:

2004: With 4 DNA grades analyzed, using both continuous and class variable analyses, elevation was significant (Fig. 5).

2005: With 4 DNA grades analyzed, using class analysis, developed/remote was significant (Fig. 6).

2006: With 4 DNA grades analyzed, no significance with any independent variable was seen; it is noted that all sites sampled in this year were at low elevation (Fig. 7).

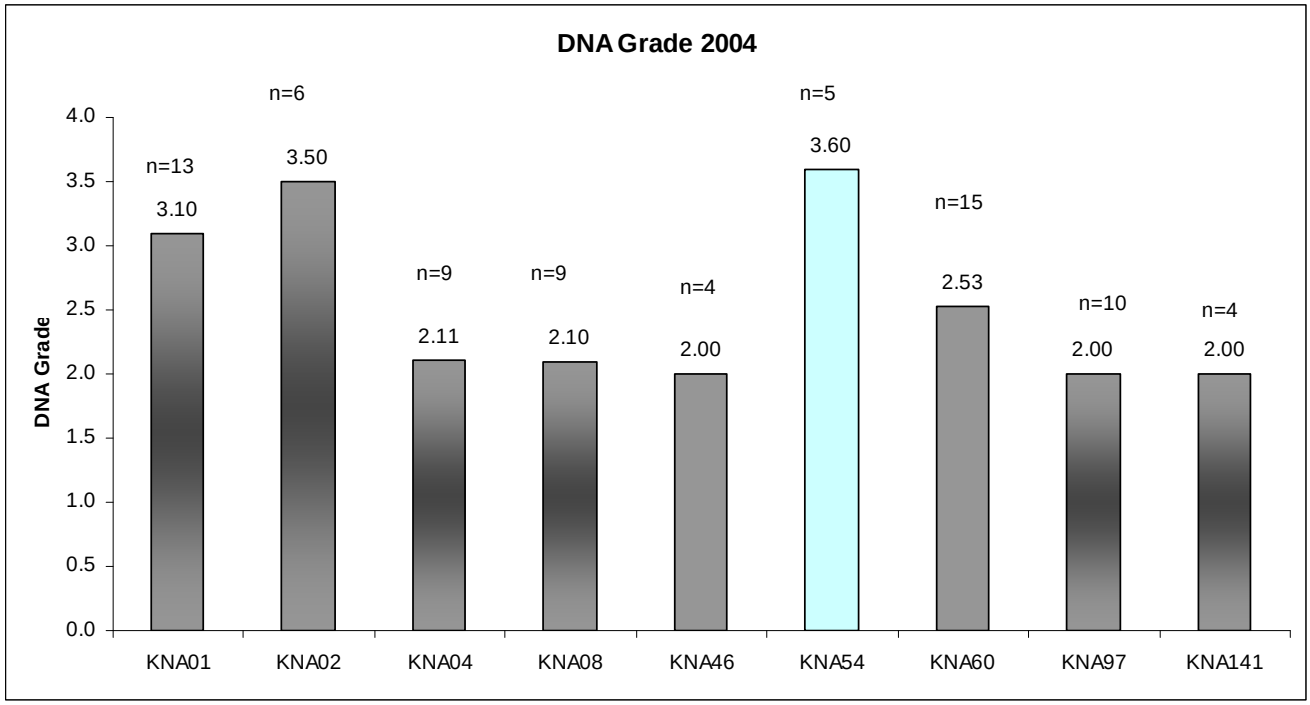


Fig. 5. Grades of DNA integrity with "1" assigned to the highest grade. Bars designating remote locations are not shaded, low elevation is grey, high elevation is blue.

Snout-to-vent length was not significant in any year, whereas road distance, elevation and Gosner were not consistently significant. Abnormalities were not significantly influenced by any other variable.

Caspase 3

To test whether the detection of caspase 3 protein was different by using the histogram or dot plot cytogram types, a paired t-test was applied independent of year. There was no significant difference between methods ($t_{(129)}$ = - 1.296, *P*=0.1972). The gating method was chosen as the preferable method for analysis.

Data were arcsine (sqrt) transformed and correlations performed with continuous variables of elevation, distance to road, and Gosner stage regardless of year. Abnormalities were treated as continuous variables. Caspase 3 was positively correlated with skeletal malformations (r = 0.22158; *P* = 0.0134).

For the field data from 2005 (Fig. 6) and 2006 (Fig. 7), continuous and class variable models were employed as above. Data were arcsine (sqrt) transformed and both models were run using PROC GENMOD and PROC GLM. Length was included in the class variable model. The polynomial of road distance*elevation was included in the continuous model.

2005: Using the continuous model with the polynomial term, elevation and the polynomial term were significant. Using the class model, no significant variables were shown (Fig. 6).

2006: Length was significant with and without the polynomial term using the continuous model. Using the class model, length was again significant (Fig. 7).

DNA Repair Protein

Data were arcsine (sqrt) transformed and correlations performed with continuous variables of elevation, distance to road, and Gosner stage regardless of year. Abnormalities were treated as continuous variables. The PK was positively correlated with Gosner stage (r = 0.2017; *P* = 0.0073).

Data were analyzed as with caspase above (Fig. 6; Fig. 7; Table 1).

2005: Using the continuous model without the polynomial term, elevation, road distance, Gosner stage, and length were significant. Using the class model, developed/remote, elevation, and Gosner stage were significant (Fig. 6).

2006: Using the continuous model, Gosner stage was significant with and without the use of the polynomial term. Using the class model, Gosner stage was significant. Elevation class was noted as being Low for all samples (Fig. 7).

No significant effects were found for any abnormality for either year.

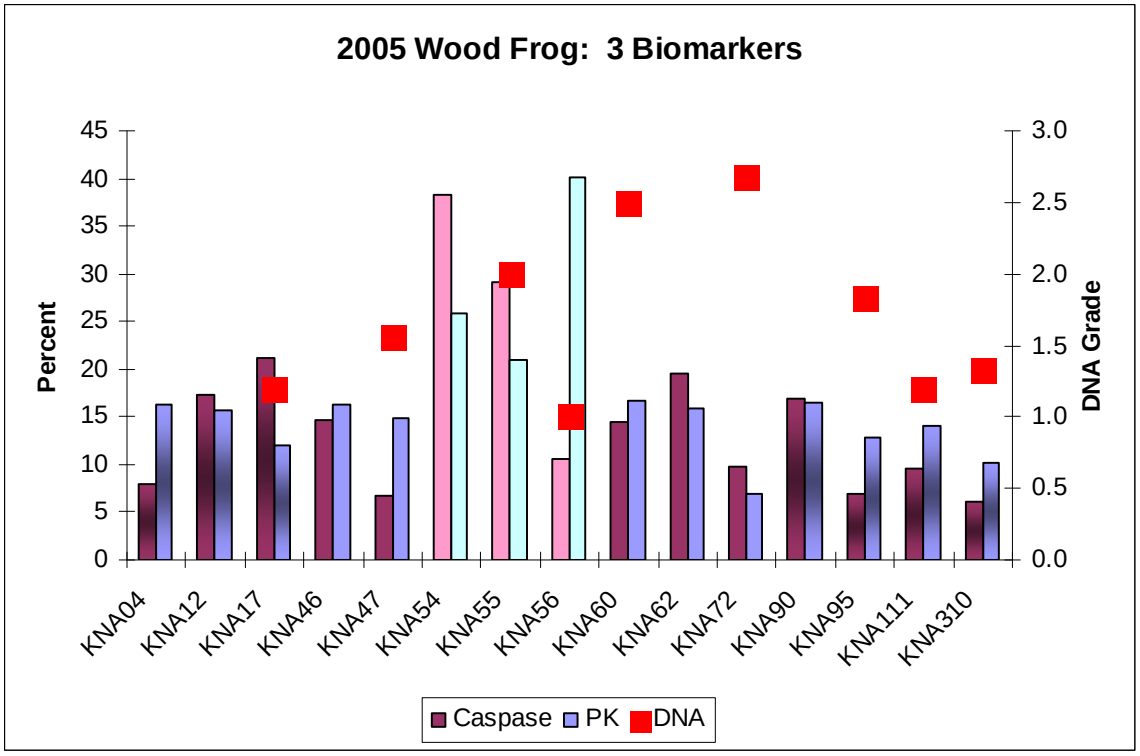


Fig. 6. Average of biomarker values per site collected from wood frog blood in 2005. Shaded bars indicate developed sites, non-shaded bars are remote, purple bars are percent caspase-3 enzyme detected per total cell population, blue bars indicate percent DNA PK repair protein detected. Light bars are sites at high elevation. For N values, see Table 1.

Table 1. Summary of Cytogenetic Biomarker Values (N), Site Elevation and Development Status

Year	Site	D or R	Elevation	Casp (n)	Casp (%)	PK (n)	PK (%)	DNA (n)	DNA Grade
2004	KNA01	D	Low	.		.		13	3.10
	KNA02	D	Low	.	.	.	.	6	3.50
	KNA03	D	Low	.	.	.	.	1	1.00
	KNA04	D	Low	.		.		9	2.11
	KNA08	D	Low	.	.	.	.	9	2.10
	KNA46	R	Low	.	.	.	.	4	2.00
	KNA54	R	High	.		.		5	3.60
	KNA60	R	Low	.	.	.	.	15	2.53
	KNA97	D	Low	.	.	.	.	10	2.00
	KNA141	D	Low					4	2.00
	Sum (n)							76	
2005	KNA04	D	Low	1	7.88	1	16.36	.	.
	KNA12	D	Low	2	17.32	2	15.74	.	.
	KNA17	D	Low	7	21.09	7	12.09	5	1.20
	KNA46	R	Low	2	14.6	2	16.35	.	.
	KNA47	R	Low	26	6.73	25	14.95	9	1.56
	KNA54	R	High	2	38.3	2	25.77	.	.
	KNA55	R	High	21	29.13	20	21.05	.	2.00
	KNA56	R	High	12	10.64	12	40.21	11	1.00
	KNA60	R	Low	7	14.47	8	16.67	4	2.50
	KNA62	R	Low	10	19.51	10	15.86	5	2.00
	KNA72	R	Low	3	9.8	4	6.94	3	2.67
	KNA90	D	Low	3	17	3	16.55	.	.
	KNA95	D	Low	8	6.83	8	12.92	6	1.83
	KNA111	D	Low	6	9.55	7	14	5	1.20
	KNA310	D	Low	11	6.04	11	10.11	9	1.33
	Sum (n)			121		122		57	
2006	KNA01	D	Low	13	12.19	13	16.42	13	1.54
	KNA03	D	Low	9	19.62	9	27.76	8	1.38
	KNA08	D	Low	13	13.71	13	18.8	13	1.46
	KNA12	D	Low	12	8.29	12	21.91	11	1.64
	KNA14	D	Low	5	19.1	5	21.54	5	1.40
	KNA17	D	Low	7	9.08	7	15.72	7	1.71
*	KNA21	unknown	Low	2	11.92	2	20.58	2	2.00
	KNA31	R	Low	14	9.88	13	12.4	13	1.31
	KNA46	R	Low	11	26.24	11	27.87	10	1.40
	KNA47	R	Low	11	19.02	11	18.2	9	1.56
	KNA60	R	Low	8	8.01	8	24.19	8	1.50
	KNA90	D	Low	13	27.21	13	23.08	11	2.08
	KNA97	D	Low	13	6.24	13	22.99	12	1.75
	Sum (n)			131		130		122	

* Frog noted by FWS as having large abnormality.

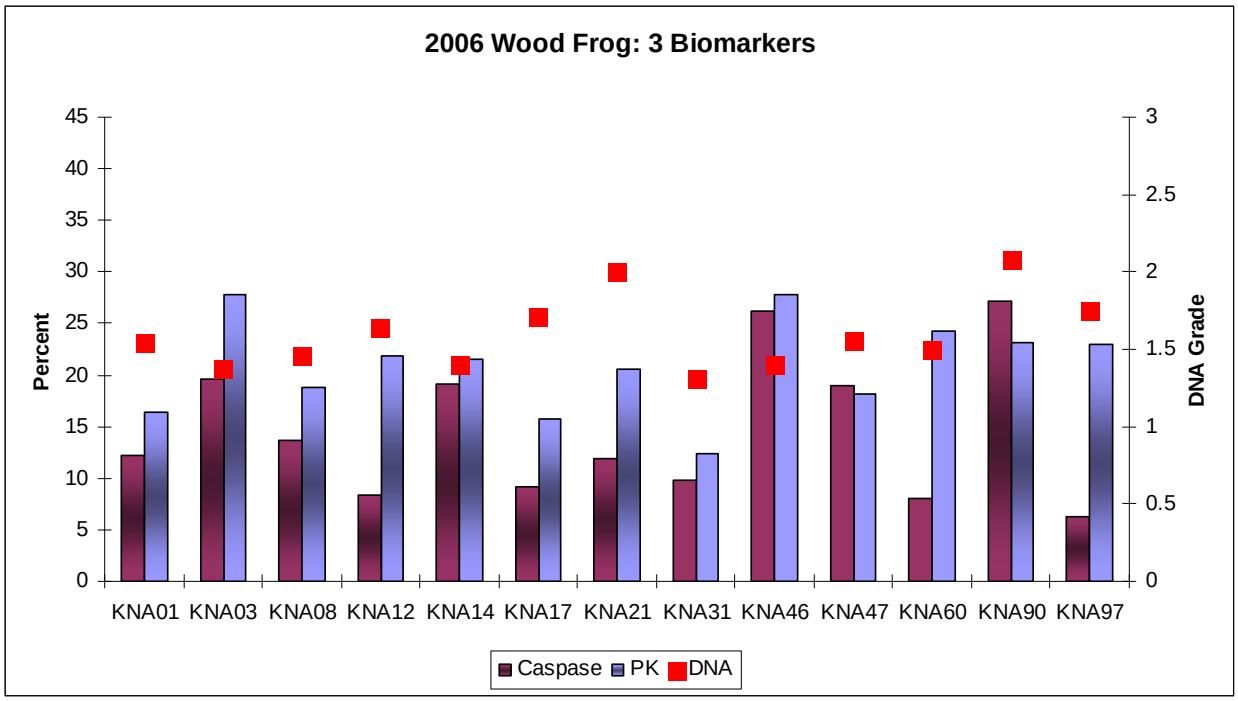


Fig. 7. Average of biomarker values per site collected from wood frog blood in 2006. Shaded bars indicate developed sites, non-shaded bars are remote, purple bars are percent caspase-3 enzyme per total population detected, blue bars indicate percent DNA repair protein detected. All sites were at low elevation. (Site KNA21 was not designated by FWS as developed or remote, but plotted above as developed.) N values are in Table 1.

Notes on Problematic Samples

Data for all analyses for samples transmitted to NWRC are not shown in the tables and figures in this report because these tubes contained either zero (List 1) or low cell numbers. In the case of low cell numbers, less than the 3 biomarkers were run (List 2). These tubes are listed here:

List 1: Empty tubes

KENAI SED TOX 2006-RASY CWATRD2B
KENAI SED TOX 2006-RASY KNA02S3B
KENAI SED TOX 2006-RASY KNA03S2B
R7-KNA01-072306-RASY-096N
R7-KNA12-071806-RASY-003
R7-KNA46-080306-RASY-031
R7-KNA46-080306-RASY-055
R7-KNA46-080306-RASY-079
R7-KNA60-071406-RASY-047

List 2: Low cell numbers
R7-KNA03-072506-RASY-026B
R7-KNA90-073106-RASY-061N
R7-KNA90-073106-RASY-070
All other samples are accounted for in the tables and figures.

Another problem was with the labeling on the tubes, where samples were received but critical sample ID information was missing or unidentifiable by FWS personnel. Sample identification numbers were not consistently recorded or generated for each of the three years of the study with regard to the frogs collected in the field and tubes received at NWRC. Reasons for inconsistency include changing of personnel, methodology for labeling, and illegible hand-writing on some tubes. In 2004, a master frog ID and host ID were assigned by FWS, and a shortened NWRC ID needed to be assigned for use. In 2005, as far as was possible, a reconciliation of IDs was made for work done at NWRC (Appendix 1 and 2) and at other locations. In 2006, a masterfrog ID and NWRC ID were assigned, but no host ID needed to be assigned.

For 2004, no site but only host ID numbers were available for the samples below; nevertheless, DNA values were collected:

Host ID	**DNA Integrity**
04-914	4
04-958	1
04-959	1
04-960	1
04-961	1
04-964	2
04-965	2

For 2005, only host ID numbers were available for the samples below; nevertheless, biomarker data were obtained with the cells available.

Host ID	% Casp	% PK	DNA Grade
05-211	7.89	4.54	.
05-415	3.25	.	.
05-420	5.41	11.29	4
05-422	8.67	12.68	.
05-430	8.08	34.96	4
05-461	23.07		.
05-479	2.12		.
05-472	.	14.13	.
05-474	.	11.03	.

Discussion

Bioaccumulation markers and biomarkers have been used to demonstrate exposure to and effects of environmental contaminants, and their feasibility for use in environmental risk assessments (ERA) has been reviewed (van der Oost et al. 2003). Among the most promising for ERA were liver biotransformation enzymes, reproductive parameters (e.g., vitellogenin), and genotoxic parameters (e.g. DNA abnormalities) (van der Oost et al. 2003). Not only can genotoxins shorten the life expectancy of organisms, they can result in alterations in population dynamics, initiating adverse ecological consequences such as lowering biodiversity (Atienzar et al. 2001).

Few studies have been done using amphibians to look at DNA strand breakage; most have been done to study DNA content or genome size (Borkin et al. 2004; Freeman and Rayburn 2004b). Lowcock et al. (1997) showed genotoxic damage by using flow cytometry of blood from Green frogs (*Rana clamitans*) from farming areas, which revealed abnormal DNA profiles. Also by flow cytometry, Freeman and Rayburn (2005) showed nuclei of *X. laevis* tadpoles were altered by atrazine exposure; however, their whole cell preparations were suspect due to use of whole animal preparations (Freeman et al. 2005). Freeman and Rayburn (2004a) used flow cytometric DNA CVs to assess damage by atrazine to anuran larvae in a controlled study, yet damage was assessed by nuclear heterogeneity and not by direct study of DNA, therefore conclusions of no damage found might be reassessed by using DNA biomarkers. The comet assay was used to demonstrate genotoxicity of herbicides in *R. catesbeiana* tadpoles (Clements et al. 1997). Although DNA damage was not detected in three species of ranid frogs from an agricultural study, the potential of flow cytometry for use in screening amphibians was indicated by Bly et al. (2004).

From reviews of the three biomarker mechanisms and their interactions within impacted cells, one would expect that, if DNA damage is present, then the repair proteins would be increased. If there is much DNA damage, then apoptosis would be occurring at a high rate, thereby lowering DNA repair because of inactivation by caspase-3 (Fig. 8). In other words, ds breaks in DNA initiate repair. If repair cannot overcome DNA damage to overcome insults, the apoptosis process starts and cells are removed from the population.

From the results of this study, further investigation into impacts of elevation status would be useful. If abnormalities found were not primarily bilaterally symmetrical, UV exposure was not

a likely cause of cytogenetic damage (Taylor 2005). Data on histological sex will be instructive, and categorizations of chemical types and individual contaminants data, summary levels, and relationships will be analyzed. Statistical analyses can be performed on data pooled among the years.

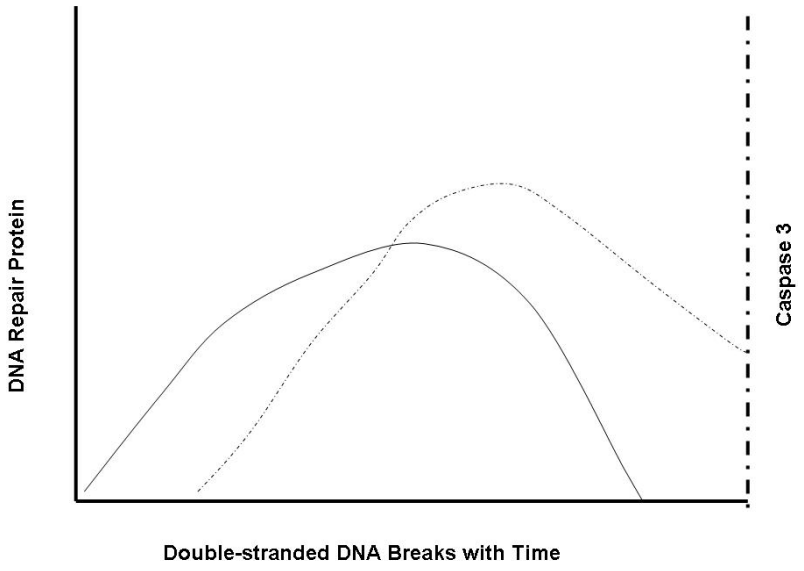


Fig. 8. Hypothetical levels of caspase-3 and DNA PKcs with increasing numbers of ds DNA breaks. Increasing DNA grades indicating increased integrity loss go along the x-axis.

Organisms function as integrators of exposure to contaminants and other stressors, accounting for abiotic and physiological factors that modulate the doses of toxicants taken up, with the resulting magnitude of change in cytogenetic properties providing an estimate of the severity of exposure and impact. We have optimized a protocol for the collection of blood for fixation and consequent analysis by flow cytometry of three reliable biomarker assays developed for assessing cytogenetic condition in anurans. The protocols and data presented herein can be used to address questions related to the high level of malformations of wood frogs at KNWR once data generated by research at NWRC are combined with other information collected by FWS staff and their colleagues, and included in more extensive statistical analyses. This will be the primary focus of continuing collaborations and joint publication.

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Appendix 1. DNA Integrity Results from 2006 Controlled Study with Dependent Variables

MASTERFROG_Frog ID	NWRC Frog ID	#	Site	Depth	Parent	Replicate	Block	DNA Integrity
KENAI SED TOX 2006-RASY CSAND1A	WF07-001CSAND1A	1	CSAND	D	1	A	2	1
KENAI SED TOX 2006-RASY CSANDD2A	WF07-002 CSANDD2A	2	CSAND	D	2	A	4	1
KENAI SED TOX 2006-RASY CSANDS2B	WF07-003 CSANDS2B	3	CSAND	S	2	B	1	1
KENAI SED TOX 2006-RASY CSANDS3B	WF07-004 CSANDS3B	4	CSAND	S	3	B	4	2
KENAI SED TOX 2006-RASY CWATRD1A	WF07-005 CWATRD1A	5	CWATR	D	1	A	4	1
KENAI SED TOX 2006-RASY CWATRD2A	WF07-006CWATRD2A	6	CWATR	D	2	A	4	2
KENAI SED TOX 2006-RASY CWATRD2B	WF07-007CWATRD2B	7	CWATR	D	2	B	2	ND
KENAI SED TOX 2006-RASY CWATRD3A	WF07-008 CWATRD3A	8	CWATR	D	3	A	3	1
KENAI SED TOX 2006-RASY KNA011D1B	WF07-009 KNA011D1B	9	KNA01	D	1	B	2	3
KENAI SED TOX 2006-RASY KNA01D1A	WF07-010 KNA01D1A	10	KNA01	D	1	A	1	1
KENAI SED TOX 2006-RASY KNA01D2A	WF07-011 KNA01D2A	11	KNA01	D	2	A	3	1
KENAI SED TOX 2006-RASY KNA01D2B	WF07-012 KNA01D2B	12	KNA01	D	2	B	2	1
KENAI SED TOX 2006-RASY KNA01D3A	WF07-013 KNA01D3A	13	KNA01	D	3	A	4	1
KENAI SED TOX 2006-RASY KNA01S1B	WF07-014 KNA01S1B	14	KNA01	S	1	B	1	1
KENAI SED TOX 2006-RASY KNA01S2A	WF07-015 KNA01S2A	15	KNA01	S	2	A	3	1
KENAI SED TOX 2006-RASY KNA01S2B	WF07-016 KNA01S2B	16	KNA01	S	2	B	4	1
KENAI SED TOX 2006-RASY KNA01S3A	WF07-017 KNA01S3A	17	KNA01	S	3	A	4	1

Appendix 1, Continued

MASTERFROG_Frog ID	NWRC Frog ID	#	Site	Depth	Parent	Replicate	Block	DNA Integrity
KENAI SED TOX 2006-RASY KNA01S3B	WF07-018 KNA01S3B	18	KNA02	S	3	B	2	1
KENAI SED TOX 2006-RASY KNA0202A	WF07-018 KNA0202A	19	KNA02	D	2	A	1	1
KENAI SED TOX 2006-RASY KNA02D3A	WF07-020 KNA02D3A	20	KNA02	D	3	A	4	2
KENAI SED TOX 2006-RASY KNA02D3B	WF07-021 KNA02D3B	21	KNA02	D	3	B	1	2
KENAI SED TOX 2006-RASY KNA02S1B	WF07-022 KNA02S1B	22	KNA02	S	1	B	2	2
KENAI SED TOX 2006-RASY KNA02S3B	WF07-023 KNA02S3B	23	KNA02	S	3	B	3	ND
KENAI SED TOX 2006-RASY KNA03D1B	WF07-024 KNA03D1B	24	KNA03	D	1	B	3	1
KENAI SED TOX 2006-RASY KNA03D2A	WF07-025 KNA03D2A	25	KNA03	D	2	A	2	2
KENAI SED TOX 2006-RASY KNA03D2B	WF07-026 KNA03D2B	26	KNA03	D	2	B	2	1
KENAI SED TOX 2006-RASY KNA03D3B	WF07-027 KNA03D3B	27	KNA03	D	3	B	4	1
KENAI SED TOX 2006-RASY KNA03S1A	WF07-028 KNA03S1A	28	KNA03	S	1	A	2	1
KENAI SED TOX 2006-RASY KNA03S2B	WF07-029 KNA03S2B	29	KNA03	S	2	B	1	ND
KENAI SED TOX 2006-RASY KNA03S3B	WF07-030 KNA03S3B	30	KNA03	S	3	B	1	2
KENAI SED TOX 2006-RASY KNA08D1A	WF07-031 KNA08D1A	31	KNA08	D	1	A	4	2
KENAI SED TOX 2006-RASY KNA08D1B	WF07-032 KNA08D1B	32	KNA08	D	1	B	2	2
KENAI SED TOX 2006-RASY KNA08S2A	WF07-033 KNA08S2A	33	KNA08	S	2	A	2	1
KENAI SED TOX 2006-RASY KNA08S3A	WF07-034 KNA08S3A	34	KNA08	S	3	A	4	2

Appendix 1, Continued

MASTERFROG_Frog ID	NWRC Frog ID	#	Site	Depth	Parent	Replicate	Block	DNA Integrity
KENAI SED TOX 2006-RASY KNA08S3B	WF07-035 KNA08S3B	35	KNA08	S	3	B	2	1
KENAI SED TOX 2006-RASY KNA12S1A	WF07-036 KNA12S1A	36	KNA12	S	1	A	3	3
KENAI SED TOX 2006-RASY KNA21D2B	WF07-037 KNA21D2B	37	KNA90	D	2	B	2	1
KENAI SED TOX 2006-RASY KNA21D3A	WF07-038 KNA21D3A	38	KNA90	D	3	A	4	1
KENAI SED TOX 2006-RASY KNA21S2A	WF07-039 KNA21S2A	39	KNA90	S	2	A	3	4
KENAI SED TOX 2006-RASY KNA21S2B	WF07-040 KNA21S2B	40	KNA90	S	2	B	4	ND
KENAI SED TOX 2006-RASY KNA21S3B	WF07-041 KNA21S3B	41	KNA90	S	3	B	3	1

Randomized block design layout.

Block 1 Layout

CSANDD3B	KNA03S3B	KNA02D2A	KNA12D2A	KNA01D1A	CSANDS1A
KNA08D3A	KNA12S3A	KNA03S2B	KNA12S2B	KNA01S1A	KNA02S2B
CWATRS2B	CWATRS1B	CWATRS2A	KNA01S1B	KNA03D3A	KNA02D3B
CSANDS2B	KNA08D2B	KNA08S1A	KNA90D1B	KNA90D1A	KNA90D3B

Block 2 Layout

KNA12D3A	KNA01D1B	KNA03D2A	KNA01D2B	KNA03D2B	CWATRS3A
CSANDS2A	KNA02D2B	KNA01S3B	CSANDD1A	CSANDS1B	KNA12S2A
KNA02S1B	KNA08D1B	KNA12S1B	KNA03S1A	KNA02S1A	KNA08S2A
KNA08S3B	KNA90S1A	KNA90D2B	CWATRS1A	CWATRD2B	KNA90S1B

Block 3 Layout

KNA01D2A	KNA01D3B	KNA01S2A	KNA02S3A	KNA12D1A	KNA12D2B
KNA08S1B	KNA08S2B	CSANDD3A	CSANDS3A	KNA12S1A	KNA03S1B
KNA02D1A	KNA03D1A	KNA03D1B	CWATRS3B	KNA02S3B	KNA90S2A
KNA08D3B	CSANDD1B	CWATRD3A	KNA90S3B	CWATRD1B	KNA90D2A

Block 4 Layout

KNA12S3B	KNA03S3A	CSANDS3B	KNA08D2A	KNA02S2A	CWATRD1A
CWATRD3B	KNA12D1B	KNA08S3A	KNA90S3A	KNA03D3B	KNA01S3A
KNA90S2B	KNA08D1A	CSANDD2A	CWATRD2A	KNA03S2A	CSANDD2B
KNA02D1B	KNA01S2B	KNA90D3A	KNA01D3A	KNA12D3B	KNA02D3A

Key: Unit ID = Site-Parentage-Replicate, eg. KNA12S3B is water and sediment from Site KNA12, Parent Pair S3 (Shallow site, pair # 3), and is Replicate B of this combination.

Appendix 2. USFWS ID's matched with NWRC IDs.

MASTERFROG_Frog ID	NWRC ID
R7-KNA01-RASY-7102004-5N	199
R7-KNA01-072306-RASY-014	44
R7-KNA01-072306-RASY-050	45
R7-KNA01-072306-RASY-054	46
R7-KNA01-072306-RASY-071	47
R7-KNA01-072306-RASY-084N	48
R7-KNA01-072306-RASY-088N	49
R7-KNA01-072306-RASY-089N	50
R7-KNA01-072306-RASY-091N	51
R7-KNA01-072306-RASY-092N	52
R7-KNA01-072306-RASY-094	53
R7-KNA01-072306-RASY-095N	54
R7-KNA01-072306-RASY-097N	56
R7-KNA01-072306-RASY-098N	57
R7-KNA03-072506-RASY-014N	58
R7-KNA03-072506-RASY-015	59
R7-KNA03-072506-RASY-019	60
R7-KNA03-072506-RASY-024N	61
R7-KNA03-072506-RASY-026	62
R7-KNA03-072506-RASY-026B	63
R7-KNA03-072506-RASY-031N	64
R7-KNA03-072506-RASY-057N	65
R7-KNA03-072506-RASY-058N	66
R7-KNA04-RASY-6282004-17N	377
R7-KNA04-RASY-6282004-43N	378
R7-KNA04-RASY-6282004-79N	379
R7-KNA04-RASY-6282004-34N	380
R7-KNA04-RASY-6282004-75N	381
R7-KNA04-RASY-6282004-15N	382
R7-KNA04-RASY-6282004-69N	383
R7-KNA04-RASY-6282004-83N	384
R7-KNA04-RASY-6282004-40N	385
R7-KNA04-RASY-6302005-26N	1739
R7-KNA08-072506-RASY-009	67
R7-KNA08-072506-RASY-021	68
R7-KNA08-072506-RASY-055	69
R7-KNA08-072506-RASY-065	70
R7-KNA08-072506-RASY-069N	71
R7-KNA08-072506-RASY-070N	72
R7-KNA08-072506-RASY-073N	73
R7-KNA08-072506-RASY-074N	74
R7-KNA08-072506-RASY-076N	75
R7-KNA08-072506-RASY-077	76
R7-KNA08-072506-RASY-080	77
R7-KNA08-072506-RASY-083	78
R7-KNA08-072506-RASY-089	79

R7-KNA111-RASY-06212005-33N	1850
R7-KNA111-RASY-06212005-52N	1851
R7-KNA111-RASY-06212005-40N	1852
R7-KNA111-RASY-06212005-27N	1853
R7-KNA111-RASY-06212005-53N	1854
R7-KNA111-RASY-06212005-7N	1855
R7-KNA111-RASY-06212005-51N	1856
R7-KNA111-RASY-06212005-1N	1857
R7-KNA12-070405-RASY-053N	1879
R7-KNA12-070405-RASY-019	1934
R7-KNA12-071806-RASY-005N	81
R7-KNA12-071806-RASY-007N	82
R7-KNA12-071806-RASY-012N	83
R7-KNA12-071806-RASY-015N	84
R7-KNA12-071806-RASY-040N	85
R7-KNA12-071806-RASY-050	86
R7-KNA12-071806-RASY-058N	87
R7-KNA12-071806-RASY-061N	88
R7-KNA12-071806-RASY-062N	89
R7-KNA12-RASY-071806-008N	90
R7-KNA12-RASY-071806-022N	91
R7-KNA12-RASY-071806-037N	92
R7-KNA12-RASY-071806-039N	93
R7-KNA13-072606-RASY-040N	94
R7-KNA13-072606-RASY-048	95
R7-KNA13-072606-RASY-055N	96
R7-KNA13-072606-RASY-058N	97
R7-KNA14-072606-RASY-023	98
R7-KNA141-RASY-752004-25N	838
R7-KNA141-RASY-752004-43N	839
R7-KNA141-RASY-752004-28N	840
R7-KNA141-RASY-752004-26N	841
R7-KNA17-071805-RASY-038	1990
R7-KNA17-071805-RASY-025	1991
R7-KNA17-071805-RASY-011	1992
R7-KNA17-071805-RASY-023	1993
R7-KNA17-071805-RASY-030	1994
R7-KNA17-071805-RASY-021	1995
R7-KNA17-071805-RASY-054	1996
R7-KNA17-080306-RASY-004	99
R7-KNA17-080306-RASY-006	100
R7-KNA17-080306-RASY-015	101
R7-KNA17-080306-RASY-023	102
R7-KNA17-080306-RASY-035	103
R7-KNA17-080306-RASY-037	104
R7-KNA17-080306-RASY-059	105
R7-KNA21-072306-RASY-010N-TAD	106
R7-KNA21-072306-RASY-011N-TAD	107
R7-KNA31-071206-RASY-001N	108

R7-KNA31-071206-RASY-007N	109
R7-KNA31-071206-RASY-009N	110
R7-KNA31-071206-RASY-010	111
R7-KNA31-071206-RASY-011N	112
R7-KNA31-071206-RASY-016N	113
R7-KNA31-071206-RASY-018	114
R7-KNA31-071206-RASY-022N	115
R7-KNA31-071206-RASY-023N	116
R7-KNA31-071206-RASY-026N	117
R7-KNA31-071206-RASY-040	118
R7-KNA31-071206-RASY-056	119
R7-KNA31-071206-RASY-064	120
R7-KNA31-071206-RASY-070N	121
R7-KNA310-RASY-742005-15N	2474
R7-KNA310-RASY-742005-46N	2475
R7-KNA310-RASY-742005-49N	2476
R7-KNA310-RASY-742005-41N	2477
R7-KNA310-RASY-742005-18N	2478
R7-KNA310-RASY-742005-62N	2479
R7-KNA310-RASY-742005-59N	2480
R7-KNA310-RASY-742005-7N	2481
R7-KNA310-RASY-742005-61N	2482
R7-KNA310-RASY-742005-58N	2483
R7-KNA310-RASY-742005-22N	2484
R7-KNA310-RASY-742005-52N	2485
R7-KNA46-RASY-7132005-39N	2569
R7-KNA46-RASY-7132005-18N	2570
R7-KNA46-080306-RASY-038	123
R7-KNA46-080306-RASY-086N	126
R7-KNA46-080306-RASY-087N	127
R7-KNA46-080306-RASY-091N	128
R7-KNA46-080306-RASY-092N	129
R7-KNA46-080306-RASY-093N	130
R7-KNA46-080306-RASY-093N1	131
R7-KNA46-080306-RASY-095N	132
R7-KNA46-080306-RASY-096N	133
R7-KNA46-RASY-080306-007N	134
R7-KNA46-RASY-080306-010N	135
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R7-KNA47-070605-RASY-054	2625
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R7-KNA47-RASY-762005-93N	2629
R7-KNA47-RASY-762005-34N	2630
R7-KNA47-RASY-762005-18N	2631
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R7-KNA47-RASY-762005-45N	2633
R7-KNA47-RASY-762005-33N	2634
R7-KNA47-RASY-762005-42N	2635
R7-KNA47-RASY-762005-87N	2636
R7-KNA47-RASY-762005-51N	2675
R7-KNA47-RASY-762005-47N	2676
R7-KNA47-RASY-762005-56N	2677
R7-KNA47-RASY-762005-91N	2678
R7-KNA47-RASY-762005-74N	2679
R7-KNA47-072406-RASY-015N	136
R7-KNA47-072406-RASY-026	138
R7-KNA47-072406-RASY-036N	139
R7-KNA47-072406-RASY-088N	140
R7-KNA47-072406-RASY-089N	141
R7-KNA47-072406-RASY-090N	142
R7-KNA47-072406-RASY-091N	143
R7-KNA47-072406-RASY-093N	144
R7-KNA47-072406-RASY-094N	145
R7-KNA47-072406-RASY-095N	146
R7-KNA47-072406-RASY-042N	147
R7-KNA54-RASY-892004-23N	1149
R7-KNA54-RASY-892004-41N	1150
R7-KNA54-RASY-892004-15N	1151
R7-KNA54-RASY-892004-26N	1152
R7-KNA54-RASY-892004-32N	1153
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R7-KNA54-080205-RASY-001N	2710
R7-KNA55-RASY-7252005-50N	2725
R7-KNA55-072505-RASY-038	2726
R7-KNA55-RASY-7252005-1N	2727
R7-KNA55-RASY-7252005-16N	2728
R7-KNA55-RASY-7252005-45N	2729
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R7-KNA55-RASY-7252005-12N	2731
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R7-KNA55-RASY-7252005-11N	2737
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R7-KNA55-080205-RASY-066N	4167
R7-KNA55-080205-RASY-068N	4168
R7-KNA55-080205-RASY-069N	4169
R7-KNA55-080205-RASY-070N	4170
R7-KNA55-080205-RASY-071N	4171
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R7-KNA56-081505-RASY-028N	2766
R7-KNA56-081505-RASY-007	2767
R7-KNA56-081505-RASY-026N	2768
R7-KNA56-081505-RASY-024	2769
R7-KNA56-081505-RASY-044N	2770
R7-KNA56-081505-RASY-002N	2771
R7-KNA56-081505-RASY-027N	2772
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R7-KNA56-081505-RASY-012N	2775
R7-KNA56-081505-RASY-009N	2776
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R7-KNA60-RASY-792004-8N	1328
R7-KNA60-RASY-792004-33N	1329
R7-KNA60-RASY-792004-50N	1330
R7-KNA60-RASY-792004-16N	1331
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R7-KNA60-RASY-792004-9N	1333
R7-KNA60-RASY-792004-42N	1334
R7-KNA60-RASY-792004-32N	1335
R7-KNA60-RASY-792004-53N	1336
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R7-KNA60-071505-RASY-020	2818
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R7-KNA60-RASY-7152005-29N	2828
R7-KNA60-RASY-7152005-38N	2831
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R7-KNA60-RASY-7152005-33N	2833
R7-KNA60-RASY-7152005-35N	2834
R7-KNA60-071406-RASY-005	148
R7-KNA60-071406-RASY-021	149
R7-KNA60-071406-RASY-046N	150
R7-KNA60-071406-RASY-050	152
R7-KNA60-071406-RASY-051N	153
R7-KNA60-071406-RASY-054	154
R7-KNA60-071406-RASY-057	155
R7-KNA60-071406-RASY-096	156
R7-KNA62-071805-RASY-012N	2865
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R7-KNA62-071805-RASY-004	2867

R7-KNA62-071805-RASY-029	2868
R7-KNA62-071805-RASY-008	2869
R7-KNA62-071805-RASY-001	2870
R7-KNA62-071805-RASY-005N	2871
R7-KNA62-071805-RASY-035	2872
R7-KNA62-071805-RASY-033N	2873
R7-KNA62-071805-RASY-014	2874
R7-KNA62-071805-RASY-010	2875
R7-KNA62-071805-RASY-018	2877
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R7-KNA72-RASY-7142005-56N	2974
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R7-KNA90-062805-RASY-018	3062
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R7-KNA90-073106-RASY-012	159
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R7-KNA90-073106-RASY-049	164
R7-KNA90-073106-RASY-050	165
R7-KNA90-073106-RASY-052	166
R7-KNA90-073106-RASY-061N	167
R7-KNA90-073106-RASY-063	168
R7-KNA90-073106-RASY-067	169
R7-KNA90-073106-RASY-070	170
R7-KNA95-071805-RASY-023	3112
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R7-KNA95-071905-RASY-059N	4176
R7-KNA95-071905-RASY-062N	4177
R7-KNA97-072006-RASY-012N	171
R7-KNA97-072006-RASY-019N	172
R7-KNA97-072006-RASY-035	173
R7-KNA97-072006-RASY-037N	174
R7-KNA97-072006-RASY-042N	175
R7-KNA97-072006-RASY-042N2	176
R7-KNA97-072006-RASY-044N	177
R7-KNA97-072006-RASY-046N	178
R7-KNA97-072006-RASY-048N	179
R7-KNA97-072006-RASY-050N	180
R7-KNA97-072006-RASY-051N	181

R7-KNA97-072006-RASY-052N	182
R7-KNA97-072006-RASY-053N	183
host # 05-211	4178
host # 05-415	4179
host # 05-420	4180
host # 05-422	4181
host # 05-430	4182
host # 05-461	4183
host # 05-479	4184
host # 05-472	4185
host # 05-474	4186

Appendix C – Sediment/Water/UVB Testing

ASSESSING THE TOXICITY AND TERATOGENICITY TO AMPHIBIANS OF WETLANDS IN THE SWANSON OIL FIELDS OF KENAI NATIONAL WILDLIFE REFUGE

A report prepared for:
Fish and Wildlife Service
Environmental Quality Field Office Anchorage
605 West 4th Avenue, Suite G-61
Anchorage, Alaska 99501

Interagency Agreement Number; 701811N121

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ABSTRACT

Deformed amphibians have been observed in the Kenai National Wildlife Refuge, Alaska. Environmental variables such as contaminants, ultraviolet radiation [UV], or parasites may cause deformities. It is important to identify the environmental cause of deformities in order that effective management strategies can be implemented to enable the recovery of affected populations. To determine whether waterborne chemicals are responsible for deformities reported in wood frogs (*Rana sylvatica*) in wetlands (Oil Fields 1 and 3) in the Swanson River Oil Field in the Kenai National Wildlife Refuge, we deployed semipermeable membrane devices (SPMDs) to accumulate lipophilic contaminants. We then exposed tadpoles (spring peepers, *Pseudacris crucifer*) to the SPMD extracts at two levels of UV-B radiation. Frogs exposed to SPMD extracts from Oil Field 3 were smaller upon metamorphosis and took longer to reach metamorphosis than frogs reared in other treatments. We found no effect attributable to UV-B or the interaction between UV-B and SPMD extract. We noted no deformities throughout the experiment.

INTRODUCTION

Reports of amphibian deformities have increased in recent years, especially in the northern United States, and including the Kenai National Wildlife Refuge in Alaska. Many causes have been proposed for the observed deformities (Ouelette, 2000; Lannoo, 2000), including contaminants (Ouelette et al,. 1997; Bonin et al., 1997), ultraviolet radiation (UV) (Blaustein et al, 1997), and parasites (Johnson et al., 1999) either singly or in combinations. Analysis of water and sediment samples in central Minnesota have revealed that many types of

contaminants are present in amphibian habitats and have the potential to trigger developmental abnormalities in some species (e.g., *Xenopus*; Fort et al., 1999a, b).

It is often difficult to determine what specific contaminants are present at a site or their concentrations, which often hinders determining the influence of exposure to contaminants on the populations of organisms inhabiting the area. Semipermeable membrane devices (SPMDs) are integrative samplers containing lipid that accumulate lipophilic organic compounds from the environment in a manner that is similar to aquatic organisms (Huckins et al., 2001). The uptake rates of fat-soluble compounds by SPMDs can be used to define the approximate daily exposure to lipophilic compounds by aquatic organisms (Huckins et al., 2001). Thus, the composite waterborne environmental contaminant exposure of organisms to the bioavailable lipophilic compounds present at the site can be accomplished by using SPMD extracts as toxicant solutions for bioassays (Petty et al., 2000a, b; Huckins et al., 2001) based on the duration of deployment in the environment. Acute tests with extracts obtained from SPMDs offer a convenient means of screening the toxicity of hydrophobic contaminants in aquatic habitats (Huckins et al., 1993).

Exposure to UV-B radiation at larval and egg stages has been suspected of negatively impacting some amphibian species directly by reducing hatching success and increasing rates of embryonic deformity (Lizana and Pedraza, 1998; Blaustein et al., 1998; Broomhall et al., 2000; Starnes et al., 2000) as well as generating adult deformities (Ankley et al,. 2000). UV radiation is also known to increase the toxicity of various compounds to amphibians in aquatic habitats, including polycyclic aromatic hydrocarbons (Little et al., 2000; Zaga et al., 1998), which may have been released into the Swanson River Field as a result of petroleum extraction activities (Parsons 2001). Therefore, another objective of our study was to investigate whether UV radiation can affect developing frogs or whether radiation can act synergistically with lipophilic

compounds present in the environment (i.e., in SPMD extracts) to increase toxicity and teratogenicity.

We exposed spring peeper tadpoles (*Pseudacris crucifer*) throughout development to concentrated SPMD extracts from two sites at the Swanson Oil Field on the Kenai National Wildlife Refuge (KNWR) to determine whether the chemicals potentially present in either of these sites are acutely toxic. We measured tadpole growth, the duration of the larval period, and recorded the incidence of deformities upon metamorphosis. Experimental UV-B intensities similar to those found in the field were used to determine whether UV-B radiation can potentiate the toxicity or teratogenicity of chemicals found at either site.

MATERIALS AND METHODS

SPMD exposure and extraction

To determine whether contaminants present in two wetlands in the KNWR in Alaska can cause mortality or deformities, we deployed several SPMDs at each of two sites in the Swanson oil field (Oil Fields 1 and 3 [OF1 and OF3, respectively]). Five standard SPMDs (described elsewhere, [Huckins et al., 2001]) were placed in each of three replicate stainless-steel canisters attached to a steel cable anchored to the shore. The SPMDs were submerged at a depth of at least 1 m for 28 d between 11 July and 8 August 2002. The water at each site was visually clear, lentic (i.e., quiescent exposure condition), and had an average temperature of approximately 16°C. Biofouling of the SPMDs was minimal.

At the time of deployment a metal can containing SPMDs (hereafter, field blank) was opened and exposed to the air at each site. These provided a control for any airborne contaminants present while the SPMDs were exposed to the air (i.e., before being placed into the

water). Once the SPMDs were in the water, the cans containing the field blanks were sealed and stored at 0-4°C for the 28 d between deployment and retrieval to halt contaminant uptake (from the site air that had filled the can). During retrieval, field blanks were once again exposed to the air at the site for as long as it took to remove the SPMDs from the water and seal them in cans. After retrieval, both SPMDs and field blanks were shipped on ice and remained frozen until they were processed at the Columbia Environmental Research Center (CERC, Columbia, MO) using techniques outlined in Petty et al. (2000).

SPMD extracts from each site were pooled into a single composite sample. Field exposure and control extracts were dissolved into sterile dimethylsulfoxide (DMSO) by solvent exchange from high purity hexane to a total volume of 0.5 mL for acute tests. SPMD extracts for the chronic test were added to 90 mL of sterile DMSO so that each mL contained the approximate equivalent of a 1-d exposure (i.e., represents the amount of bioavailable residues extracted from site water by a standard SPMD in one days' time) when added to 1 L of water.

Assuming lentic conditions, an ambient temperature between 10 and 18° C, and minimal biofouling, 1-gram equivalent of a standard 1-mL triolein SPMD will clear or extract hydrophobic organic contaminants (e.g., PAHs, PCBs, organochlorines, pyrethroids) from about 0.01 to 2.0 L g of water daily (Huckins et al., 2001). Although much greater variability exists in the uptake rate constants of aquatic organisms for the same chemicals, the values for invertebrates and fishes generally range from 0.03 to 8.0 L/d/g (Mackay et al., 1992a, 1992b, 1997). Thus, it is reasonable to expect that aliquots of SPMD extracts, which represent the daily volume of water extracted by a whole standard SPMD, are representative of the amounts of chemicals to which aquatic organisms (e.g., tadpoles) are exposed daily.

Solar radiometric readings

Solar radiation throughout the water column was measured at each site during SPMD deployment using a Macam Photometrics broadband UV meter. This instrument measures total UV-B with a peak spectral response at 311 nm and a bandwidth of 292 to 330 nm. It also measures total UV-A with a peak spectral response at 369 nm and a bandwidth of 332 to 406 nm. The instrument was calibrated using standards traceable to the British Standard Institute. Measurements were taken at the surface, just below the surface, and at 10, 20, 30 and 40 cm below the surface (Table 1).

Experimental exposure

Three pairs of adult spring peepers (*Pseudacris crucifer*) were collected from a pond at the University of Missouri-Columbia Baskett Wildlife Research Area in Ashland, MO. The frogs were brought to the CERC where they were allowed to oviposit in separate aquaria filled with well water. Eggs were allowed to hatch and after 6 d water was changed and tadpoles were placed into a single aquarium and fed *ad libitum* Tetra-Min® fish flakes. Tadpoles were used in the experiment 11 d after oviposition and had reached stage 25 (Gosner, 1960).

The experiment was designed as a 2×5 complete factorial: tadpoles were exposed to one of two UV light treatments and to one of five SPMD treatments, replicated three times. Test solutions were created by filling 3-L beakers with 2 L of well water, and adding 1.0 mL of the appropriate SPMD extract treatment (hereafter, SPMD treatment) or field blank SPMD. We also used a well water (pH 7.8; hardness 286 mg/L $CaCO_3$; alkalinity 258 mg/L $CaCO_3$) control. Our high UV-treatment (16.2 $\mu W/cm^2$ UV-B) was achieved by wrapping the sides of each chamber polycarbonate plastic (0.030 inch thickness, Cope Plastics, Inc. St. Louis, Mo) and covering the top of the chamber with two pieces of cellulose acetate (0.015 inch thickness, Cope Plastics, Inc.,

St. Louis, Mo) and one piece of and shade cloth (50% shading, Lowe's Home Improvement Center). The low-UV treatment (<1 $\mu W/cm^2$ UV-B) was created by wrapping the sides of the chambers with Mylar D (0.005 inch thickness, Cope Plastics, Inc., St. Louis, Mo) and covering the tops of the chambers with two pieces of polycarbonate plastic and one piece of shade cloth. Prior to testing, the irradiance level of each treatment was measured with a spectroradiometer at a 10-cm depth in the solar simulator, as described in Little and Fabacher (1996). The high irradiance value we used throughout the experiment in the solar simulator fell just above the mean value we measured at the lowest depths we measured in the field. The simulator was programmed on a 16L:8D photoperiod. UV-B lights were activated five hours into the light cycle for five hours to simulate midday solar intensity. This lighting regime provides for the induction of cellular photorepair functions and prevents the over-estimation of UV-induced injuries.

Groups of ten tadpoles were placed in each beaker, and these were randomly arranged under a solar simulator in a 17° C flow-through water bath. Tadpoles were fed ground fish flakes (Aquaria® brand) *ad libitum* at each test water change (every 3 d) until metamorphosis, which was defined as the emergence of at least one forelimb (stage 42, Gosner [1960]). At metamorphosis individuals were housed in the lab until tail resorption (about 4 d), when they were weighed to the nearest 0.1 mg.

Statistical Analyses

Mean values derived from each replicate chamber were used in all analyses. Mass at metamorphosis and days to metamorphosis were log transformed, after which a Shapiro-Wilk test showed the data were normally distributed. Two-way analysis of variance (ANOVA) was used to determine the effects of UV light treatment and SPMD treatment (and their interaction) on mass at and days to metamorphosis. In the analysis of mass at metamorphosis, days to

metamorphosis was used as a covariate and vice versa. When necessary, significant pairwise differences were determined by comparing least-square means.

RESULTS

Tadpoles reared in SPMD extract from OF3 took significantly longer to reach metamorphosis (Fig 1) and were smaller upon metamorphosis (Fig 2) than tadpoles from any other treatment (Table 2). Tadpoles reared in SPMD extract from OF1 were not significantly different from the water control or any of the field blank controls (Fig 2). Responses from the field blanks were not significantly different than the controls. None of the endpoints we examined were significantly affected by UV-B alone or by an interaction between SPMD and UV-B (Table 2). No deformities were noted.

DISCUSSION

Frogs exposed to SPMD extract from OF3 were smaller at metamorphosis and took longer to reach metamorphosis than frogs exposed to any other treatment. Why there were effects at OF3 and not OF1 remains unclear, particularly because OF1 is closer in proximity to an oil pad than OF3. The fact that the growth and development of the frogs reared in OF3 field blank SPMD did not differ from the water control indicates that the contaminant in question is aquatic and not airborne. The duration of the larval period and the mass at metamorphosis are important life history traits for amphibians exhibiting complex life cycles. Wilbur and Collins (1973) suggest that amphibian larvae make trade-offs between growth and development based on the quality of the larval environment. In stressful environments amphibians will metamorphose

sooner at the expense of larger body size. Under favorable conditions, larvae will remain in the aquatic environment and achieve a larger body size at metamorphosis.

Although there may be no correlation between larval life history traits and adult fitness, many researchers have demonstrated such a link (Berven and Gill, 1983; Smith, 1987; Semlitsch et al., 1988). Amphibian larvae with rapid growth rates can more quickly escape gape-limited predation (Heyer et al., 1988) and can leave the aquatic environment sooner, thereby reducing the time exposed to aquatic predators. Furthermore, individuals metamorphosing at a large size may have the benefit of greater overwintering success and ultimately greater fitness. Semlitsch et al. (1988) found that juveniles that metamorphosed early and at a large size were larger adults at reproduction. Larger females have greater fitness because they can produce more eggs (Berven, 1982). In species that must compete to secure territories and/or females (e.g., bullfrogs), larger males will attain better territories and more mates (Howard, 1978a) and females will be able to reproduce more frequently (Howard, 1978b). These characteristics can be especially important in species occurring in northern climates with short growing seasons, such as the wood frog (*Rana sylvatica*) in Alaska.

We selected the spring peeper *Pseudacris crucifer* as a surrogate test species for the wood frog because of its availability from uncontaminated habitat, its comparatively short developmental time to metamorphosis, and its high sensitivity to environmental contaminants. Birge et al. (2000) ranked the spring peeper as highly sensitive based upon acute toxicity tests of 34 inorganic elements and 27 organic chemicals. In these tests the spring peepers consistently exhibited lower tolerance for such exposures than any of the ranid species tested.

A number of oil spills in the Swanson River Oil Fields have likely contributed to contamination by petroleum hydrocarbons including PAHs (Parson, 2001), which have been shown to be made more toxic in the presence of UV radiation (Little et al., 2000; Zaga et al., 1998). In our study, there were no differences in growth and development attributable to the interaction of UV-B and SPMD extracts. This suggests that compounds responsible for reduced growth and delayed development at these sites may not be photoactivated. However, since SPMDs selectively absorb lipophilic substances, the observed biological effects were likely caused by fat-soluble compounds.

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Table 1. Ultraviolet-A and –B intensities from various depths at Oil Fields 1 and 3. Each value is derived from two replicate measurements at each depth.

Oil Field 1	UV-A (mW/M^2)	UV-B (mW/M^2)
Surface	21,950	868
10 cm	1,970	28.8
20 cm	852.5	24.2
30 cm	654	20.9
40 cm	4.51	11.0

Oil Field 3	UV-A (mW/M^2)	UV-B (mW/M^2)
Surface	43,710	304.5
10 cm	3,082	55.3
20 cm	2,035	21.2
30 cm	1,285	10.5
40 cm	705	6.5

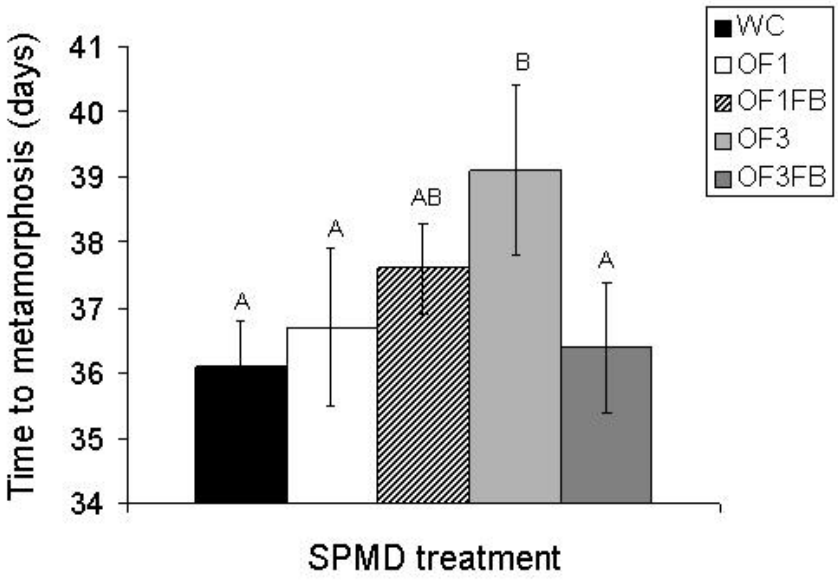
Table 2. Univariate analyses of variance on the effects of SPMD and UV-B treatments (and their interaction) on the mass and days to metamorphosis of *Pseudacris crucifer* tadpoles. Type III mean squares are reported.

Response variable	Source	df	MS	F	P
Mass at metamorphosis	Days	1	0.0842	6.31	0.0212
	SPMD treatment	4	0.0496	3.72	0.0212
	UV-B treatment	1	0.0009	0.07	0.7887
	SPMD x UV-B	4	0.0300	2.25	0.1020
	Error	19	0.0133		
Days to metamorphosis	mass	1	32.3956	6.15	0.0227
	SPMD treatment	4	15.6095	2.96	0.0464
	UV-B treatment	1	1.1937	0.23	0.6394
	SPMD x UV-B	4	7.9893	1.52	0.2371
	Error	19	5.2656		

Figure legend

Figure 1. The time it took *Pseudacris crucifer* tadpoles to reach metamorphosis. Bars with the same letters are not significantly different from one another. Vertical bars represent ± 1 SE.

Figure 2. The mass of *Pseudacris crucifer* tadpoles upon metamorphosis. Bars with the same letters are not significantly different from one another. Vertical bars represent ± 1 SE.


WC
OF1
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OF3
OF3FB
Time to metamorphosis (days)
41
40
39
38
37
36
35
34
A
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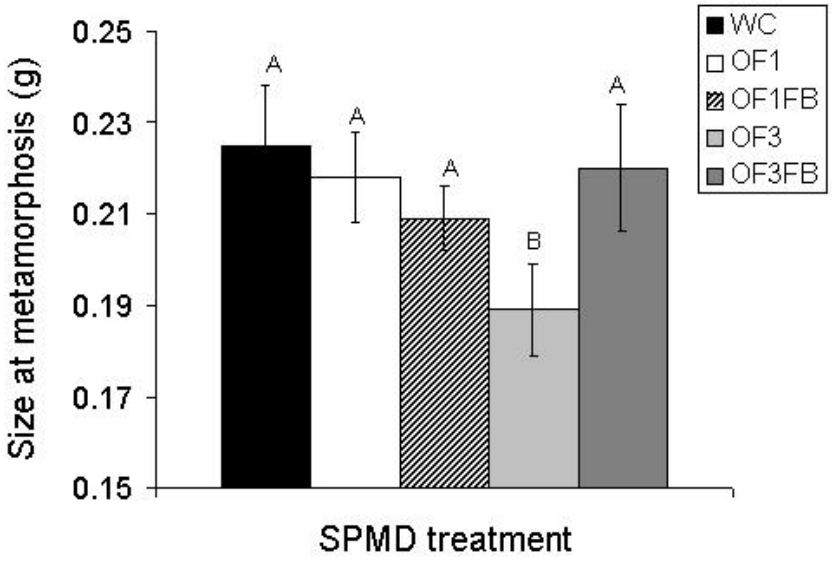
AB
B
A
SPMD treatment


Size at metamorphosis (g)
0.25
0.23
0.21
0.19
0.17
0.15
A
A
A
B
A
WC
OF1
OF1FB
OF3
OF3FB
SPMD treatment

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Examining the Toxicity and Teratogenicity of Sites on the Kenai Peninsula using Semi-permeable Membrane Device Extracts and Sediments to Wood Frogs

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Administrative Report

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INTRODUCTION

Declining amphibian populations and increasing incidences of amphibian malformations have been recorded in many locations throughout the United States. Many suggested causes for amphibian declines also are associated with amphibian 89deformities (Ouelette, 2000; Lannoo, 2000), including contaminants (Ouelette and others, 1997; Bonin and others, 1997), ultraviolet radiation (UV) (Ankley and others, 2000), and parasites (Johnson and others, 1999) either singly or in combination. Analysis of water and sediment collected from amphibian habitat in central Minnesota documented the presence of multiple contaminants that have the potential to trigger developmental abnormalities in some species (e.g., *Xenopus*; Fort and others, 1999a, 1999b).

The Kenai National Wildlife Refuge (KNWR) covers more than 800,000 hectares (ha) on Alaska's Kenai Peninsula, south of Anchorage. In the past, activities on the KNWR such as oil and gas development, pesticide application, military and recreational uses, mining, and fire and fire retardant application have resulted in chemical contamination (Parson, 2001). Little or no remedial efforts have been undertaken, primarily because of Alaska's remoteness.

KNWR provides habitat for the region's only amphibian species, the wood frog (*Rana sylvatica*). Recently, surveys have identified several populations of wood frogs on KNWR with several deformed individuals. These surveys prompted contaminants studies on and around KNWR in an attempt to determine causality (Bridges and Little, 2005).

Bioassays using extracts obtained from semipermeable membrane devices (SPMDs) offer a means of evaluating contaminants in amphibian habitats (Huckins and others, 1993). The SPMDs are integrative passive samplers containing lipid that accumulate contaminants from the environment similar to aquatic organisms (Huckins and others, 2001; Petty and others, 2000). Toxicity tests using SPMD extracts can be a useful tool in determining whether or not environmental compounds or complex mixtures of contaminants can have effects on development and the incidence of deformities of larval anurans (Bridges and Little, 2003; Bridges and Little, 2004).

Bridges and Little (2005) indicated that tadpoles exposed under laboratory conditions to water containing a complex mixture of organic chemicals collected from several aquatic environments on KNWR delayed metamorphosis. In addition, exposed organisms reached metamorphosis at a smaller size than control tadpoles; however, these tests evaluated only the effects of water exposure using extracts collected with SPMDs that sampled only lipid-soluble contaminants. Because developing tadpoles spend much time in (even ingesting) the sediment, it is important to examine whether or not sediment exposure can adversely affect larval amphibian survival, growth, and development.

This report summarizes toxicity assessments of water and sediments collected from the KNWR. The objectives of this investigation were to determine whether or not site water and sediments from eight sites on the KNWR contained compounds that negatively affect developing wood frog tadpoles by measuring tadpole growth, the duration of the larval period, and the incidence of malformations upon metamorphosis of tadpoles; and to determine whether or not exposure to UV-B intensities similar to those measured in the field could alter the toxicity or teratogenicity of sediments to wood frog tadpoles.

METHODS

SPMD Specifications: The SPMDs used in this project consisted of a 90-centimeter (cm) length of 2.5-cm wide layflat low density polyethylene tubing containing 1 milliliter (mL) of purified triolein. The

resulting membrane surface area to total SPMD volume ratio was approximately 90 centimeter squared per milliliter (cm^2/mL) or approximately 460 cm^2/mL of triolein, where the SPMD consisted of ≈ 20 percent triolein. The SPMDs weighed 4.4 to 4.6 gram (g) with an active exchanging or sampling surface of approximately 440 centimeter squared (cm^2). The SPMDs were placed into labeled, solvent rinsed, gas-tight cans, which were flushed with argon and then sealed. These cans were shipped overnight in coolers to the field for deployment.

SPMD Deployment for Water Sampling: SPMDs were deployed during August 2004 at eight sites (KNA01, KNA03, KNA62, KNA31, KNA97, KNA08, KNA46, KNA51) within the KNWR. These sites were selected by the U. S. Fish and Wildlife Service (USFWS) and included "developed sites" within 1.6 kilometers (km) of towns, roads, mines, and areas of oil development and "remote sites" that were beyond 1.6 km of human activities. Three plots, defined at each site, were searched for frogs. Ponds supporting wood frog populations were randomly selected as sampling sites. At each site, five standard SPMDs were placed in each of three replicate stainless-steel canisters attached to a steel cable anchored to the shore. At the time of deployment, a metal can containing SPMDs (hereafter, field blank) was opened and exposed to the air at each site. These provided a control for any airborne contaminants present while the SPMDs were exposed to the air (i.e., before being placed into the water). Once the SPMDs were in the water, the cans containing the field blanks were sealed and stored at 0 to 4 degrees Celsius (°C) for the time between SPMD deployment and retrieval to halt contaminant uptake (from the site air that had filled the can). During retrieval, field blanks were again exposed to the air at the site for as long as it took to remove the SPMDs from the water and seal them in cans. After retrieval, the SPMDs and field blanks were shipped on ice and remained frozen until they were processed at the Columbia Environmental Research Center (CERC, Columbia, MO) using techniques outlined in Petty and others, (2000).

SPMD extracts from each site were pooled into a single composite sample. Field exposure and control extracts (i.e. field blanks) were dissolved into sterile dimethylsulfoxide (DMSO) by solvent exchange. Extracts from the SPMDs were added to 90 mL of sterile DMSO so that each milliliter contained the approximate equivalent of a 2-day exposure (i.e. represents the amount of bioavailable residues extracted from site water by a standard SPMD in two days' time) when added to 1-liter (L) of water.

Sediment and Animal Collection: Sediment was collected with a clean plastic shovel to a depth of 10 cm at two different times from each of the eight sites. The first set of sediment samples was collected in August, 2004, and the second set of samples was collected in September, 2006. The sediments were extremely organic, primarily composed of submerged peat. Sediments were placed in acid washed, solvent rinsed 1-L glass jars. The jars were covered with acetone- and hexane-rinsed aluminum foil, sealed with a screw-top lid and shipped overnight to CERC in coolers packed with blue ice. Upon arrival at CERC, the jars were refrigerated (4 °C) until use in the exposures.

During May 2005 and 2007, wood frog (*Rana sylvatica*) tadpoles were collected in the field in Alaska by the USFWS and shipped overnight to CERC. Upon arrival, tadpoles were in good condition and were allowed to slowly acclimate (7 days) to temperature (17 °C) in CERC blended water (25 percent well water, 75 percent deionized water) to mimic the water quality of KNWR natural conditions (alkalinity approximately 75 milligrams per liter (mg/L) as calcium carbonate ($CaCO_3$, pH=8.0). These wood frogs from Alaska were used in tests assessing site water. During April 2005, additional wood frog tadpoles were collected from the Daniel Boone National Forest in Missouri for use in the sediment exposures. These tadpoles were at stage 25 of development with an average weight of 0.25 g. In 2007, sediment/UV tests were conducted with wood frog tadpoles collected from Alaska.

Site Water Evaluation with SPMD Extract Exposures: The SPMD extract exposures were conducted during the summer of 2005 and were designed as a 2 × 16 complete factorial: tadpoles from Alaska were exposed in one of two solar simulators (Little and Fabacher, 1996) to one of two UV light treatments and to one of 20 treatments including: four well water controls (one for each simulator during each trial), SPMD extracts from eight sites within Kenai, and field blank extracts from each of the eight sites. SPMD treatments were replicated three times. Groups of three tadpoles were placed in the 3-L chambers randomly arranged under one of two solar simulators in a 20 ° C flow-through water bath. Two solar simulators were used to accommodate the large number of exposure chambers. Every time water was changed (every third day), 1 mL of the appropriate extract (the equivalent to a 2-day dose) was added to the chambers. Tadpoles were fed ground fish flakes (Tetra-Min® brand) *ad libitum* at each test water change until metamorphosis, which was defined as the emergence of at least one forelimb. At metamorphosis individuals were removed from the experimental chamber and housed in the laboratory polypropylene sandwich containers with a wet paper towel substrate until tail resorption (about 4 days), when they were weighed to the nearest 0.1 milligram (mg).

2005 Sediment Exposures: Sediment exposures were conducted during the summer of 2005 using wood frog tadpoles from Missouri because no Alaskan tadpoles were available. Tadpoles were exposed to sediments retrieved from each of eight KNWR sites (corresponding to the sites at which SPMDs were deployed) throughout development. Sediment (35 g) was squeezed dry, wrapped in a double layer of unbleached cheesecloth, tied with unbleached natural string, and placed in a 3-L glass chamber containing 2 L of water. This method of exposure limited the direct contact of tadpoles with the sediment, thus increasing the likelihood that exposure would be to chemicals that diffuse into the water. Groups of three tadpoles were placed into each chamber. Tadpoles were fed ground fish flakes *ad libitum*, water was changed every third day and sediment was changed every 2 weeks. This exposure was conducted under

general laboratory lighting. At metamorphosis individuals were removed from the experimental chamber and housed in the laboratory until tail resorption (about 4 d), when they were weighed to the nearest 0.1 mg.

2007 Sediment Exposure: A second experiment with sediments was conducted during summer, 2007. This exposure was designed as a 2 x 8 complete factorial, replicated four times. Tadpoles from Alaska were exposed to one of two UV intensities and one of 10 treatments (two controls and eight unique sites). Exposure began on June 20, 2007 (day 0) and ended on day 74, at which time nearly all tadpoles had reached metamorphosis.

Sediments were prepared in cheesecloth as described above and placed in 2 L of water. The sediment was not changed during the exposure. One set of control chambers contained only CERC blended water. Another set of chambers (started at day 13 of the experiment) contained bags of commercially-purchased, clean peat moss prepared in cheesecloth. The tadpoles stocked in these chambers were of comparable size to the organisms in the ongoing exposure.

Glass chambers (3-L) were filled with 2 L of CERC blended water, prepared by mixing 25 percent well water and 75 percent deionized water to create an alkalinity of about 75 mg/L as $CaCO_3$, before adding the sediment bags to each. Tadpoles were added in groups of three to the exposure chambers, which were then placed into a circulating water bath in the laboratory, and maintained at 17 ^{o}C. Developing tadpoles were on a 16:8 light:dark (L:D) cycle. UV lights were activated between 10 am and 2 pm each day. The control (low UV) chambers were covered and wrapped with a sheet of polycarbonate plastic, which yielded a UV-B intensity of less than 0.001 microWatts per centimeter squared ($\mu W/cm^2$). The high UV treatments were covered and wrapped with mylar, resulting in a UV-B intensity of about 4 µW/cm2. The treatment intensities were comparable to UV levels measured at the collection sites in KNWR (table 1, at the back of this report).

A 90 percent water change was completed twice weekly throughout the duration of the exposure. Tadpoles were fed a diet of ground Tetra-Min® brand fish flakes at each water change. Tadpoles were fed 20 mg of food the first week; food volume was increased by 20 mg each week to a maximum of 200 mg of food. The amount of food added to each chamber was proportionally adjusted when tadpoles died or metamorphosed. The test was checked daily for metamorphed tadpoles [those reaching forelimb emergence, or stage 42 (Gosner, 1960)] and for mortality. Each week, 100-mL water samples were collected from 16 random chambers to assess water quality, including eight high UV and eight low UV treatments.

Upon metamorphosis (defined as foreleg emergence, stage 42, Gosner, 1960), tadpoles were weighed, and held in the laboratory until tail absorption, then weighed again. Tadpoles generally are weighed only at tail absorption because they tend to lose weight between stages 42 and 46 (Gosner, 1960). However, weighing them at stage 42 was the only weight that could be attained for many individuals because many died before completing tail absorption. Upon emergence, tadpoles were examined for malformations.

Statistical Analysis: Mass and time to metamorphosis for the three tadpoles in each replicate chamber were pooled to attain a single data point. Analysis of variance (ANOVA) considered differences in response to solar simulators and to UV treatment. Separate ANOVAs were conducted for the extract and sediment exposures. There were no significant differences in responses between the two solar simulators during the 2005 tests with extracts and sediments, so the effect of this factor was dropped from the model. For each site ANOVA was used to determine whether or not survival, mass at metamorphosis, the length of the larval period, or the proportion of tadpoles in the treatment reaching metamorphosis were dependent on SPMD extract treatment (i.e., site), UV treatment, or the interaction of these factors. Mass and time to metamorphosis were log transformed, whereas survival and proportion reaching metamorphosis were

arcsin square-root transformed to increase normality. When there were significant differences among SPMD extract treatments, Bonferroni multiple comparison tests were conducted to discern differences among specific treatments at $\alpha = 0.05$.

An ANOVA was conducted to determine treatment effects among sediment and UV treatments with respect to mortality that occurred between the time they reached metamorphosis and the time tail absorption was complete during the 2007 tests.

RESULTS

UV intensity (table 1) applied during this study, and water quality (table 2, at the back of this report), were within acceptable limits for the test organisms.

Exposures to Extracts: In the SPMD extract study tadpoles exposed to the low-intensity UV treatment had significantly higher survival $F_{1,80}=4.36$; $p<0.0400$ than tadpoles reared in high UV treatments (fig. 1, table 3, at the back of this report). Tadpole mass at metamorphosis was also significantly affected by UV radiation (fig. 2, table 4, at the back of this report)

There was a significant effect due to site with respect to time to metamorphosis, the mass at metamorphosis, and survival. Sites significantly affected metamorphosis ($F_{18,65}= 4.72$, $p < 0.0001$). Tadpoles exposed to extracts from the KNA01 and KNA46 sites, took 14 and 8 days longer, respectively, on average to metamorphose than tadpoles from the controls, whereas tadpoles exposed to extracts from KNA03 metamorphosed on average about 4 days earlier than the controls. The responses from tadpoles in most field blank extracts were similar to those of controls (fig. 3, table 5, at the back of this report) although those exposed to extracts of the field blanks from site KNA46 metamorphosed on average, 5 days later than controls. Site affected the size of the organisms at metamorphosis ($F_{17,51} = 1.86$; $p \leq 0.0446$; fig. 4, table 6, at the back of this report). Tadpoles exposed to extract from site KNA97 were

smaller upon metamorphosis than controls as were those exposed to field blanks from sites KNA62 and KNA31. Tadpoles exposed to extracts from sites KNA08 and KNA51 and to field blanks from site KNA46 were larger than controls at metamorphosis (fig. 3). Site also affected tadpole survival ($F_{19,80}$=4.36. p<0.0400; fig. 5, table 7, at the back of this report). Survival among tadpoles exposed to extracts from sites KNA31, KNA 97, KNA 62, and KNA01 and to the field blank extract from site KNA08 was lower than controls. The proportion of tadpoles exposed to extracts from sites that reached metamorphosis by the end of the study ($F_{19,70}$=1.78, p<0.0424) also was lower than controls.

The only significant interactions between UV exposure and site extract toxicity occurred during exposures to KNA01 and KNA 62 ($F_{19,70}$=1.98; p<0.0207), which resulted in a reduced proportion of tadpoles reaching metamorphosis compared to controls. (fig. 6, table 8, at the back of this report), and reduced survival ($F_{19,70}$ = 2.73; p<0.0400: fig. 7, table 9, at the back of this report).

2005 Sediment Exposures: In the 2005 sediment study, site had a significant effect on the time to metamorphosis ($F_{9,11}$=3.68; p<0.0229; fig. 8, table 10, at the back of this report) Tadpoles exposed to sediments from sites KNA08 and KNA51 required an average of 9 and 4 days, respectively, longer to reach metamorphosis than controls. Mass at metamorphosis was also affected by sediment exposures ($F_{9,11}$=4.55; p<0.0107; fig. 9, table 11, at the back of this report). Tadpoles exposed to sediment from site KNA46 were larger than controls, whereas tadpoles from site KNA97 were smaller than controls at metamorphosis. Tadpoles exposed to sediment from sites KNA62, KNA97, KNA01, and KNA46 had lower survival than controls (fig. 10, table 12, at the back of this report). This indicates the presence of compounds within the sediments of some sampling sites in the KNWR may have been detrimental to tadpole growth, development, and survival.

2007 Sediment Exposures: For the 2007 sediment study, the number of days for tadpoles to reach metamorphosis was significantly affected by sediment collection site ($F_{7,39}$=2.96; $p \leq 0.0138$; fig. 10, at the back of this report). Individuals exposed to sediments from site KNA02 metamorphosed about 7 days earlier than tadpoles exposed to control sediments and or sediments from site KNA08. No other endpoints, including incidence of malformations, were significantly affected by site.

Although tadpoles exposed in the highest UV intensity completed metamorphosis more quickly than those exposed to low UV intensity ($F_{1,39}$=3.34; $p \leq 0.0755$), this trend was not significant. Furthermore, there was no significant interaction between UV intensity and sediment site suggesting that compounds within the sediment are not photoactivated or photodegraded ($F_{1,39}$.= 0.32; p= 0.938).

DISCUSSION

Effective management for the restoration of amphibian populations requires identification of causal factors contributing to their declines. A systematic approach to determine causality especially is important because initial impressions may be misleading or ambiguous (Little and others, 2003). The common model is to test for specific compounds based on environmental sampling, site history, or chemical use in the watershed, when in fact there may be a complex mixture of substances that may be additive or synergistic in their injury to amphibians. Using environmental extracts (i.e., contaminants sequestered using SPMDs) provides a rapid means of detecting the presence of toxicity that would justify further chemical analysis to identify the causal agent (Bridges and Little 2004).

Most chemical agents do not elicit outright mortality to amphibians, which is likely part of the reason it is so difficult to determine specific causal agents of declines; however, subtle shifts in life-history characteristics (or even increases in the incidence of malformations) can have population-level effects with time, making it important to examine more subtle endpoints than mortality. A habitat that yields smaller amphibians at metamorphosis may realize declines in population sizes with time when compared

with a healthy population (Smith, 1987; Semlitsch and others, 1988) because smaller individuals will have fewer eggs Similarly, delays in metamorphosis (i.e., longer larval periods) can have the same population-level impacts (Smith, 1987; Semlitsch and others, 1988).

No limb malformations were observed during the laboratory exposures; however, examine newly transformed frogs were note examined for non-limb (internal) malformations. Frogs with limb malformations observed in the field at these sites may have been caused by factors unrelated to contamination. Also, deformities may be attributable to a contaminant not sequestered by SPMDs and, therefore, not sampled in our study (e.g., metals, polar organics).

UV radiation was included as a factor in the study because UV-B exposure of larval and egg stages has been suspected of negatively impacting some amphibian species by reducing hatching success and increasing embryonic malformation rates (Lizana and Pedraza, 1998; Blaustein and others, 1998; Broomhall and others, 2000; Starnes and others, 2000) as well as generating larval malformations (Ankley and others, 2000). UV radiation also is known to increase the toxicity of various compounds to aquatic amphibians, including some polycyclic aromatic hydrocarbons (Little and others, 2000; Zaga and others, 1998), which are known to have been released within the Kenai Refuge as a result of petroleum extraction activities (Parsons, 2001). Tadpoles exposed to the low UV treatment had greater survival than those exposed to the elevated treatment during the extract exposures; during sediment exposures, UV did not affect tadpole survival. It did cause tadpoles to reach metamorphosis at a smaller size and at a later date than low-UV controls. Higher UV radiation also decreased the number of individuals reaching metamorphosis before the conclusion of the experiment. The enhanced survival during the sediment exposures in the high UV treatment may have resulted from shading by the sediment bags, or the presence of UV-absorbing humic acids released by the sediments. Bridges and Boone (2003) suggested that in a laboratory setting larvae may not benefit from naturally-occurring dissolved organic carbon that normally would filter much of the UV radiation entering a water column. During this study, the intensities of UV

radiation measured in the laboratory were similar to if not lower than those measured in the field at 10 cm deep.

With one exception, there were no significant interactions between UV and the toxicity of extracts or sediments of the study sites. The lack of significant interaction between UV intensity and sediment treatment indicates that any contaminants present in the sediments are not photoactivated or photodegraded. Photoenhanced toxicity was expected to be evident if polyaromatic hydrocarbons (PAHs) were present at the site.

Significant effects on survival, time to metamorphosis, and mass at metamorphosis resulted from exposure to extracts and to sediments from certain sites. This outcome indicates the possibility of the presence of compounds in certain sites that are harmful to tadpoles in terms of time to metamorphosis and mass at metamorphosis. The tests with extracts and sediments during the 2005 investigations indicate consistent mortality among tadpoles exposed to extracts or to sediments from sites KNA01, KNA62, and KNA97. Previous studies have been conducted on the toxicity of site water and sediments at three of the sites used in this study: KNA01, KNA03, and KNA08 (Bridges and Little, 2003). Even though these studies are not directly comparable because they were conducted during different years with a different species, trends can be compared. With reference to days to metamorphosis, SPMD extracts and sediments from the site KNA01 had a relatively long developmental period. This indicates that the water and sediment at this site have at least a low level of toxicity. Tadpoles exposed to SPMD extracts and sediments from KNA03 never significantly differed from controls, indicating a lack of toxicity for this site. Interestingly, tadpoles exposed to sediments from site KNA08 had long developmental periods, whereas tadpoles exposed to SPMD extracts from this site had relatively short developmental periods, indicating that the contaminants at this site that lengthen the larval period were more associated with sediment rather than water. During sediment exposures in 2007, it was determined that tadpoles exposed to sediment from site KNA08 had the longest development time compared to the other Kenai sites. It is

important to note that the 2005 and 2007 sediment exposures occurred 10 to 11 months after sediment collection as test organisms became available; therefore, there may have been changes in their chemical composition during storage.

Field blanks are intended to detect airborne substances present while the SPMDs are being deployed, and are a type of control. Exposure to the field blank from site KNA08 seemed to decrease survival, whereas exposure to the KMA46 field blank appeared to increase the number of days it took tadpoles to reach metamorphosis and increased mass at metamorphosis. Field blanks from KNA31 and KNA62 appeared to decrease mass at metamorphosis. This may indicate the presence of a contaminant in the air while the SPMD was being deployed.

Many researchers have demonstrated a link between larval life history traits and adult fitness in amphibians (Berven and Gill, 1983; Smith, 1987; Semlitsch and others, 1988). The length of the larval period particularly is important in species occurring in northern climates, such as the wood frog, with short growing seasons; however, the absence of malformations would tend to rule out potential contaminant causality in the KNWR sites.

From a management perspective, contamination issues often are difficult to address. This is particularly true in the case where a contamination event has occurred is not ongoing, such as at the Kenai NWR. It is important to continue to monitor amphibian population status for several seasons to determine the extent to which contamination is affecting the population, and whether or not other factors are involved at such sites. Regardless, it is important to continue to protect such sites from further contamination events. It also might be possible to determine whether or not contaminants present at a site are interacting with other, more controllable, factors or to undertake measures to minimize the availability of contaminants to amphibians in the water column.

Disclaimer: Any mention of trade names does not constitute government endorsement.

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Figure legend

Figure 1. The effect of UV radiation on tadpole survival. Vertical lines represent ± 1 SE.

Figure 2. The effect of SPMD site extract on tadpole time to metamorphosis. Vertical lines represent ± 1 SE.

Figure 3. The effect of SPMD site extract on tadpole mass at metamorphosis. Vertical lines represent ± 1 SE.

Figure 4. The effect of SPMD site extract on tadpole survival. Vertical lines represent ± 1 SE.

Figure 5. The effect of site sediment on tadpole larval period length. Vertical lines represent ± 1 SE. Control (+)= contained control sediment; Control (-) = contained no sediment. The effect of mass at metamorphosis is factored into this analysis.

Figure 6. The effect of site sediment on tadpole mass at metamorphosis. Vertical lines represent ± 1 SE. Control (+)= contained control sediment; Control (-) = contained no sediment. The effect of the length of the larval period is factored into this analysis.

Figure 7. The effect of site sediment on tadpole survival. Vertical lines represent ± 1 SE. Control (+)= contained control sediment; Control (-) = contained no sediment.

Figure 8. Mean days to metamorphosis for tadpoles reared in each of the site sediments. C = Controls without sediment; C+ = controls with sediment; Bars with * and ** differ significantly ($P < 0.05$) from each other.

Figure 9. The effect of site sediment on Missouri wood frog tadpole mass at metamorphosis. Vertical lines represent ± 1 SE. Control (+)= contained control sediment; Control (-) = contained no sediment.

Figure 10. The effect of site sediment on Missouri wood frog tadpole survival. Vertical lines represent ± 1 SE. Control (+) = contained control sediment; Control (-) = contained no sediment.

Figure 11. Mean days to metamorphosis for Alaska wood frog tadpoles exposed in 2007 KNWR site sediments. C= Controls without sediment; C+ = controls with sediment. Vertical bars represent ± 1 SE

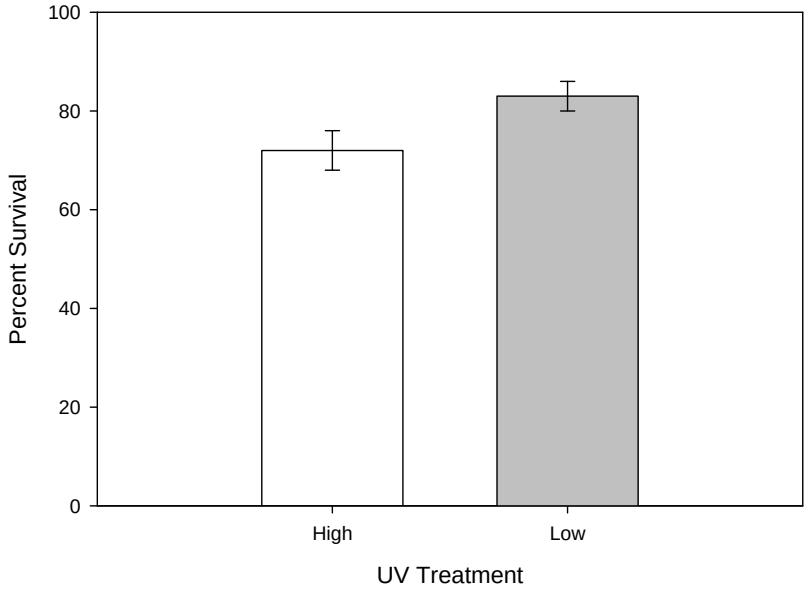

Figure 1. Effects of UV treatment on Alaska wood frog tadpole survival during SPMD extract exposures. Vertical lines represent ± 1 standard error of the mean.

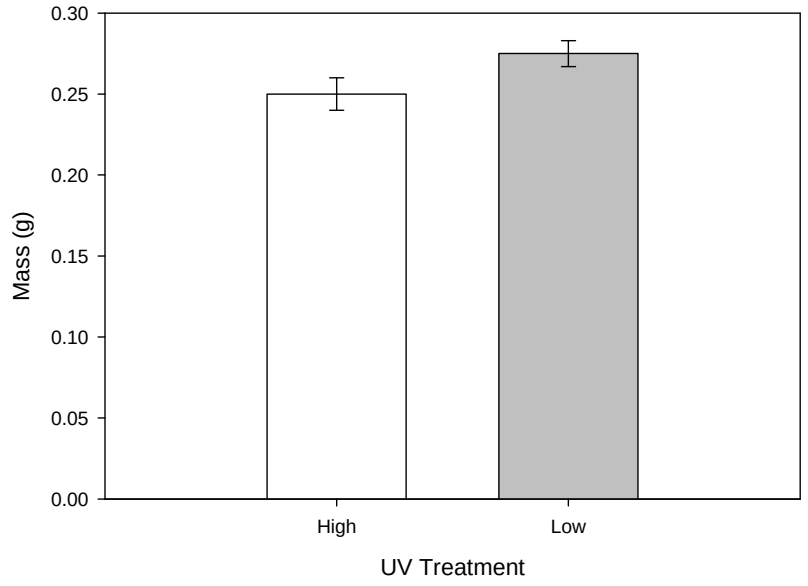

Figure 2. Effects of UV treatment on mass of Alaska wood frog tadpoles during SPMD extract exposures. Vertical lines represent ± 1 standard error of the mean.

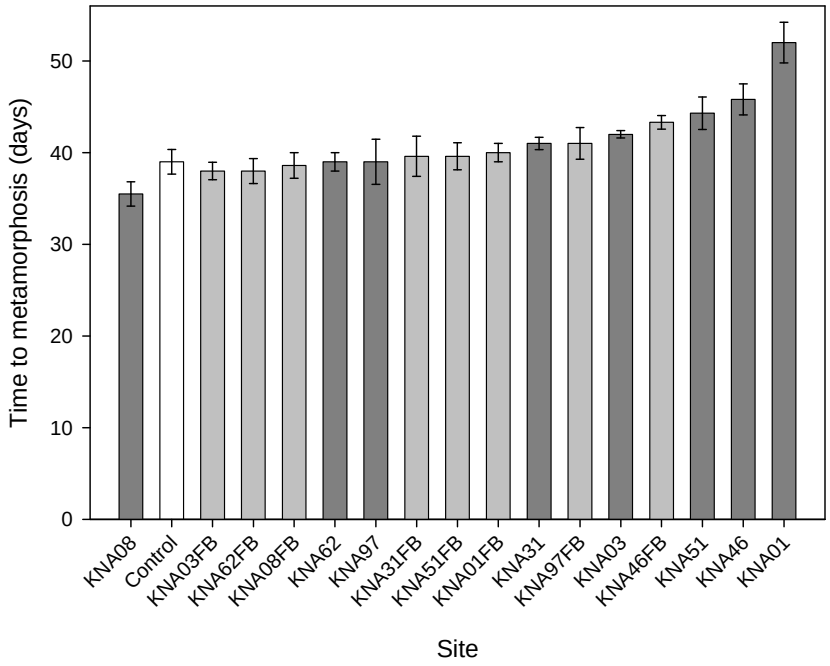

Figure 3. The effect of SPMD site extract on Alaska wood frog tadpole time to metamorphosis. Vertical lines represent ± 1 standard error of the mean.

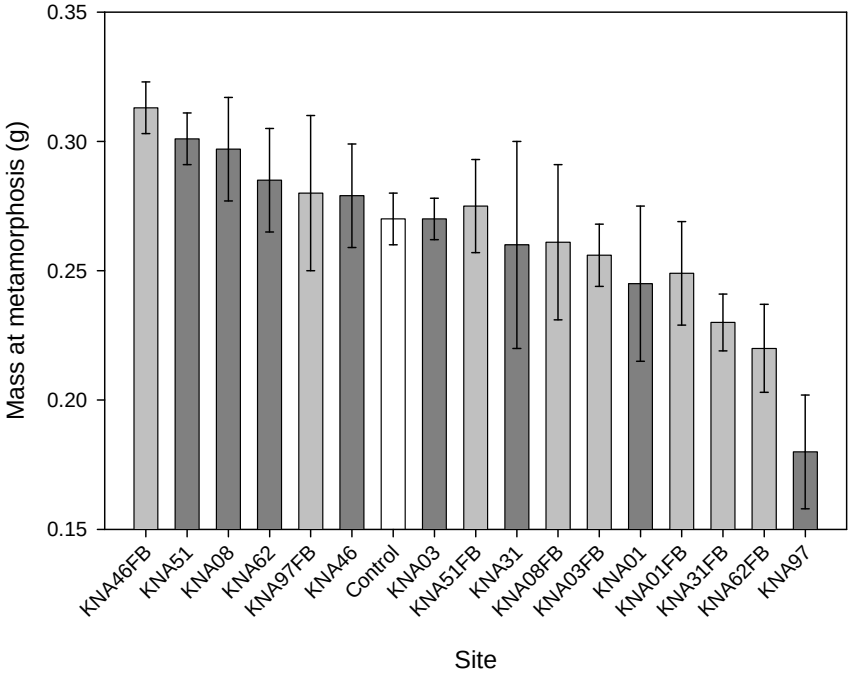

Figure 4. The effect of SPMD site extract on Alaska wood frog tadpole mass at metamorphosis. Vertical lines represent ± 1 standard error of the mean.

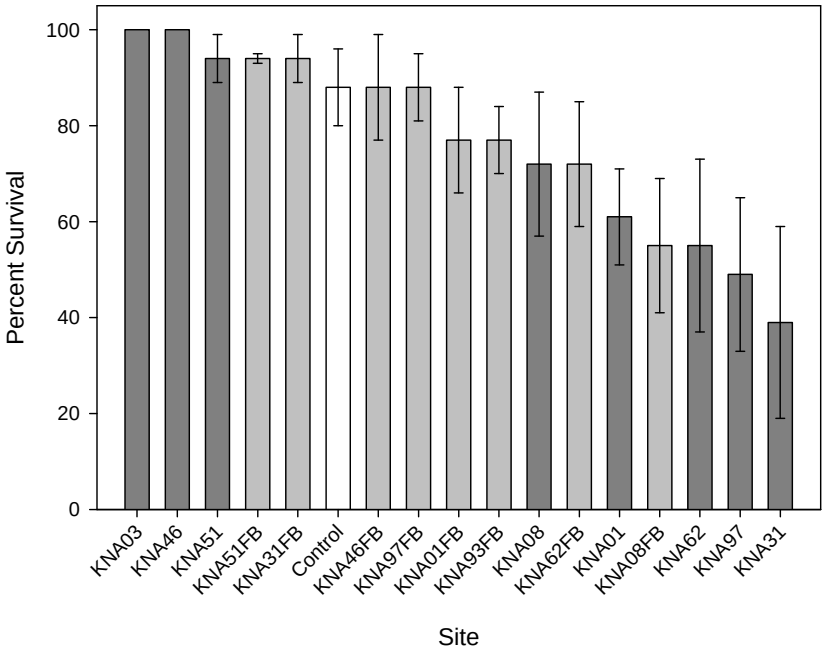

Figure 5. The effect of SPMD site extract on Alaska wood frog tadpole survival. Vertical lines represent ± 1 standard error of the mean.

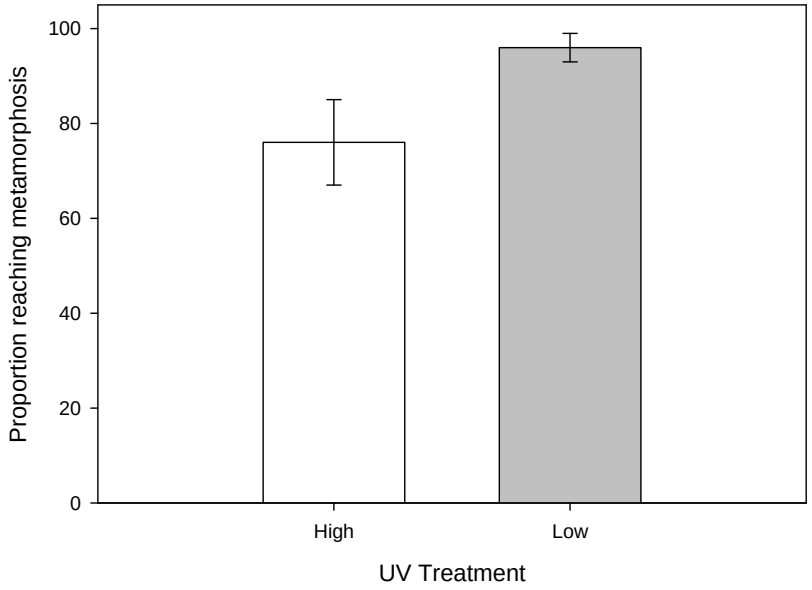

Figure 6. Effects of UV treatment on the proportion of Alaska wood frog tadpoles reaching metamorphosis from sites KNA97 and KNA 08 during SPMD extract exposures. Vertical lines represent ± 1 standard error of the mean.

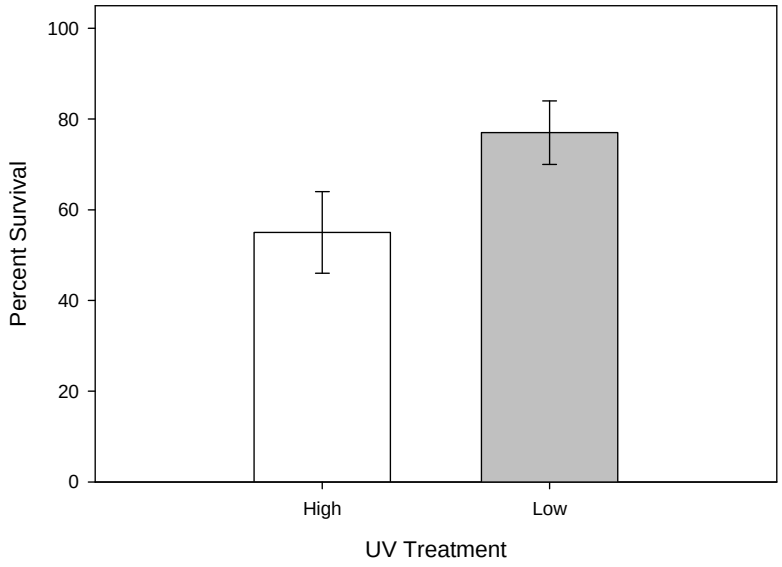

Figure 7. Effects of UV treatment on the survival of Alaska wood frog tadpoles from sites KNA01 and KNA62 during SPMD extract exposures. Vertical lines represent ± 1 standard error of the mean.

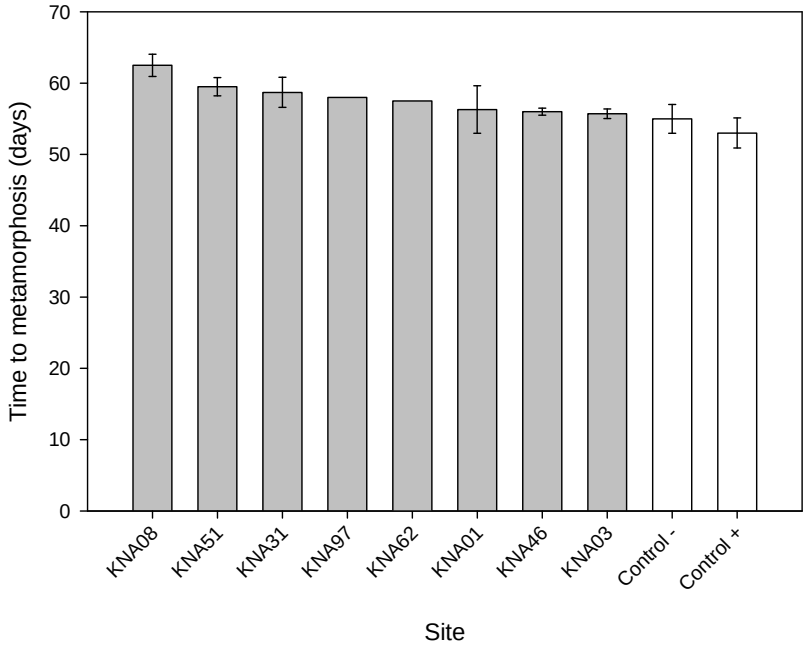

Figure 8. The effect of 2005 site sediment on tadpole time to metamorphosis. Vertical lines represent ± 1 standard error of the mean. Control (+)= contained control sediment; Control (-) = contained no sediment.

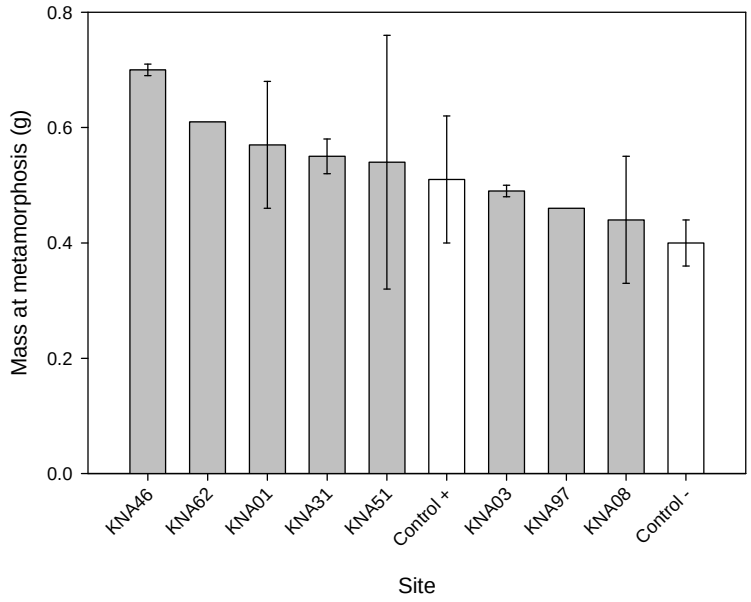

Figure 9. The effect of 2005 site sediment on Missouri wood frog tadpole mass at metamorphosis. Vertical lines represent ± 1 standard error of the mean. Control (+)= contained control sediment; Control (-) = contained no sediment.

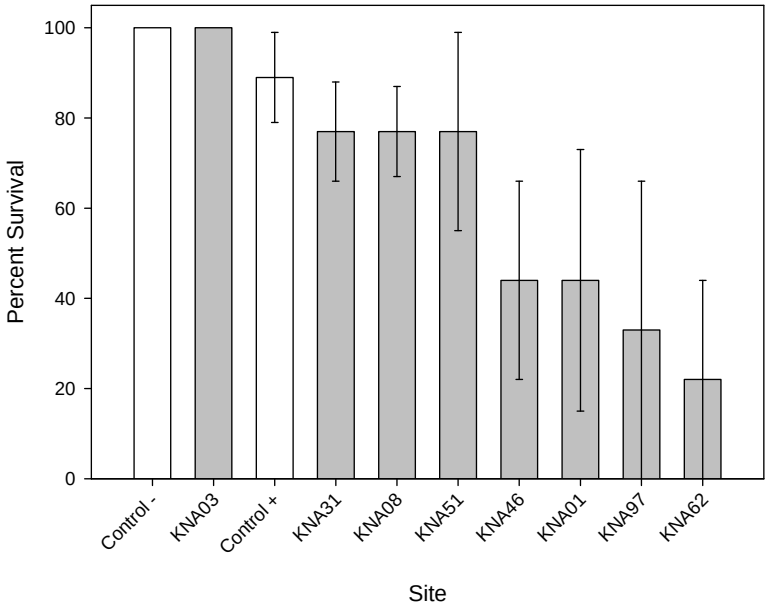

Figure 10. The effect of 2005 site sediment on Missouri wood frog tadpole survival. Vertical lines represent ± 1 standard error of the mean. Control (+)= contained control sediment; Control (-) = contained no sediment.

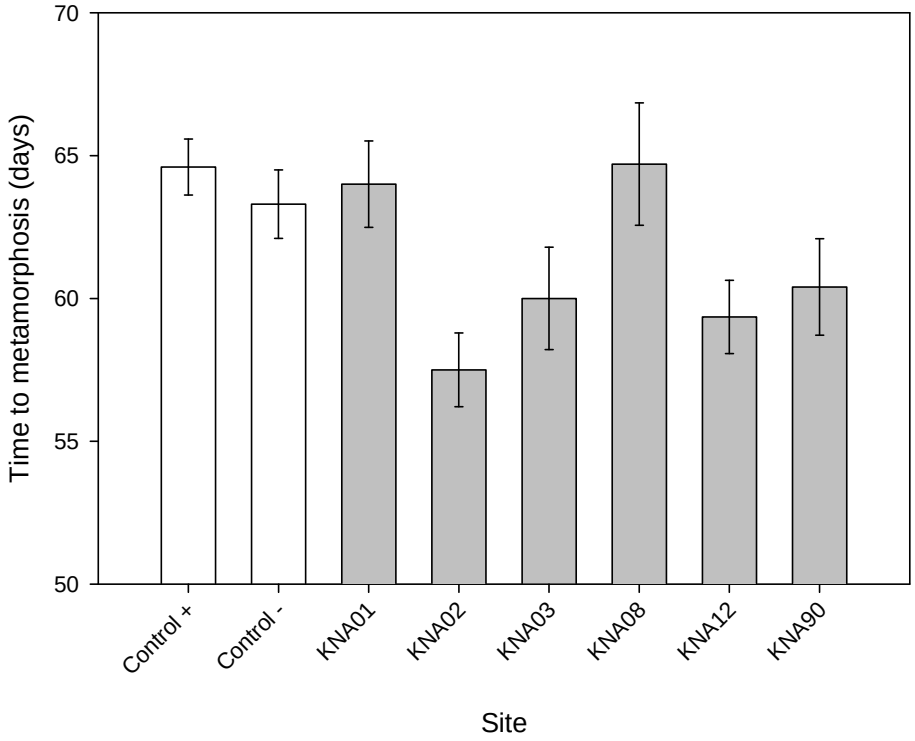

Figure 11. Mean days to metamorphosis for Alaska wood frog tadpoles reared in 2007 KNWR site sediments. C = Controls without sediment; C+ = controls with sediment. Vertical bars represent $\pm$ 1 standard error of the mean.

Table 1 . Spectral irradiance at the surface and at 10 cm depth in water of the eight sites in Kenai National Wildlife Refuge.

[UVB, ultraviolet-B; UVA, ultraviolet-A; cm, centimeter; µW/cm^2, microwatts per centimeter squared]

Site	2004 Irradiance[1] (µW/cm^2)					
	UVB Surface	**UVB 10 cm**	**UVA Surface**	**UVA 10 cm**	**VIS Surface**	**VIS 10 cm**
KNA01	141	7.6	1492	128	16,320	11,220
KNA03	152.3 (6.12)	9.9 (0.53)	1462 (19.3)	293.7 (33.6)	17,030 (1416.2)	10,920 (402.0)
KNA08	289	192	1178	70.8	26,400	12,300
KNA31	38.6 (9.58)	4.02 (0.99)	610 (15.8)	121.1 (10.02)	8487 (471.9)	5421 (421.6)
KNA46	na	na	na	na	Na	na
KNA62	210	17.2	1890	540	19,900	3640
KNA51	98.8 (8.99)	14.2 (2.50)	947.1 (44.9)	233.5 (38.3)	12,517 (683.7)	7611 (262.2)
KNA97	41.2 (2.95)	4.1 (2.11)	451.8 (21.7)	11.2 (5.33)	5784	538

[1]Measurements represent an average of the data taken during July 17-19, 2004.

Table 2. Water quality characteristics during 2007 Alaska wood frog sediment exposure.

[UV, ultraviolet; mg/L, milligrams per liter; ºC, degrees Celsius; µS/cm^2, microsiemens per centimeter squared; $CaCO_3$, calcium carbonate]

UV treatment	**DO (mg/L)**	**Temp (°C)**	**Cond. (µS/cm^2)**	**pH**	**Alkalinity (mg/L $CaCO_3$)**	**Hardness (mg/L $CaCO_3$)**	**NH4 (mg/L)**
UV	7.37 (1.13)	16.8 (0.41)	165.3 (10.6)	7.98 (0.39)	73.4 (35.6)	68.2 (32.9)	0.1855 (0.14)
NoUV	7.41 (1.05)	16.8 (0.39)	166.6 (13.1)	7.94 (0.32)	77.3 (36.4)	70.2 (31.0)	0.2632 (0.20)

Table 3. Percent survival of Alaska wood frog tadpoles exposed to SPMD extracts under two UV light treatments. Treatments are significantly different from one another ($p<0.0285$).

[Standard errors in parentheses]

UV treatment	**Percent survival**
Low	83 (0.03)
High	72 (0.04)

Table 4. Average weight of Alaska wood frog tadpoles exposed to SPMD extracts under two UV light treatments. Treatments are significantly different from one another ($p<0.0285$).

[UV, ultraviolet; g, grams; standard errors in parentheses]

UV treatment	**Mass (g)**
Low	0.275 (0.008)
High	0.250 (0.010)

Table 5. Time to metamorphosis of Alaska wood frog tadpoles exposed to SPMD extracts.

[Asterisks denote significance from control; standard deviations in parentheses]

Site	**Time to metamorphosis (days)**
KNA08	35.5* (1.33)
Control	39 (1.35)
KNA03 Field Blank	38 (0.95)
KNA62 Field Blank	36 (1.36)
KNA08 Field Blank	38.6 (1.39)
KNA62	39 (1.0)
KNA97	39 (2.46)
KNA31 Field Blank	39.6 (2.18)
KNA51 Field Blank	39.6 (1.48)
KNA01 Field Blank	40 (1.0)
KNA31	41 (0.67)
KNA97 Field Blank	41 (1.73)
KNA03	42* (0.41)
KNA46 Field Blank	43.3* (0.74)
KNA51	44.3* (1.78)
KNA46	45.8* (1.69)
KNA01	52* (2.22)

Table 6. Mass at metamorphosis for Alaska wood frog tadpoles exposed to SPMD extracts.

[Asterisks denote significance from control; standard deviations in parentheses]

Site	**Mass at metamorphosis (g)**
KNA46 Field Blank	0.313* (0.01)
KNA51	0.301* (0.01)
KNA08	0.297 (0.02)
KNA62	0.285 (0.02)
KNA97 Field Blank	0.280 (0.03)
KNA46	0.279 (0.02)
Control	0.270 (0.01)
KNA03	0.270 (0.008)
KNA51 Field Blank	0.275 (0.018)
KNA31	0.260 (0.04)
KNA08 Field Blank	0.261 (0.04)
KNA03 Field Blank	0.256 (0.012)
KNA01	0.245 (0.03)
KNA01 Field Blank	0.249 (0.02)
KNA31 Field Blank	0.230* (0.011)
KNA62 Field Blank	0.220* (0.017)
KNA97	0.180* (0.022)

Table 7. Percent survival of Alaska wood frog tadpoles exposed to SPMD extracts.

[Asterisks denote significance from control; standard deviations in parentheses]

Site	**Percent survival**
KNA03	100
KNA46	100
KNA51	94 (0.05)
KNA51 Field Blank	94 (0.01)
KNA31 Field Blank	94 (0.05)
Control	88 (0.08)
KNA46 Field Blank	88 (0.11)
KNA97 Field Blank	88 (0.07)
KNA01 Field Blank	77 (0.11)
KNA03 Field Blank	77 (0.07)
KNA08	72 (0.15)
KNA62 Field Blank	72 (0.13)
KNA01	61* (0.10)
KNA08 Field Blank	55* (0.14)
KNA62	55* (0.18)
KNA97	49* (0.16)
KNA31	39* (0.20)

Table 8. Proportion reaching metamorphosis of Alaska wood frog tadpoles from sites KNA97 and KNA08 exposed to SPMD extracts under two UV light treatments. Treatments are significantly different from one another ($p<0.0412$).

[Standard errors in parentheses]

UV treatment	**Percent survival**
Low	96 (0.03)
High	76 (0.09)

Table 9. Percent survival of Alaska wood frog tadpoles from sites KNA01 and KNA62 exposed to SPMD extracts under two UV light treatments. Treatments are significantly different from one another ($p<0.0412$).

[Standard errors in parentheses]

UV treatment	**Percent survival**
Low	77 (0.07)
High	55 (0.09)

Table 10. Time to metamorphosis of Alaska wood frog tadpoles exposed to sediments.

[Standard deviations in parentheses]

Site	**Time to metamorphosis (days)**
KNA08	62.5 (1.56)
KNA51	59.5 (1.28)
KNA31	58.7 (2.11)
KNA97	58.0
KNA62	57.5
KNA01	56.3 (3.33)
KNA46	56.0 (0.50)
KNA03	55.7 (0.67)
Control -	55.0 (2.02)
Control +	53.0 (2.11)

Table 11. Mass at metamorphosis for Alaska wood frog tadpoles exposed to sediment.

[Standard deviations in parentheses]

Site	**Mass at metamorphosis (g)**
KNA46	0.70 (0.01)
KNA62	0.61
KNA01	0.57 (0.11)
KNA31	0.55 (0.03)
KNA51	0.54 (0.22)
[1]Control +	0.51 (0.11)
KNA03	0.49 (0.01)
KNA97	0.46
KNA08	0.40 (0.11)
[2]Control -	0.40 (0.04)

[1]contained control sediment
[2]contained no sediment

Table 12. Percent survival of Alaska wood frog tadpoles exposed to sediments.

[Standard deviations in parentheses; * treatments differ significantly from ** treatments]

Site	**Percent survival**
Control -	100*
KNA03	100*
Control +	89 (0.10)
KNA31	77 (0.11)
KNA08	77 (0.10)
KNA51	77 (0.22)
KNA46	44 (0.22)
KNA01	44 (0.29)
KNA97	33 (0.33)
KNA62	22** (0.22)

Appendix D – Study Plan for USFWS Sediment and Water Toxicity Study

Title. *Toxicity Assessment of Sediment and Water from Deep and Shallow Wood Frog Breeding Sites on the Kenai National Wildlife Refuge.*

Background.

Abnormal wood frogs have been found at sites on the Kenai National Wildlife Refuge (Refuge) between 2000 and 2005. The causes of most abnormalities are unknown.

Preliminary data from the Kenai Refuge show three interesting trends. First, deeper sites have more developmental malformations than do shallow sites. Second, a subset of abnormal frogs from Kenai had more damaged DNA than normal frogs from the same sites. Third, wood frogs were found with gonadal abnormalities, such as testicular oocytes and hermaphroditic tissue, along with a skewed sex ratio of 3:1 females:males.

Contaminants in site water and sediment could cause all of the abnormalities observed in the field: developmental malformations (Hatch and Burton, 1998), DNA damage (Matson *et al.*, 2005), abnormal gonads (Hayes, 2004), and skewed sex ratios (Qin *et al.*, 2003).

A controlled study will allow us to test site sediment and water while limiting exposure to other stressors that also cause frog abnormalities: UV radiation (Ankley *et al.*, 2002), parasites (Johnson, *et al.*, 2002), and invertebrate predators (Formanowicz, 1986). Other studies have replicated field abnormalities with controlled exposure to site sediment and water (Fort, 1999; and Levey, 2003).

We propose to expose developing wood frogs to water and sediments from deep and shallow sites, and to clean water and sand, to see if we can replicate field observations. This experiment will let us assess the relationship between site sediment and water and frog abnormalities including malformations, DNA degradation, and abnormal gonads. Finally, our control treatments will give us a baseline for DNA damage, sex ratio, and intersex prevalence in Alaskan wood frogs.

Goal. To determine whether sediment and water from Kenai Refuge breeding sites can cause negative effects on wood frogs exposed from eggs to metamophosis. End points will include hatching success, mortality, size at metamorphosis, time to metamorphosis, gross physical abnormalities, DNA damage, sex ratio, and gonadal abnormalities.

Null Hypotheses.

Null hypotheses are listed below. Null Hypotheses marked with an asterix (*) will be tested only if funding to run diagnostics becomes available.

H_{01}: Hatching success is the same in wood frog eggs exposed to site water and sediment versus controls.

H_{02}: The prevalence of physical abnormalities is the same in wood frog eggs, tadpoles, and metamorphs exposed to site water and sediment versus controls.

H_{03}: Mortality is the same in wood frog tadpoles and metamorphs exposed to site water and sediment versus controls.

H_{04}: Wood frog metamorphs exposed to site water and sediment from the egg stage are the same size at metamorphosis as controls (SVL and mass).

H_{05}: Wood frogs exposed to site water and sediment take the same amount of time to develop from eggs to metamorphs as controls.

*H_{06}: Wood frog metamorphs exposed to site water and sediment and to control water and clean sand all have a 1:1 female:male sex ratio.

*H_{07}: Wood frog metamorphs exposed to site water and sediment do not manifest gonadal abnormalities, such as testicular oocytes or hermaphroditic tissue.

*H_{08}: Wood frog metamorphs exposed to control water and clean sand do not manifest gonadal abnormalities, such as testicular oocytes or hermaphroditic tissue.

*$H0_{09}$: Wood frog metamorphs exposed to site water and sediment show the same DNA grade by flow cytometry as metamorphs reared in control water and clean sand.

Materials and Methods.

The study will take place during summer, 2006, in a fully-fenced area on the Swanson River Oil Fields in the Kenai Refuge. The entire experiment will be covered with shade cloth to limit UV exposure. Experimental units will be screened with window screen to prevent oviposition by invertebrate predators.

Experimental Unit. An experimental unit will be one chemically-clean, 2.5 L glass bubble bowl, washed with alconox and rinsed with distilled water, before site sediment and water are added. Site sediment (250 mL) and site water (2.5 L) will be added to each bowl. The sediment will settle to the bottom of the bowl before eggs are added. Tadpole exposure in the experimental units is described below.

Experimental Design. A randomized block design will be used to test the following factors: *Block; Site; Site Type; Parentage.*

Block. Four blocks, water baths with 24 units each, will be used to control temperature of the experimental units (Figure 1). Temperature in each bath will be recorded using a tidbit temperature logger, set to record each half-hour. Treatments will be randomized within each block.

Site. We will test water and sediment from 6 wood frog breeding sites at which abnormal frogs have been found. Two controls will also be used: one with only well water (tested for chemical quality) and the other with well water and clean sand, which has been washed in distilled water. Twelve tadpoles will be tested per site.

Site Type. Three deep and 3 shallow sites will be tested to determine whether water depth explains any of the variation in response variables.

Parentage. Three amplecting pairs of wood frogs will be caught with dip nets at 1 deep and 1 shallow site. This will result in 6 egg masses from 12 individual parents from 2 sites, one deep and one shallow. KNA01 and KNA04 will be used to provide parents. Parentage is being tested to control for heritable traits associated with specific individuals or with site depth. Each site treatment will include two tadpoles from each parent pair (Figure 2). Parents will be measured (SVL and mass) and the number of eggs in each mass will be estimated based on the mass of three subsamples of 20 eggs from each mass.

Egg Collection. Amplecting pairs will be caught with dipnets and taken to the bunkhouse to lay eggs in well water in chemically-clean, covered glass bowls. After oviposition, adults will be released at their breeding sites. Extra eggs will be placed back in the breeding ponds from which adults were collected. These two sites are not formal study sites, so this should not interfere with field study results from our primary project.

Sediment Collection. Sediment will be collected in late April from 6 sites with hand-held stainless-steel scoops or Eckman dredges. Sediment samples will be composites of 3 locations in a pond, and will be homogenized in 5 gallon stainless steel buckets in the field. Sampling equipment will be decontaminated by washing with Alconox and water, rinsing with DI water, then rinsing with hexane, then acetone, to remove organic contaminants and prevent cross-contamination between sites.

Water Collection and Changes. Water in each bowl will be changed every 4-6 days to prevent tadpoles from fouling the water. Old water will be drained with site-dedicated siphon hoses, taking care to not harm the tadpole or disturb the sediment. It will be replaced with temperature-equilibrated site water collected either that day or the day before. We will collect site water with lab-certified, chemically-clean, 5-gallon cubitainers. The same cubitainer will be used to collect water from each site, at each water change. The cubitainer will be rinsed once with site water before it is filled at each water change. Water in 4 randomly selected units (1 per block) will be monitored for ammonia and dissolved oxygen concentrations prior to each water change. Ammonia concentrations will be tested using a commercially-available aquarium test kit. Temperature, pH, DO and conductivity (Water Quality) will be recorded in the randomly-selected units before each water change with a Hydrolab. Water quality and tadpole developmental stage will be measured in all units four times: at hatching, twice during development, and during tadpole metamorphosis.

Assignment of Treatments to Blocks. The treatments were assigned to blocks in a stratified, random design. Equal numbers of animals from each site are assigned to each block. A list of desired treatments was put into Excel. Each of the 96 animals were assigned a random number between 0 and 1, using the =RAND() function. Then, animals were sorted by site. Within each site, the data were again sorted by the random number column. Blocks were assigned in ascending order of random numbers within each site (ie. The animals assigned the 3 lowest numbers at each site were assigned to block 1, the animals with the next 3 numbers were assigned to block 3, the next 3 to block 3, the highest 3 numbers to block 4. This method resulted in 9 cases of animals with the same

parent exposed to water and sediment from the same site being assigned to the same block, which seems acceptable.

Hatching Success. At the start of the experiment, 20 eggs (Gosner stage 8) will be placed in each experimental unit. Hatching success will be evaluated at each water change. Once tadpoles are free-swimming (Gosner stage 20) all except one will be removed from each bowl. The largest and most vigorous tadpole was left in each container. At removal, tadpoles will be examined for skeletal and eye abnormalities.

Tadpole Rearing. Once tadpoles are free-swimming (Gosner, 1960, stage 20), all animals will be 1 ml of food (NASCO frog brittle for tadpole *xenopus*) just after each water change. When tadpoles reach Gosner stage 42, site vegetation will be provided in the bowl for them to climb up on, because they cannot swim once their tails resorb.

Endpoint Assessment. Tadpole mortality will be assessed at each water change. We will examine any dead tadpoles for the presence of physical abnormalities and preserve them in 70% ethanol. At metamorphosis (Gosner stage 46), each metamorph will be weighed, measured, and assessed for the presence of abnormalities by standard protocols (USFWS, 2005). The date of metamorphosis will be recorded. They will then be anesthetized in 1% MS-222 solution, bled by cardiac puncture for analysis of DNA quality by flow cytometry at the USGS National Wetlands Research Center, and evaluated for parasite loads. The gonads will be excised, placed in individual microfuge tubes in 10% neutral-buffered formalin for later analysis at McNeese State University.

Data Analysis. We will analyze experimental data using general linear models in SAS. The hypotheses will be tested at a significance level of $\alpha = 0.05$.

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Figure 1. Randomized block design layout – see key below for sample IDs.

Block 1 Layout

CSANDD3B	KNA03S3B	KNA02D2A	KNA12D2A	KNA01D1A	CSANDS1A
KNA08D3A	KNA12S3A	KNA03S2B	KNA12S2B	KNA01S1A	KNA02S2B
CWATRS2B	CWATRS1B	CWATRS2A	KNA01S1B	KNA03D3A	KNA02D3B
CSANDS2B	KNA08D2B	KNA08S1A	KNA21D1B	KNA21D1A	KNA21D3B

Block 2 Layout

KNA12D3A	KNA01D1B	KNA03D2A	KNA01D2B	KNA03D2B	CWATRS3A
CSANDS2A	KNA02D2B	KNA01S3B	CSANDD1A	CSANDS1B	KNA12S2A
KNA02S1B	KNA08D1B	KNA12S1B	KNA03S1A	KNA02S1A	KNA08S2A
KNA08S3B	KNA21S1A	KNA21D2B	CWATRS1A	CWATRD2B	KNA21S1B

Block 3 Layout

KNA01D2A	KNA01D3B	KNA01S2A	KNA02S3A	KNA12D1A	KNA12D2B
KNA08S1B	KNA08S2B	CSANDD3A	CSANDS3A	KNA12S1A	KNA03S1B
KNA02D1A	KNA03D1A	KNA03D1B	CWATRS3B	KNA02S3B	KNA21S2A
KNA08D3B	CSANDD1B	CWATRD3A	KNA21S3B	CWATRD1B	KNA21D2A

Block 4 Layout

KNA12S3B	KNA03S3A	CSANDS3B	KNA08D2A	KNA02S2A	CWATRD1A
CWATRD3B	KNA12D1B	KNA08S3A	KNA21S3A	KNA03D3B	KNA01S3A
KNA21S2B	KNA08D1A	CSANDD2A	CWATRD2A	KNA03S2A	CSANDD2B
KNA02D1B	KNA01S2B	KNA21D3A	KNA01D3A	KNA12D3B	KNA02D3A

Key: Unit ID = Site-Parentage-Replicate, eg. KNA12S3B is water and sediment from Site KNA12, Parent Pair S3 (Shallow site, pair # 3), and is Replicate B of this combination.

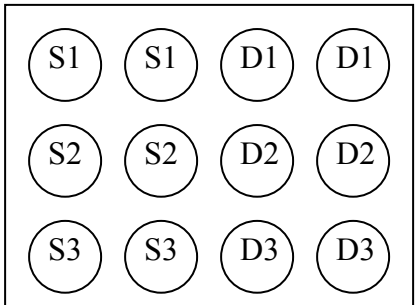

Figure 2. Animals assigned to each site. Each site will have a replicate of each parent pair

Appendix E – Study Plan for Heritable Abnormality Study

Study Plan for Wood Frog Heritable Abnormality Microcosm Study

Contact: Mari K. Reeves, *US Fish and Wildlife Service, 605 W. 4th Avenue, Anchorage, AK 99501. (907) 271-2785*

Objective: To determine whether wood frogs from the Kenai National Wildlife Refuge manifest skeletal abnormalities when reared from eggs to metamorphosis in controlled conditions and clean water.

Background: Abnormal wood frogs have been documented at breeding sites on the Kenai National Wildlife Refuge between 2000 and 2006. The causes of the abnormalities are unknown. One hypothesis for the observed abnormalities is that adult frogs are exposed to mutagens, and their offspring bear chromosomal damage that leads to abnormal growth. A controlled experiment, beginning at fertilization and continuing through metamorphosis, will allow us to assess whether malformations occur when the tadpoles are reared in a controlled setting in clean water and in the absence of other stressors known to cause abnormalities, like UV radiation, high temperatures, predatory invertebrates, and parasites.

Goal. To determine whether wood frogs from the Kenai Refuge develop normally when reared in clean water from fertilization to metamophosis. Endpoints include hatching success, skeletal and eye malformations, and survival.

Null Hypothesis.
H_0: The prevalence of skeletal and eye abnormalities is the same in wood frogs reared under controlled conditions as the seven-year average abnormality prevalence from the breeding site from which the parents were collected.

Materials and Methods.
The study will take place during summer, 2007, in Anchorage.

Experimental Design.
Parentage. Ten amplecting pairs of wood frogs will be caught with dip nets and placed in drinking water (Alaska's Best Water Company) in chemically-clean buckets onsite to lay eggs. Fecundity of each parent pair will be estimated by sub-sampling 20 eggs from each mass 3 times, then weighing the total mass. This will result in 10 egg masses from 20 individual parents from 1 site at which abnormalities are consistently found. Once egg masses are obtained, the adults will be weighed, measured, swabbed for disease and genetic screening, and released at the breeding site. We will retain a total of 1500 eggs for our research (mortality can be high at the early developmental stages) and an equal number of eggs from each mass will be deployed into each of the experimental units. Wood frogs lay an average of 778 eggs per mass (Herreid and Kinney, 1966), therefore only a subset of each of the 10 egg masses will be used. The remaining eggs will be deposited onto the existing egg mass cluster at the breeding site.
Experimental Unit. An experimental unit will be one 20-gallon artificial pond with 8 tadpoles in it. The tank will be covered with window screen to keep out predators. The experimental unit will be replicated 50 times. A temperature logger will be deployed in the center of 20 randomly-selected tanks out of the 50.
Tadpole Rearing. Once tadpoles are free-swimming, eight will be placed in each experimental unit. They will be fed NASCO frog brittle for tadpole *Xenopus,* until volunteer algae colonize the ponds, then they will subsist off of the algae. The tank will be covered with window screen to keep out predators. A temperature logger will be deployed in the center of each tank.
Abnormality Assessment. At metamorphosis, tadpoles will be examined for skeletal and eye abnormalities. USFWS Abnormal Amphibian Assessment Standard Operating Protocols will be used to assess abnormalities in the metamorphs.
Data Analysis. Frogs will be classed as normal or abnormal and assigned an abnormality type, using USFWS standard operating procedures. We will use general linear models in SAS to test whether parentage is a significant predictor of each of the different classes of abnormalities: skeletal abnormalities, eye abnormalities, and other abnormalities.

Appendix F – Environmental Health Perspectives Paper

Road Proximity Increases Risk of Skeletal Abnormalities in Wood Frogs from National Wildlife Refuges in Alaska

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Background: Skeletal and eye abnormalities in amphibians are not well understood, and they appear to be increasing while global populations decline. Here, we present the first study of amphibian abnormalities in Alaska.

Objective: In this study we investigated the relationship between anthropogenic influences and the probability of skeletal and eye abnormalities in Alaskan wood frogs (*Rana sylvatica*).

Methods: From 2000 to 2006, we examined 9,269 metamorphic wood frogs from 86 breeding sites on five National Wildlife Refuges: Arctic, Innoko, Kenai, Tetlin, and Yukon Delta. Using road proximity as a proxy for human development, we tested relationships between skeletal and eye abnormalities and anthropogenic effects. We also examined a subsample of 458 frogs for the trematode parasite *Ribeiroia ondatrae*, a known cause of amphibian limb abnormalities.

Results: Prevalence of skeletal and eye abnormalities at Alaskan refuges ranged from 1.5% to 7.9% and were as high as 20% at individual breeding sites. Proximity to roads increased the risk of skeletal abnormalities ($p = 0.004$) but not eye abnormalities. The only significant predictor of eye abnormalities was year sampled ($p = 0.006$). *R. ondatrae* was not detected in any Alaskan wood frogs.

Conclusions: Abnormality prevalence at road-accessible sites in the Kenai and Tetlin refuges is among the highest reported in the published literature. Proximity to roads is positively correlated with risk of skeletal abnormalities in Alaskan wood frogs.

Key words: abnormality, Alaska, amphibian, *Lithobates sylvaticus*, malformation, national wildlife refuge, *Rana sylvatica*, wood frog. *Environ Health Perspect* 116:1009–1014 (2008). doi:10.1289/ehp.10963 available via *http://dx.doi.org/* [Online 21 April 2008]

Amphibian populations, often considered sentinels of ecologic health and indicators of environmental change (Van der Schalie et al. 1999), are declining worldwide (Stuart et al. 2004). Concurrent with this decline is an apparent increase in morphologic abnormalities (Hoppe 2000). Although the background rate of abnormalities in wild amphibian populations has been described as between 0% and 5% (Converse et al. 2000; Eaton et al. 2004; Gurushankara et al. 2007; Hoppe 2000; Johnson et al. 2002; Ouellet 2000; Schoff et al. 2003; Taylor et al. 2005), recent studies of frogs in some areas have documented rates as high as 6–22% (Bacon et al. 2006; Levey 2003; McCallum and Trauth 2003). Established causes of limb abnormalities in amphibians include parasites, chemical contaminants, ultraviolet-B radiation (UVB), and invertebrate predators (Blaustein and Johnson 2003). Causes of eye abnormalities are less well understood, but authors have suggested chemical contaminants and early-season temperature extremes (Vershinin 2002) or a recessive genetic mutation (Nishioka 1977). In field studies, high abnormality prevalence has been correlated with human activities such as urbanization and agricultural and industrial land use (Gurushankara et al. 2007; Hopkins et al. 2000; Ouellet et al. 1997; Taylor et al. 2005; Vershinin 2002). In assessing current trends in environmental health, pivotal questions remain about the extent to which human activities are driving amphibian abnormalities in different parts of the world (Johnson et al. 2007; Skelly et al. 2007; Taylor et al. 2005).

The prevalence of abnormalities in Alaskan amphibians had not been examined before this study. The highest-latitude studies of this type were in central Canada (55°7′48″N; Eaton et al. 2004) and Russia (56°51′00″N; Vershinin 2002). Alaska represents an important place to examine hypotheses about amphibian abnormalities for a number of reasons. In contrast to the contiguous 48 states—where ecologic cause-and-effect relationships are confounded by multiple broad-scale land-use alterations—Alaska is characterized by vast stretches of wilderness punctuated by local and self-contained disturbances such as roads and small towns. As such, it offers a unique opportunity to isolate the effects of human activities on amphibian populations. Second, the extreme northern latitude of Alaska allows for consideration of the UVB hypothesis for limb abnormalities (Ankley et al. 2004), because long summer days increase the duration of UVB exposure during tadpole development. Finally, because Alaska contains the largest tracts of protected land in the country, it is important from a natural resource management and conservation perspective to develop a baseline understanding of amphibian health in the region.

Here, we present the first study of abnormal amphibians in Alaska: a large, systematic, multiyear sampling effort, which documents the prevalence and types of abnormalities in wood frogs (*Rana sylvatica*, also called *Lithobates sylvaticus*) from five different National Wildlife Refuges. We also analyzed the relationship between anthropogenic landscape alterations, approximated by the presence of roads, and abnormality prevalence to assess the effect of human activities on Alaskan amphibians.

Materials and Methods

Species, refuge, and site selection. *R. sylvatica* (Hillis 2007) is the only amphibian common in most of Alaska, and the only amphibian in the refuges we studied. Wood frogs breed explosively just after snowmelt, laying eggs in late April or early May and metamorphosing in late June or July (Herreid and Kinney 1967). After metamorphosis, young frogs migrate up to 2 km from breeding wetlands to adult habitat in adjacent woods (Berven and Grudzien 1990). This synchronous breeding and development at each site cause larvae to metamorphose within a 5- to 7-day window (Herreid and Kinney 1967;

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Reeves MK, unpublished data). We examined frogs for abnormalities only during this time.

We chose five refuges in Alaska for this study—Arctic, Innoko, Kenai, Tetlin, and Yukon Delta (Figure 1, Table 1)—based on known frog presence and geographic location in the state. We chose sampling sites within each refuge based on proximity to roads and logistics of site access. We used geographic information systems (ESRI) and site latitude/longitude data (World Geodetic System 1984) to calculate distance to the nearest road.

All sites in the Arctic and Innoko refuges are in remote wilderness areas, accessible only by float plane or river boat. Sites in these refuges are clustered along rivers or lakes, near permanent camps or cabins from which sampling was based.

All sites within the Yukon Delta Refuge were in the town of Bethel (population 6,262) and were accessed by road. Bethel is a shipping and transportation hub for western Alaska, but it is not on the main highway system and lacks road access to other Alaskan cities. Potential contaminant sources associated with roads in Bethel include gravel operations, landfills, sewage treatment facilities, and defunct military communications sites.

In the Kenai and Tetlin refuges, we sampled both road-accessible and wilderness sites. The Kenai Refuge has 345 km of roads, including the only major highway bisecting the Kenai Peninsula. Many of these roads were developed to support the two operating oil and gas fields in the refuge, the first of which began drilling in the 1950s. Oil and gas development and other road-associated human activities in the Kenai Refuge have led to the release of contaminants, including pentachlorophenol, petroleum products, polychlorinated biphenyls, mercury from historic mining, and historic herbicide applications (Parson 2001). The site farthest from any road in the Kenai Refuge is 10 km. In the Tetlin Refuge, approximately half the sites lie along the Alaska–Canada highway (the only highway connecting Alaska to the coterminous United States), and half are near Jathamund Lake, between 35 and 40 km from the nearest road. At Tetlin, former military installations, transportation corridors, and a natural gas pipeline (which parallels the highway) have all led to environmental contamination. Contaminants associated with former military activities include petroleum products and pesticides (Rocque 2007). The pipeline route was sprayed with dioxin-containing herbicides in the 1960s (Rocque 2007).

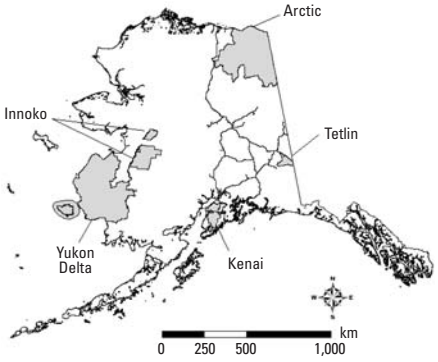

Figure 1. Map of Alaska showing locations of refuges sampled for abnormal wood frogs.

Animal collection. Between 50 and 100 metamorphic frogs, stage 42–46 (Gosner 1960), were assessed for abnormalities at each site. Stages 42–44, which are mainly aquatic, were captured with dip nets, and stages 45–46, which are primarily terrestrial, were caught by hand at the pond edge. Frogs were placed in buckets at the capture site until they were examined for abnormalities using standard protocols [U.S. Fish and Wildlife Service (FWS) 1999]. Snout-to-vent length (SVL) and tail length were measured, and developmental stage was recorded. Abnormal frogs were euthanized with tricaine methanesulfonate (MS-222; Argent Chemical Laboratories, Redmond, WA), photographed, and sent to the U.S. Geological Survey, National Wildlife Health Center, or Ball State University for radiographs to aid in abnormality classification. A subset of normal and abnormal frogs from Kenai (n = 448) and Tetlin (n = 10) were examined for parasites, including *R. ondatrae*, at the University of Wisconsin, La Crosse. All normal frogs not collected for parasitology were released at the capture site after field examination. Equipment was disinfected with 5% bleach solution between sites to prevent disease spread. All animals were treated humanely with regard to alleviation of suffering and according to U.S. government principles for the use and care of vertebrate animals used in testing, research, and training.

Abnormality classification. According to Johnson et al. (2001), "abnormality" is a general term referring to "any gross deviation from the normal range in morphological variation" and includes both "malformations" (permanent structural defects resulting from abnormal development) and "deformities" (alterations, such as amputation, to an otherwise correctly formed organ or structure). We categorized abnormalities for analysis using standard protocols (U.S. FWS 2007) and published guides (Meteyer 2000), and subdivided them into the following categories: skeletal abnormalities, eye abnormalities, surface abnormalities (e.g., wounds, skin discolorations, cysts), and diseases. Animals with only surface abnormalities or diseases were considered normal in this analysis. Skeletal abnormalities include three subcategories: malformations, injuries, and abnormalities of unknown origin (Table 2). A single researcher classified all frogs in this data set from pictures, radiographs, and field notes.

Statistical analysis. To examine potential risk factors associated with abnormality prevalence in Alaskan wood frogs, we performed a regression analysis of skeletal and eye abnormalities as a function of breeding site characteristics and covariates. Explanatory variables included frog length, frog developmental stage, year the frogs were found, and refuge in which the frogs were found. We used frog length and stage as covariates; the refuge parameter represented large-scale geographic patterns; and year represented environmental variables that change annually (e.g., temperature, UVB). As a surrogate for human disturbance (chemical habitat alteration or predator, pathogen, or parasite introduction) we also included distance from breeding sites to the nearest road, which we log-transformed before analysis to make the relationship with abnormalities linear. In our study areas, distance to road is a better predictor of chemical contamination than is distance to nearest population center (Parson 2001; Rocque 2007).

We first used logistic regression with stepwise selection to identify factors that were significant predictors for each abnormality

Table 1. Skeletal and eye abnormality and breeding site information by refuge.

Refuge	Years sampled	No. sites[b]	Abnormalities (%)[a] Mean	Median	Range	Latitude	Longitude	Site elevation (m)	Distance to road (km)
Arctic (7,932,000 ha)	2001–2002	9	2.0	1.4	0–6	67°10′48″–67°13′12″N	142°7′48″–142°11′59″W	195–200	151–155
Innoko (1,558,000 ha)	2002–2006	13	3.0	1.5	0–19	63°36′0″–63°38′24″N	158°1′48″–158°8′24″W	25–30	14–139
Kenai (797,200 ha)	2000–2006	38	7.9	7.6	0–20	60°8′42″–60°46′48″N	150°3′36″–151°5′24″W	60–520	0–10
Tetlin (95,426 ha)	2003–2006	19	5.9	4.0	0–14	62°38′24″–62°58′12″N	141°1′48″–141°51′36″W	500–700	0–40
Yukon Delta (6,555,850 ha)	2002–2004	7	1.5	0.0	0–5	60°46′48″–60°47′24″N	161°48′36″–161°52′48″W	15–30	0–5

Data for site latitude and longitude from WGS (1984).
[a]Mean refuge overall abnormality prevalence = number abnormal frogs/total frogs sampled at all sites over all years. [b]Number of breeding sites sampled. [c]For the median and range of breeding site abnormality prevalence we did not calculate prevalence of skeletal and eye abnormalities for ponds at which < 50 individuals were examined.

type (skeletal and eye abnormalities). We then used a generalized linear model (GENMOD in SAS, version 9.1; SAS Institute Inc., Cary, NC) to perform a repeated-measures analysis, which specified that individuals from the same collection event (animals at the same site in the same year) were correlated. This second analysis tended to reduce the significance values of factors in the original model. We dropped variables if they were significant during the stepwise selection but not significant once the repeated-measures analysis accounted for autocorrelation in the data. After we dropped nonsignificant factors, we reran the repeated-measures analysis a final time to obtain *p*-values and odds ratios (ORs).

The original sample contained 9,268 metamorphs examined between 2000 and 2006; we excluded 272 from statistical analysis because we lacked information about the site, frog length, or stage, leaving 8,997 for the skeletal abnormality and malformation analyses. We used only data from 2003–2006 for the eye abnormality analysis (n = 7,136) because of a change in eye abnormality protocols in 2003. We performed all analyses using SAS software. Error bars in figures were calculated based on the underlying binomial distribution

$$s(\hat{p}) = \frac{\sqrt{\hat{p}(1-\hat{p})}}{n}, \qquad [1]$$

where $s(\hat{p})$ is the standard error estimate, $\hat{p}$ is the proportion abnormal in that category, and n is the number sampled in each category.

Results

We examined a total of 9,268 metamorphic wood frogs from 86 breeding sites at five Alaskan refuges in this study. Abnormalities were observed at all refuges sampled. Kenai had the highest prevalence of abnormal individuals (7.9%), followed by Tetlin (5.9%), Innoko (3.0%), Arctic (2.0%), and Yukon Delta (1.5%). The overall prevalence of abnormal frogs was 6.2%.

The highest abnormality prevalence at any single breeding site was 20%, at a Kenai site in 2005 (Table 2). In Innoko, the highest single-site abnormality prevalence was 19%; in Tetlin, 14%; Arctic, 6%; and Yukon Delta, 5%. Each refuge had sites at which no abnormal frogs were found, but this was not the norm; 88% of the 161 sampling events yielded at least one abnormal frog.

More than 20 different types of abnormalities were documented (Table 2). Ectromelia (partial limb), micromelia (shrunken limb or limb element), amelia (limb totally missing), and unpigmented iris (eye totally black) were the four most common, collectively accounting for 73% of the abnormalities across all refuges (Figure 2; Table 2). These abnormalities were also the most common at each individual refuge, with some exceptions. Black-eyed frogs were common at Innoko, Kenai, and Tetlin, making up ≥ 20% of the abnormalities at each refuge, but only one black-eyed frog was found in Yukon Delta, and none were found in Arctic. Shrunken and partial limbs were among the most common abnormality types at all refuges except Innoko, which had a high proportion of partial limbs (27% of the abnormalities were of this type) but fewer shrunken limbs (only 7% of the abnormalities at this refuge). Several abnormality types occurred only in Kenai, including anteversion (twisted long bones), microcephaly (shrunken head), scoliosis (curved spine), cutaneous fusion (skin webbing), and kinked tail (Table 2). The rarest abnormality type was polymelia (extra limb); only one specimen had an extra limb, and this frog was also found in Kenai. Injuries comprised 12–36% of the skeletal abnormalities at each refuge, with the lowest proportion at Innoko, the highest at Yukon Delta, and more intermediate values at Arctic (17%), Kenai (17%), and Tetlin (20%).

The trematode parasite *R. ondatrae* is known to induce skeletal malformations in amphibians (Johnson and Sutherland 2003). To investigate whether *R. ondatrae* could be implicated in the abnormalities identified, we kept a subset of normal and abnormal frogs collected in the field for parasite analysis. We examined a total of 448 specimens from Kenai and 10 from Tetlin for parasites. None of these frogs were infected with *R. ondatrae*, nor were planorbid snail hosts seen at any sampling site.

In the regression analysis, prevalence of skeletal abnormalities increased with site proximity to the nearest road [p = 0.004, odds ratio (OR) = 0.8809; Figure 3). With one exception, all sites with abnormality prevalence > 6% were within 10 km of a road. One remote site in Innoko deviated from this trend (shown as an outlier in Figure 3, with an abnormality prevalence of 19%). This site, located > 100 km from any road, is adjacent to a historic mining and trapping cabin, now used as the base of Innoko Refuge field operations. This outlier did not affect our result

Table 2. Summary of abnormalities in wood frog populations at five national wildlife refuges in Alaska.

	No. of abnormalities					
Abnormality	Arctic	Innoko	Kenai	Tetlin	Yukon Delta	Total
Eye abnormality						
Anophthalmia (missing eye)	0	2	12	6	0	20
Unpigmented iris (black eye)	0	15	118	20	1	154
Microphthalmia (small eye)	0	0	1	1	0	2
Other[a]	0	2	6	2	0	10
Skeletal injury[b]						
Brachydactyly (short digits)	2	0	7	0	2	11
Ectrodactyly (missing digits)	1	1	4	3	0	9
Ectromelia (partial limb)	0	0	44	6	0	50
Limb crushed	0	0	14	1	2	17
Other[c]	0	2	2	0	0	4
Skeletal malformation						
Amelia (missing limb)	0	1	31	3	0	35
Anteversion (twisted long bones)	0	0	9	0	0	9
Brachygnathia (short jaw)	1	4	6	0	0	11
Microcephaly (shrunken head or blunt snout)	0	0	4	0	0	4
Micromelia (shrunken limb or limb element)	5	3	126	17	3	154
Polymelia (extra limb)	0	0	1	0	0	1
Polydactyly (extra digits)	2	0	2	0	0	4
Scoliosis or lordosis (curved spine)	0	0	2	0	0	2
Cutaneous fusion (skin webbing)	0	0	3	0	0	3
Syndactyly (digits fused)	0	0	11	2	0	13
Taumelia (bone bridge or triangle)	0	0	4	0	0	4
Skeletal unknown origin						
Kinked tail	0	0	3	0	0	3
Brachydactyly (short digits)	0	1	27	1	2	31
Ectrodactyly (missing digits)	0	0	26	3	0	29
Ectromelia (partial limb)	7	12	90	14	2	125
Other[d]	0	2	5	0	0	7
Overall						
Eye total	0	19	137	29	1	186
Injury total	3	3	71	10	4	91
Malformation total	8	8	199	22	3	240
Unknown origin total	7	15	151	18	4	195
Total abnormalities	18	45	558	79	12	712
Total abnormal individuals	12	39	450	68	7	576
Total individuals examined	615	1,309	5,716	1,146	482	9,268
Percent individuals abnormal	2.0%	3.0%	7.9%	5.9%	1.5%	6.2%

[a]Includes oversized eyes, abnormally shaped pupils, and cataracts. [b]Either fresh blood or exposed bone was noted for the injury category. [c]Includes dissociated and dangling limb. [d]Includes apparent dislocations.

interpretation, so we retained it during statistical analysis. Frogs in our study were also more likely to have skeletal abnormalities if they were smaller ($p = 0.002$, OR = 0.8831; Figure 4) and at a later developmental stage ($p < 0.0001$, OR = 1.2812; Figure 5). The preliminary logistic regression analysis identified significant differences in skeletal abnormalities among refuges; however, once we accounted for autocorrelation in our data with the repeated-measures analysis, refuge was no longer a significant predictor of any abnormality type. We found no relationship between skeletal abnormalities and year sampled. Eye abnormalities varied with year ($p = 0.006$), although they were not correlated with refuge, frog size, Gosner stage, or distance to the nearest road. Significantly fewer eye abnormalities were found in 2003 than in 2004 (OR = 0.1969) or 2005 (OR = 0.2078).

In our data, frogs found closer to roads were smaller. In a simple linear regression of frog size against distance to the nearest road, the equation is SVL (millimeters) = 19.2 + [0.02 × distance to road (kilometers)] ($p < 0.0001$; $R^2 = 0.20$). By this equation, the average SVL of frogs in a site adjacent to the road is 19 mm, whereas the average SVL at 150 km is 22 mm. Despite this collinearity between size and distance to roads, we included both in our final regression model because both were significant during stepwise model selection, suggesting this collinearity was overcome. Additionally, both factors could independently influence abnormality prevalence, so we avoided choosing one or the other to represent both.

Figure 2. The four most common abnormalities in Alaskan wood frogs: (*A*) micromelia, (*B*) ectromelia, (*C*) amelia, and (*D*) unpigmented iris.

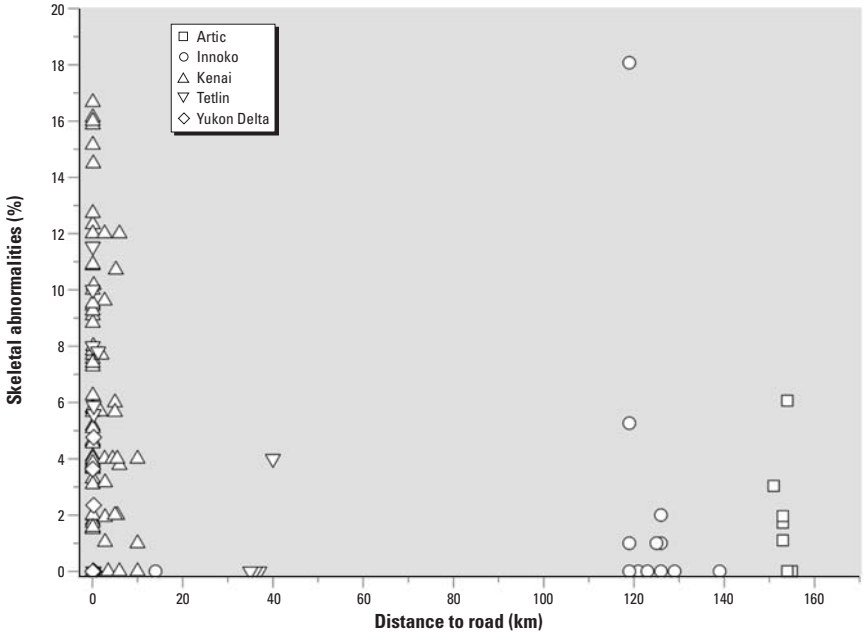

Figure 3. Skeletal abnormalities and malformations versus distance to the nearest road. Symbols indicate prevalence of frogs with skeletal abnormalities during single collection events at different refuges.

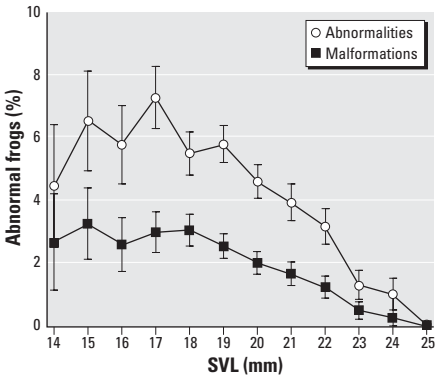

Figure 4. Skeletal abnormalities and malformations shown as the proportion of abnormal frogs at each SVL (mean ± SE, where SE is based on Equation 1).

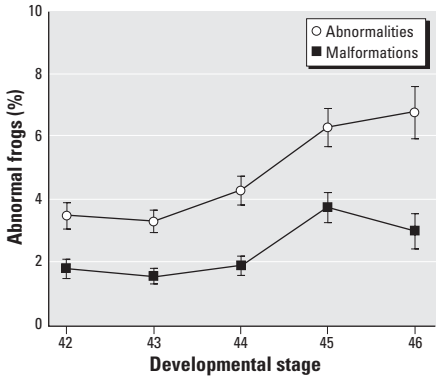

Figure 5. Skeletal abnormalities and malformations shown as the proportion of abnormal frogs at each developmental stage (mean ± SE, where SE is based on Equation 1).

Discussion

The average abnormality prevalence in this study (6.2%) is higher than background levels of 0–5% reported for other areas (Ouellet 2000). The average in this study is high, however, because of hotspots in some areas. Specifically, the abnormality prevalence at road-accessible sites in the Kenai and Tetlin refuges is among the highest reported to date. Remote areas in Alaskan refuges exhibited abnormality prevalence closer to 2% and within the published range for background levels in other places in North America (Converse et al. 2000; Eaton et al. 2004; Hoppe 2000; Schoff et al. 2003; Taylor et al. 2005).

We observed higher abnormality prevalence in sites closer to roads. Ostensibly, road proximity could increase the prevalence of frog abnormalities by contributing to chemical contamination of the habitat (Parson 2001; Rocque 2007) or by facilitating introduction of predators, parasites, or pathogens (Reeves 2008; Urban 2006). If contaminants caused abnormal development in Alaskan amphibians, then proximity to roads should result in malformations but not injuries in the absence of other stressors (Loeffler et al. 2001). If predators caused the limb abnormalities, we should see fresh and healed injuries and possibly developmental malformations if limbs were amputated early enough in tadpole development to partially regenerate (Forsyth 1946; Fry 1966). The prevalence of both malformations and injuries in our data suggests that predators were almost certainly responsible for some proportion of the skeletal abnormalities. Either early limb amputation by predators (Forsyth 1946; Fry 1966) or exposure to chemical contaminants (Gardiner et al. 2003) may have caused the developmental malformations. Road-associated contaminants may also reduce tadpole size or fitness, increasing the risk of predation injury (Boone and James 2003). Nevertheless, both chemical contaminants (Relyea 2005) and invertebrate predators (Relyea 2001) can decrease frog size at metamorphosis. Thus, we cannot discern whether road effects on skeletal abnormalities are mediated through chemical contaminants, shifts in predator community composition, or a combination of these two stressors.

Our data do not support the parasite or UVB hypotheses for skeletal abnormalities in Alaskan wood frogs. We did not detect the malformation-inducing parasite *R. ondatrae* in any of the frogs in this study, and the lack of bilateral malformations is atypical of UVB exposure (Ankley et al. 2002). It is possible that UVB induced the eye abnormalities in our study, based on the correlation with year sampled, yet we cannot find any report that associates UVB with amphibian eye abnormalities. Causes of eye abnormalities in amphibians are not well understood, but others have proposed chemical contaminants, temperature extremes, and genetic mutations as causes (Nishioka 1977; Vershinin 2002). We cannot rule out temperature extremes or genetic mutations as causes of the eye abnormalities in this study, but chemical contaminants are unlikely candidates, based on the lack of correlation with roads and associated environmental contamination.

A number of limitations are associated with using road proximity as the only means by which to quantify the effects of human disturbance on Alaskan wood frogs. For example, not all roads in this study represent the same kind of landscape disturbance. Whereas all of the Yukon Delta sites were closest to roads in the town of Bethel, a small village accessible only by air or barge, in Kenai the nearest road may have been either a major highway or a restricted-access gravel road on the oil and gas fields. Moreover, roads are not necessarily the only or even the most significant source of human disturbance to a breeding site. The high abnormality prevalence at one remote Innoko site may be an example of anthropogenic effects unrelated to roads, because this site was subject to stressors related to current refuge operations and historic land use. Clearly, further study is needed to discern whether and how human activities are related to abnormalities in Alaskan frogs.

In addition to the correlation between road proximity and abnormality prevalence, we identified other significant covariates, including frog size, frog developmental stage, and year sampled. Several mechanisms could explain the increased probability of skeletal abnormalities with smaller size. Small frogs might be more likely to suffer insults such as failed predation attempts. Size at metamorphosis has been related to adult fitness (Werner 1986), and small tadpoles and metamorphs are more vulnerable to gape-limited predation (Brodie and Formanowicz 1983). Alternatively, abnormal frogs may compete poorly for resources, leaving them smaller at metamorphosis than their normal counterparts. Finally, the stressor causing abnormalities could also reduce size at metamorphosis. Wood frogs exposed to caged predators (Relyea 2001) and chemical contaminants (Relyea 2005) were smaller at metamorphosis than unexposed controls. Size and developmental stage were not correlated in our data.

The increased prevalence of skeletal abnormalities at later developmental stages is probably sampling bias created by different capture techniques. Whereas dip netting for earlier-stage metamorphs (Gosner stage 42–44) samples abnormal and normal individuals with comparable efficiency, capturing later-stage metamorphs on land may result in the disproportionate collection of the less-mobile abnormal animals. Moreover, normal metamorphs leave the breeding area quickly, but frogs with skeletal abnormalities may stay closer to water, where they can dive from predators instead of relying on missing or misshapen limbs to escape. Care was taken to examine each limb during sampling, because the primary goal of this study was detection of morphologic abnormalities. Therefore, we do not think limb abnormalities were obscured by the longer tails of earlier-stage metamorphs (another potential source of sampling bias).

Correlative models provide results valuable for focusing future data collection. Our model identified contaminants and predators, or a synergistic interaction between them, as important areas of future research into the causes of limb abnormalities in Alaskan wood frogs. Our data also suggest that *R. ondatrae* and UVB are probably not responsible for the skeletal abnormalities we observed, but UVB or climate may cause the eye abnormalities.

Conclusion

The elevated abnormality prevalence in some areas of Alaska's National Wildlife Refuges is a striking indication that we cannot assume the size and relative remoteness of these protected areas render them immune to the influence of humans. On the other hand, although preliminary evidence points to a possible effect of anthropogenic disturbance on Alaskan wood frogs, we lack sufficient evidence to identify a specific causal agent. The results of our analyses suggest that predation injuries and some effect of roads, such as chemical contamination or shifts in predator community composition, may contribute to the skeletal abnormalities we observed. The cause of eye abnormalities is unknown, yet the lack of association with human disturbance and the significance of year sampled in our statistical model suggest that eye abnormalities in Alaskan wood frogs are more likely to be associated with something that occurs statewide and changes annually, such as UVB or climate. More study is needed to elucidate risk factors for amphibian abnormalities in Alaska, and such research is ongoing.

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Appendix G – Disease Papers

accumulated while undertaking other studies. The data, however, are important because in addition to identifying *R. pipiens* as a carrier of *Bd*, they highlight the infectivity of *Bd* and the requirement for stringent biosecurity.

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Batrachochytrium dendrobatidis in Wood Frogs (*Rana sylvatica*) from Three National Wildlife Refuges in Alaska, USA

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Batrachochytrium dendrobatidis (*Bd*) is a fungal pathogen implicated in recent amphibian declines (Pounds et al. 2006). It was first documented in Alaska, USA, in 2002, in a single dead subadult Wood Frog (*Rana sylvatica*) in the Kenai National Wildlife Refuge (Reeves and Green 2006). Since then, there have only been two other studies of *Bd* in Alaska. In one, *Bd* was found in Boreal Toads (*Bufo boreas*) and Red-legged Frogs (*Rana aurora*) in western Canada and southeast Alaska (Adams et al. 2007). In the other, *Bd* was not found in wood frogs in Denali National Park (Chestnut et al. 2008). *Bd* distribution in other parts of Alaska is unknown. In summer of 2006, we sampled adult Wood Frogs from three Alaskan National Wildlife Refuges to screen them for *Bd*.

Methods.—Wood Frogs from the Innoko, Kenai, and Tetlin refuges were sampled (Fig. 1). From these refuges, we tested 48 opportunistically-encountered adult frogs from 29 breeding ponds between 11 May and 21 July 2006 (Table 1). At Kenai, four ponds were road-accessible and six were in remote areas, 1–10 km from any road (Fig. 1). All ponds at Innoko (N = 9) and Tetlin (N = 10) were in remote areas, 35–125 km from any road. All animals were alive when sampled and appeared healthy. Frogs were swabbed

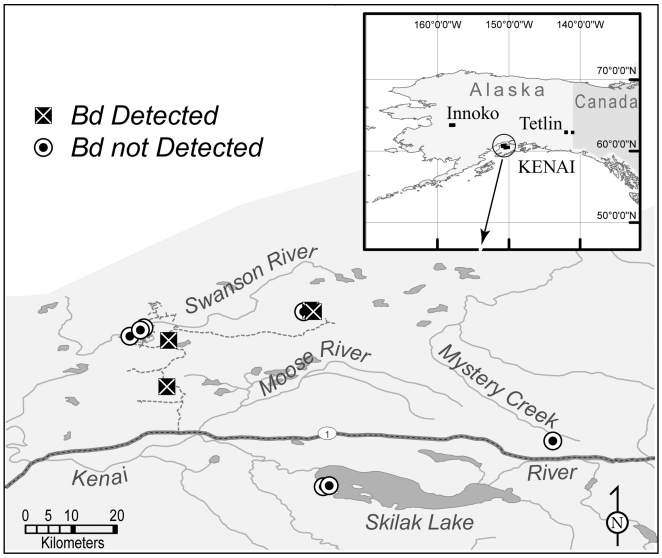

Fig. 1. Wood Frog (*Rana sylvatica*) *Bd* sampling locations in Alaska, USA, and Kenai Refuge sampling ponds and *Bd* detection locations. Dashed lines are gravel roads.

TABLE 1. Surveys for *Batrachochytrium dendrobatidis* (*Bd*) in Wood Frogs (*Rana sylvatica*) in three wildlife refuges in Alaska, USA. Prevalence indicates: number of frogs with *Bd* detected/total number of frogs sampled.

Date	Refuge	Latitude °N	Longitude °W	Prevalence
12 July 2006	Innoko	63.573080	-158.156880	0/1
6 July 2006	Innoko	63.599840	-158.136220	0/1
7 July 2006	Innoko	63.595970	-158.112640	0/1
14 July 2006	Innoko	63.615150	-158.081880	0/1
6 July 2006	Innoko	63.617460	-158.080380	0/1
13 July 2006	Innoko	63.618890	-158.020890	0/1
9 July 2006	Innoko	63.610790	-157.792040	0/1
15 July 2006	Innoko	63.648980	-157.750640	0/1
9 July 2006	Innoko	63.622410	-157.739620	0/1
23 June 2006	Kenai	60.725870	-150.889740	0/2
11 May 2006	Kenai	60.737920	-150.872070	0/8
11 May 2006	Kenai	60.743180	-150.863910	0/6
25 July 2002	Kenai	60.627410	-150.815570	1/1
20-29 June 2006	Kenai	60.714230	-150.815410	4/4
12 May 2006	Kenai	60.776810	-150.547290	0/1
28 June 2006	Kenai	60.780170	-150.543000	2/2
28 June 2006	Kenai	60.776550	-150.539150	1/1
24 May – 14 July 2006	Kenai	60.436260	-150.507710	0/3
14 July 2006	Kenai	60.439000	-150.500000	0/1
22 June 2006	Kenai	60.523690	-150.064090	0/1
18 July 2006	Tetlin	62.603330	-142.051030	0/1
13 July 2006	Tetlin	62.616140	-141.992900	0/1
15 July 2006	Tetlin	62.633420	-141.985200	0/1
12 July 2006	Tetlin	62.608420	-141.980030	0/1
12 July 2006	Tetlin	62.610000	-141.980000	0/1
21 July 2006	Tetlin	62.618210	-141.979860	0/1
17 July 2006	Tetlin	62.607280	-141.972820	0/1
13 July 2006	Tetlin	62.630000	-141.970000	0/1
17 July 2006	Tetlin	62.614990	-141.966540	0/1
17 July 2006	Tetlin	62.619140	-141.963100	0/1

30–35 times on the pelvic patch, inner thighs, and between toes with sterile, foam-tipped swabs (REF 25-1506 1PF: Puritan, Guilford ME). Swab tips were then placed in individual 1.5 ml microfuge tubes in reagent-grade ethanol and stored at room temperature until analysis. Samples were sent in late August 2006 to Pisces Molecular (J. Wood, Boulder, Colorado, USA) for analysis by polymerase chain reaction (Annis et al. 2004). Clean nitrile gloves were worn when handling each frog, and field equipment was decontaminated with 5% bleach solution between ponds. Pond coordinates were recorded with hand-held Garmin III GPS units and referenced to datum, WGS84.

Results.—*Bd* was detected in the Kenai refuge, but not in the Tetlin or Innoko refuges. In Kenai, *Bd* was found in one of four road-accessible ponds and in two of six remote ponds; in the three ponds where *Bd* was found, all sampled frogs tested positive (Table 1). All of the *Bd*-positive ponds were near the Swan Lake recreational canoe route or the gravel road used to access it (Fig. 1). One of the *Bd*-positive ponds was directly adjacent to the road, and the other two ponds were in a wilderness area within 3 km of the road and within 1 km of the canoe trails.

Discussion.—Wood Frogs are the only amphibian common in southcentral and interior Alaska (Wright and Wright 1995). In these regions, *Bd* has been found only on the Kenai refuge, in limited locations (1 pond in 2002 and 3 ponds in 2006). The dead Wood Frog found in 2002 (Reeves and Green 2006) was in a different pond along the same access road as our 2006 *Bd* detections; all positive detections in the Kenai refuge thus have been along this recreational access corridor. Of the four remote ponds that tested negative for *Bd* in 2006, two are in areas where human visitation is rare or nonexistent (access requires a motor boat and then a walk through trail-less forest), one is in a wilderness area near an established hiking trail, and one is near the recreational canoe route and the two ponds that tested positive. Amphibian researchers have visited all the remote ponds repeatedly since 2004, using hygiene protocols described above. The three road-accessible ponds that tested negative for *Bd* are on an operating oil field, where the public is not allowed. The oil field ponds have been visited by amphibian researchers since 2000, and access by other people is limited to oil field workers and other (non-amphibian) researchers. It is unlikely these other users enter the wetlands sampled in this study, although the roads pass within 100 m of each pond. The ponds in the Innoko and Tetlin refuges are all remote, requiring a combination of planes and watercraft to access, and it is unlikely that any people other than amphibian researchers enter the ponds sampled for this study. Although sample size is limited, the results from this study suggest *Bd* is not ubiquitous in southcentral and interior Alaska. More systematic research is needed on the distribution and abundance of *Bd* in Alaskan refuges, especially as it relates to recreational use.

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Amphibian Chytrid Fungus Infections in *Hyperolius* (Anura: Hyperoliidae) from Eastern Democratic Republic of Congo

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Amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) infection has been well-studied in Australia and the New World, where species seem to be especially susceptible to infection in montane, stream habitats (e.g., Carnaval et al. 2006; Hero and Morrison 2004; Lips et al. 2004; McDonald et al. 2005). The destructive fungus also has been associated with frog die-offs and mortality in similar habitats in East and South Africa (e.g., Channing et al. 2006; Hopkins and Channing 2003; Smith et al. 2007), and it is likely that the fungus is killing frogs in other highland regions throughout Africa. If present, the fungus could be potentially catastrophic in Central Africa where the species richness, endemism, and numbers of threatened amphibians are among the highest in continental Africa (Burgess et al. 2004; IUCN et al. 2006). Moreover, the amphibians of Central Africa are poorly surveyed or unknown in many areas (Broadley and Cotterill 2004; Channing and Howell 2006; Laurent 1983; Plumptre et al. 2003; Schmidt and Noble 1919), and thus, infections could wipe out species before they are identified by science. Remarkably, no published study has examined amphibians in Central Africa for the presence of chytrid fungus.

We (EG, CK, and MA) conducted a preliminary survey of the herpetofauna at sites in and near Kahuzi Biega National Park (a UNESCO World Heritage Site in Danger), South Kivu Province, Democratic Republic of Congo between 14 August and 2 September 2007. Habitats ranged from high-elevation bamboo forest to lowland rainforest, and although the timing of our collecting corresponded to the dry season for the eastern Congo highlands (Chapin, 1932) we observed some rainfall almost daily. Amphibians were collected by hand, euthanized via cutaneous contact with Orajel®, and preserved in 10% formalin solution; after a 24 h rinse in water, specimens were transferred to 75% ethanol and 1–4 mm toe clippings of 24 selected specimens (Table 1) were prepared for histological examination. Tissues were dehydrated in graded concentrations of ethanol and then xylene, paraffin-embedded, sectioned at 4 microns and stained with hematoxylin and eosin. To avoid delays from cataloging backlogs, field numbers are provided for voucher specimens, but these specimens will be deposited in an American natural history museum collection in the future. Abbreviations are as follows: EBG = Eli Greenbaum field series; SVL = snout–vent length.

Two of 24 specimens (EBG 1087 and EBG 1307) showed evidence of chytridiomycosis. The former specimen is a subadult *Hyperolius kivuensis* (19.7 mm SVL; adult size 22–39 mm according to Schiøtz 1999) with no evidence of lesions. A juvenile *H. kuligae* (EBG 1307; 10.1 mm SVL; adult size 20–31 mm according to Schiøtz 1999) has multiple small, white lesions on the venter of the hind limbs and abdomen. Infections were characterized by thickening of the superficial keratinized layers of the epidermis due to the presence of smooth-walled sporangia of *Batrachochytrium* that ranged in diameter from 10–25 microns. Most sporangia were empty, but several contained five to ten zoospores (Figs. 1A, B). No hyphae were present and there was no inflammatory cell response in the deeper layers of the epidermis and dermis. Twenty-two additional specimens representing 17 additional anuran species were negative for chytrid infection (Table 1).

To the best of our knowledge, the chytrid infections reported herein are the first positive results for any amphibian in Central Africa, where the fungus is present in both lowland (primary rainforest) and highland (secondary montane forest) habitats. Both infected individuals were collected in (EBG 1087) or near (EBG 1307) streams in close proximity to (< 1 km) human habitations and agricultural fields. The subadult and juvenile ages of the infected frogs are consistent with the high rate of infection and mortality reported for postmetamorphic frogs in Africa and Australia (Berger et al. 1999; Smith et al. 2007).

Weldon et al. (2004) hypothesized that *Batrachochytrium*

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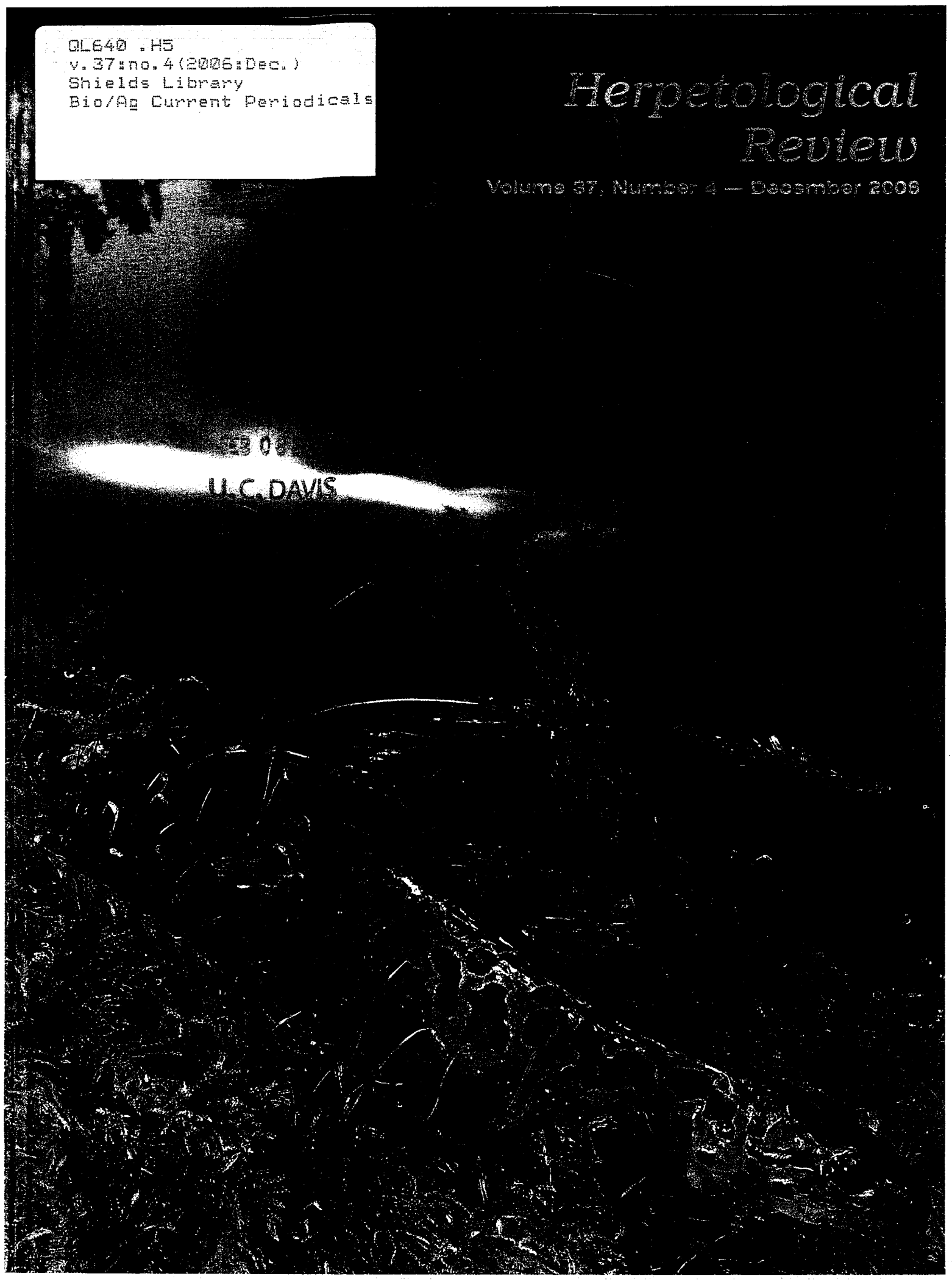

38–39 (Gosner 1960. Herpetologica 16:183–190).

Tooth row formula for most of these tadpoles is 3/4 (3 upper rows and 4 lower). Two tadpoles have 3/3 tooth rows, but one seems to have partially broken mouthparts.

These tadpoles were in a 6 × 7 m pool in bedrock, 30 cm deep, located 60 m from the St.-Lawrence River. It was bordered with Black Crowberry (*Empetrum nigrum*) and Leatherleaf (*Chamaedaphne calyculata*). The water was salty, with a marine fauna: Blue Mussel (*Mytilus edulis*), Steamer Clam (*Mya arenaria*), Periwinkle (*Littorina* sp.), Sandworm (*Nereis virens*), and barnacle (*Balanus* sp.). Coniferous forest was about 100 m N of the pool.

The major interest in this discovery is to understand why these northern shore line Wood Frog tadpoles are so big: has their growth been enhanced or their metamorphosis inhibited? It seems likely that the pool would have frozen solid during the winter, so they might have grown to this exceptional size in the two months since the breeding season.

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RANA SYLVATICA (Wood Frog). **CHYTRIDIOMYCOSIS.** The chytrid fungus, *Batrachochytrium dendrobatidis* (BD), has been implicated in mass amphibian mortalities and global population declines (Berger et al. 1998. Proc. Natl. Acad. Sci. 95:9031–9036; Pounds et al. 2006. Nature 439:161–167). On 25 July 2002, a dead subadult male *Rana sylvatica* (26 mm SVL; 1.8 g body weight after ethanol fixation) was collected near a pond on the Kenai National Wildlife Refuge, Alaska, USA (60.62741°N, 150.81557°W, WGS 84). The frog was preserved in ethanol and shipped to the U.S. Geological Survey, National Wildlife Health Center (NWHC) in Madison, Wisconsin, USA. A whole-body radiograph of the specimen showed a normal musculoskeletal system with a paucity of calcium carbonate in the paravertebral endolymphatic sacs. Histological sections of two hindlimb digits and ventral skin (pelvic patch area) showed mild hyperkeratosis of the epidermis with numerous 6–12 μ diameter empty chytrid thalli within keratinized cells of the *stratum corneum*. These epidermal lesions were diagnosed as mycotic hyperkeratotic epidermitis due to infection by BD. On 18 July 2002, five dead tadpoles were observed at this site but not submitted for disease diagnosis. Calling adult Wood Frogs, egg masses, or tadpoles were detected in 18 of 26 site visits during 2000–2005, and live frogs were documented at the site each year. This site borders a gravel road. There are no reptiles or fish at this site. No other species of amphibian has been detected on the Kenai Refuge during surveys of >100 ponds during 2000–2005. Waders and nets are disinfected with 5% bleach solution between sites. This is the first report of a BD-infected frog from Alaska. The effects of BD at such a high northern latitude, and on this population, are unknown. The specimen is stored in ethanol at the National Wildlife Health Center (Case #4848-041).

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RANA YAVAPAIENSIS (Lowland Leopard Frog). **EGG AND TADPOLE PREDATION.** Ranid frogs in the western United States have been disproportionally affected by amphibian declines (Bradford 2005. *In* M. Lannoo [ed.], Amphibian Declines: The Conservation Status of United States Species, pp. 916–925. Univ. California Press, Berkeley). Among the many causative agents for these declines, predation by non-native fishes has been strongly implicated, especially in naturally fish depauperate areas (Bradford 2005, *op. cit.*). However, first-hand descriptions of egg predation by introduced fish have rarely been reported. Here, I describe predation on a *Rana yavapaiensis* egg mass by non-native catfish in southern Arizona.

On 20 August 2002, I watched two *Ameiurus melas* (Black Bullhead; Deborah Sebesta, USFS District Biologist, Coronado National Forest, pers. comm.), each ca. 25–35 cm long, prey upon a *R. yavapaiensis* egg mass. The egg mass was located within 5–10 cm of the water's surface, ca. 2.5 m from the creek shore, in water 1.0–1.5 m deep, in a slow moving oxbow of Peck Canyon in southern Arizona (31°29'N, 111°04'W). The substrate was coarse sand and gravel. The eggs were just hatching, Gosner Stages 20–25 (Gosner 1960. Herpetologica 16:183–190), and the hatchling tadpoles were still in a tight aggregation around or within the egg mass. The fish repeatedly swam through the egg mass with mouths open, turning around for another run after passing beyond the eggs by 10–30 cm. The possibility exists that the eggs were of *R. chiricahuensis*, also recorded from the general area. However, on this day hundreds of *R. yavapaiensis* metamorphs were observed, with no *R. chiricahuensis* documented from this length of the canyon (unpubl. data).

I thank Kevin Bonine and Cecil Schwalbe for reviewing an earlier version of this note.

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RHACOPHORUS KAJAU (White-eared Tree Frog). **FOOT FLAGGING.** A population of *Rhacophorus kajau* occurs at Kubah National Park (01°33'N, 110°12'E), Matang, Sarawak, Malaysia (Borneo). While being photographed *ex-situ* indoors on 11 Dec 2005 and 22 Jan 2006, two adult males exhibited a behavior previously unreported in this species. While keeping the forelimbs planted on the substratum (in both cases, green leaves), the hind limbs were extended upward, and turned counter-clockwise at the level of the knee, with the undersurfaces of the shanks and sole touching the posterior of the dorsum of the body (Fig. 1). The entire action was performed in under 1 sec. Males of *R. kajau* are typically found in social groups of up to five in the wild. We suggest that the behavior, exhibited under stress such as handling associated with photography or the bright light from a flash, simulates the behavior, and is a warning to conspecifics, when the pale undersurfaces of limbs, black webbing, and/or the typically con-

Appendix H – Tables of Contaminants in Water and Sediment

Table 3. Polycyclic Aromatic Hydrocarbons and Alkylated Homologues in Sediment of Study Sites.

Site	1-methylphenanthrene	2,6-dimethylnaphthalene	2-methylnaphthalene	benzo(b)fluoranthene	benzo(g,h,i)perylene	biphenyl	C1-chrysenes	C1-Fluoranthenes & Pyrenes	C1-fluorenes	C1-naphthalenes	C1-Phenanthrenes & Anthracenes	C2-chrysenes
KNA01	< 0.04592	< 0.04592	< 0.04592	< 0.04592	< 0.04592	< 0.04592	< 0.04592	< 0.04592	0.07056	< 0.04592	0.08306	< 0.04592
KNA02	0.00924	< 0.00795	< 0.00795	< 0.00795	< 0.00795	< 0.00795	< 0.00795	< 0.00795	0.04463	0.01262	0.02062	< 0.00795
KNA03	< 0.05313	< 0.05313	< 0.05313	< 0.05313	< 0.05313	< 0.05313	0.45593	< 0.05313	< 0.05313	< 0.05313	< 0.05313	< 0.05313
KNA08	< 0.06743	< 0.06743	< 0.06743	< 0.06743	< 0.06743	< 0.06743	< 0.06743	< 0.06743	< 0.06743	< 0.06743	0.15522	< 0.06743
KNA08	< 0.02544	< 0.02544	< 0.02544	< 0.02544	< 0.02544	< 0.02544	0.07945	< 0.02544	0.04767	< 0.02544	0.04180	0.09914
KNA12	0.00426	< 0.00334	< 0.00334	< 0.00334	< 0.00334	< 0.00334	0.00592	0.00462	0.02076	0.00509	0.01024	0.01065
KNA14	< 0.02864	0.05029	< 0.02864	< 0.02864	< 0.02864	< 0.02864	< 0.02864	< 0.02864	0.04971	< 0.02864	0.03397	< 0.02864
KNA21	< 0.01119	< 0.01119	< 0.01119	< 0.01119	< 0.01119	0.01472	0.02029	< 0.01119	0.03703	0.01758	0.02649	0.29078
KNA31	< 0.03996	< 0.03996	< 0.03996	< 0.03996	< 0.03996	< 0.03996	0.04868	< 0.03996	< 0.03996	< 0.03996	0.11737	< 0.03996
KNA46	< 0.06412	< 0.06412	< 0.06412	< 0.06412	0.08920	< 0.06412	< 0.06412	< 0.06412	< 0.06412	< 0.06412	< 0.06412	< 0.06412
KNA47	< 0.01929	< 0.01929	< 0.01929	< 0.01929	< 0.01929	< 0.01929	0.21113	< 0.01929	0.04291	0.02464	0.03563	0.14954
KNA51	< 0.04502	< 0.04502	< 0.04502	< 0.04502	< 0.04502	< 0.04502	< 0.04502	< 0.04502	0.05647	< 0.04502	< 0.04502	< 0.04502
KNA54	< 0.01055	0.01649	< 0.01055	< 0.01055	< 0.01055	< 0.01055	0.03163	< 0.01055	0.09398	< 0.01055	< 0.01055	0.03885
KNA55	< 0.01469	0.04458	< 0.01469	< 0.01469	< 0.01469	< 0.01469	< 0.01469	< 0.01469	0.09836	< 0.01469	0.02106	< 0.01469
KNA56	< 0.01710	< 0.01710	< 0.01710	< 0.01710	< 0.01710	< 0.01710	0.09364	< 0.01710	0.04462	< 0.01710	< 0.01710	0.02566
KNA60	0.03939	< 0.02171	< 0.02171	0.02803	< 0.02171	< 0.02171	0.10045	0.05076	0.17091	< 0.02171	0.19621	0.17606
KNA62	< 0.05419	< 0.05419	< 0.05419	< 0.05419	< 0.05419	< 0.05419	< 0.05419	< 0.05419	0.06069	< 0.05419	< 0.05419	< 0.05419
KNA62	< 0.01412	0.01568	< 0.01412	< 0.01412	< 0.01412	< 0.01412	0.12236	0.01897	0.07367	< 0.01412	0.07909	0.13185
KNA90	< 0.00399	< 0.00399	< 0.00399	0.00417	< 0.00399	< 0.00399	0.02012	< 0.00399	0.00835	< 0.00399	0.01067	0.00589
KNA95	< 0.01216	< 0.01216	< 0.01216	< 0.01216	< 0.01216	< 0.01216	< 0.01216	< 0.01216	0.04223	< 0.01216	< 0.01216	0.01876
KNA97	< 0.03628	< 0.03628	< 0.03628	< 0.03628	< 0.03628	< 0.03628	0.09023	< 0.03628	0.05659	< 0.03628	0.12091	0.19045
KNA111	0.00937	< 0.00702	0.00870	0.01079	< 0.00702	0.02568	0.01859	0.00804	0.07328	0.01374	0.02539	0.26733
KNA141	< 0.00775	< 0.00775	< 0.00775	< 0.00775	< 0.00775	< 0.00775	0.01599	< 0.00775	0.02257	0.00859	0.01170	< 0.00775

All results in mg kg^{-1} dry weight

Table 3. Polycyclic Aromatic Hydrocarbons and Alkylated Homologues in Sediment of Study Sites.

Site	C2-fluorenes	C2-naphthalenes	C2-Phenanthrenes & Anthracenes	C3-chrysenes	C3-dibenzothiophenes	C3-fluorenes	C3-naphthalenes	C3-Phenanthrenes & Anthracenes	C4-chrysenes	C4-naphthalenes	C4-Phenanthrenes & Anthracenes	chrysene
KNA01	0.05444	< 0.04592	< 0.04592	< 0.04592	< 0.04592	0.10778	< 0.04592	< 0.04592	< 0.04592	< 0.04592	< 0.04592	< 0.04592
KNA02	0.03851	0.01477	0.01117	< 0.00795	< 0.00795	< 0.00795	0.02879	0.07352	< 0.00795	0.01880	< 0.00795	< 0.00795
KNA03	< 0.05313	< 0.05313	< 0.05313	< 0.05313	< 0.05313	0.09296	< 0.05313	< 0.05313	< 0.05313	< 0.05313	< 0.05313	< 0.05313
KNA08	0.10957	< 0.06743	< 0.06743	< 0.06743	< 0.06743	0.19304	< 0.06743	0.20522	< 0.06743	< 0.06743	< 0.06743	< 0.06743
KNA08	0.05734	0.03886	0.05285	< 0.02544	< 0.02544	0.09016	0.03955	0.09896	< 0.02544	< 0.02544	< 0.02544	< 0.02544
KNA12	0.01388	0.00545	0.01000	0.00776	0.00715	0.00998	0.00605	0.02623	0.00455	0.00632	< 0.00334	< 0.00334
KNA14	0.04203	0.06910	< 0.02864	< 0.02864	< 0.02864	0.05624	0.05489	0.06468	< 0.02864	0.06699	< 0.02864	< 0.02864
KNA21	0.05918	< 0.01119	< 0.01119	0.08869	< 0.01119	0.62161	< 0.01119	< 0.01119	0.01580	0.02045	0.03633	< 0.01119
KNA31	< 0.03996	< 0.03996	< 0.03996	< 0.03996	< 0.03996	0.08605	< 0.03996	0.05947	< 0.03996	< 0.03996	< 0.03996	< 0.03996
KNA46	< 0.06412	< 0.06412	< 0.06412	< 0.06412	< 0.06412	< 0.06412	< 0.06412	0.09760	< 0.06412	< 0.06412	< 0.06412	< 0.06412
KNA47	0.05947	0.03417	0.02887	0.05219	< 0.01929	0.06013	0.03007	0.12185	0.04106	0.03126	< 0.01929	< 0.01929
KNA51	0.14471	< 0.04502	< 0.04502	< 0.04502	< 0.04502	< 0.04502	< 0.04502	< 0.04502	< 0.04502	< 0.04502	< 0.04502	< 0.04502
KNA54	0.05909	0.02123	< 0.01055	0.11019	< 0.01055	0.08641	< 0.01055	0.08762	0.05952	0.01564	< 0.01055	< 0.01055
KNA55	0.04438	0.04836	0.13926	< 0.01469	0.01616	0.04775	0.02178	0.11513	< 0.01469	0.01718	< 0.01469	< 0.01469
KNA56	0.02162	< 0.01710	< 0.01710	0.04254	< 0.01710	0.04497	< 0.01710	< 0.01710	< 0.01710	< 0.01710	< 0.01710	< 0.01710
KNA60	0.19000	0.03348	0.12773	0.34091	< 0.02171	0.17333	0.02470	< 0.02171	0.12212	0.05545	< 0.02171	< 0.02171
KNA62	< 0.05419	< 0.05419	< 0.05419	< 0.05419	< 0.05419	0.21207	< 0.05419	< 0.05419	< 0.05419	< 0.05419	< 0.05419	< 0.05419
KNA62	0.05818	0.02343	0.05150	0.19855	< 0.01412	0.19971	0.02188	0.10658	< 0.01412	< 0.01412	< 0.01412	< 0.01412
KNA90	0.00937	0.00777	0.00633	< 0.00399	< 0.00399	0.01144	0.00749	< 0.00399	< 0.00399	< 0.00399	< 0.00399	< 0.00399
KNA95	0.03149	< 0.01216	< 0.01216	< 0.01216	< 0.01216	0.01793	< 0.01216	0.02355	< 0.01216	0.01661	< 0.01216	< 0.01216
KNA97	0.06023	< 0.03628	0.04773	0.09409	< 0.03628	0.11659	< 0.03628	0.09841	< 0.03628	< 0.03628	0.06773	< 0.03628
KNA111	0.04831	0.01246	0.01902	0.07646	< 0.00702	0.05164	0.01488	0.06087	0.02473	0.01921	< 0.00702	0.00908
KNA141	0.02526	0.01144	< 0.00775	< 0.00775	< 0.00775	0.01972	0.01092	0.02899	< 0.00775	0.01599	< 0.00775	< 0.00775

All results in mg kg^{-1} dry weight

Table 3. Polycyclic Aromatic Hydrocarbons and Alkylated Homologues in Sediment of Study Sites.

Site	Dibenz(a,h)anthracene	fluoranthene	fluorene	indeno(1,2,3-cd)pyrene	naphthalene	perylene	phenanthrene	pyrene
KNA01	< 0.04592	< 0.04592	< 0.04592	< 0.04592	< 0.04592	0.26250	< 0.04592	< 0.04592
KNA02	< 0.00795	< 0.00795	< 0.00795	< 0.00795	0.01214	0.09802	0.01439	< 0.00795
KNA03	< 0.05313	< 0.05313	< 0.05313	< 0.05313	< 0.05313	0.39926	< 0.05313	< 0.05313
KNA08	< 0.06743	< 0.06743	< 0.06743	< 0.06743	< 0.06743	< 0.06743	< 0.06743	< 0.06743
KNA08	< 0.02544	< 0.02544	< 0.02544	< 0.02544	< 0.02544	0.87482	< 0.02544	< 0.02544
KNA12	0.00397	< 0.00334	< 0.00334	0.00475	0.00412	< 0.00334	0.00672	< 0.00334
KNA14	< 0.02864	< 0.02864	< 0.02864	< 0.02864	< 0.02864	0.32395	< 0.02864	< 0.02864
KNA21	< 0.01119	< 0.01119	< 0.01119	< 0.01119	0.01704	< 0.01119	0.02192	< 0.01119
KNA31	< 0.03996	< 0.03996	< 0.03996	< 0.03996	< 0.03996	0.09211	< 0.03996	< 0.03996
KNA46	< 0.06412	< 0.06412	< 0.06412	< 0.06412	< 0.06412	0.20720	< 0.06412	< 0.06412
KNA47	< 0.01929	0.02755	< 0.01929	< 0.01929	0.02662	0.08467	< 0.01929	< 0.01929
KNA51	< 0.04502	< 0.04502	< 0.04502	< 0.04502	< 0.04502	< 0.04502	< 0.04502	< 0.04502
KNA54	< 0.01055	< 0.01055	< 0.01055	< 0.01055	< 0.01055	< 0.01055	< 0.01055	< 0.01055
KNA55	< 0.01469	< 0.01469	< 0.01469	< 0.01469	< 0.01469	< 0.01469	< 0.01469	< 0.01469
KNA56	< 0.01710	< 0.01710	< 0.01710	< 0.01710	< 0.01710	< 0.01710	< 0.01710	< 0.01710
KNA60	< 0.02171	0.03864	< 0.02171	< 0.02171	< 0.02171	< 0.02171	0.06106	0.03530
KNA62	< 0.05419	< 0.05419	< 0.05419	< 0.05419	< 0.05419	0.16759	< 0.05419	< 0.05419
KNA62	0.01655	< 0.01412	0.01955	0.01491	< 0.01412	0.21245	0.02982	< 0.01412
KNA90	0.00591	< 0.00399	< 0.00399	< 0.00399	< 0.00399	0.06619	0.00401	< 0.00399
KNA95	< 0.01216	< 0.01216	< 0.01216	< 0.01216	< 0.01216	0.01591	< 0.01216	< 0.01216
KNA97	< 0.03628	< 0.03628	< 0.03628	< 0.03628	< 0.03628	< 0.03628	< 0.03628	< 0.03628
KNA111	< 0.00702	0.01484	< 0.00702	< 0.00702	0.02173	< 0.00702	0.04750	0.00846
KNA141	< 0.00775	< 0.00775	< 0.00775	< 0.00775	< 0.00775	< 0.00775	0.01046	< 0.00775

All results in mg kg^{-1} dry weight

Table 4. Organochlorines in study site sediment.

Site	1,2,3,4,6,7,8-HpCDD	1,2,3,4,7,8-HxCDD	1,2,3,4-Tetrachlorobenzene	1,2,4,5-Tetrachlorobenzene	Aldrin	alpha BHC	alpha chlordane	beta BHC	chlorpyrifos	chrysene	cis-nonachlor	delta BHC
KNA01	< 0.00005	< 0.00005	< 0.00378	0.00382	< 0.00378	< 0.00378	< 0.00378	< 0.00378	< 0.00378	< 0.04592	< 0.00378	< 0.00378
KNA02	< 0.00001	< 0.00001	0.00139	0.00708	0.00307	< 0.00013	0.00406	< 0.00013	< 0.00013	< 0.00795	< 0.00013	0.00105
KNA03	< 0.00004	< 0.00004	< 0.00444	0.01647	< 0.00444	< 0.00444	< 0.00444	< 0.00444	< 0.00444	< 0.05313	< 0.00444	< 0.00444
KNA08	< 0.00004	< 0.00004	< 0.00560	0.02139	< 0.00560	< 0.00560	< 0.00560	< 0.00560	0.01061	< 0.06743	0.01723	< 0.00560
KNA08	< 0.00004	< 0.00004	0.00256	0.01819	0.00207	0.00257	0.00468	0.00107	0.01622	< 0.02544	< 0.00042	< 0.00042
KNA12	< 0.00001	< 0.00001	0.00021	0.00099	0.00171	0.00049	0.00034	0.00076	0.00325	< 0.00334	0.00019	0.00059
KNA14	< 0.00005	< 0.00005	0.00155	0.00656	< 0.00048	< 0.00048	< 0.00048	< 0.00048	0.00148	< 0.02864	< 0.00048	0.00196
KNA21	< 0.00002	0.00004	0.00122	0.00900	0.00030	0.00025	0.00263	< 0.00019	0.00366	< 0.01119	< 0.00019	0.00345
KNA31	< 0.00002	< 0.00002	< 0.00336	0.01379	< 0.00336	< 0.00336	< 0.00336	< 0.00336	< 0.00336	< 0.03996	< 0.00336	< 0.00336
KNA46	< 0.00003	< 0.00003	< 0.00539	0.01323	< 0.00539	< 0.00539	< 0.00539	< 0.00539	0.00665	< 0.06412	< 0.00539	< 0.00539
KNA47	< 0.00003	< 0.00003	0.00201	< 0.00032	0.02282	0.00201	0.00746	< 0.00032	0.01351	< 0.01929	< 0.00032	0.00469
KNA51	< 0.00003	< 0.00003	< 0.00372	0.01551	0.00452	< 0.00372	< 0.00372	< 0.00372	0.01082	< 0.04502	< 0.00372	< 0.00372
KNA54	< 0.00002	< 0.00002	0.00607	0.01784	0.00056	0.00112	0.00428	0.00176	0.00927	< 0.01055	< 0.00018	0.00154
KNA55	< 0.00002	< 0.00002	0.00264	< 0.00024	< 0.00024	0.00189	< 0.00024	0.00105	0.00244	< 0.01469	0.00348	0.00372
KNA56	< 0.00003	< 0.00003	0.00140	< 0.00029	< 0.00029	< 0.00029	0.00331	< 0.00029	< 0.00029	< 0.01710	< 0.00029	0.00068
KNA60	< 0.00004	< 0.00004	0.03467	0.06300	0.00745	0.01198	0.01070	< 0.00036	0.10379	< 0.02171	0.01774	0.05785
KNA62	< 0.00003	< 0.00003	< 0.00453	0.00488	< 0.00453	< 0.00453	< 0.00453	< 0.00453	< 0.00453	< 0.05419	< 0.00453	< 0.00453
KNA62	< 0.00002	< 0.00002	0.00543	0.01286	0.00321	0.00198	0.00273	0.00138	0.00876	< 0.01412	0.00038	0.00336
KNA90	< 0.00001	< 0.00001	0.00009	0.00058	0.00020	< 0.00007	0.00050	< 0.00007	0.00024	< 0.00399	< 0.00007	< 0.00007
KNA95	0.00006	< 0.00002	0.00152	0.00062	< 0.00020	< 0.00020	0.00176	0.00098	0.00128	< 0.01216	< 0.00020	0.00047
KNA97	< 0.00002	< 0.00002	< 0.00306	0.00970	< 0.00306	< 0.00306	< 0.00306	< 0.00306	< 0.00306	< 0.03628	< 0.00306	< 0.00306
KNA111	< 0.00001	< 0.00001	0.00167	0.00259	0.00204	0.00049	0.00146	< 0.00012	0.00459	0.00908	0.00067	0.00226
KNA141	< 0.00001	< 0.00001	0.00120	0.00548	0.00364	0.00052	0.00170	0.00023	0.00620	< 0.00775	< 0.00013	0.00214

All results in mg kg^{-1} dry weight

Table 4. Organochlorines in study site sediment.

Site	dieldrin	endosulfan II	endrin	gamma BHC	gamma chlordane	HCB	Heptachlor	heptachlor epoxide	mirex	o,p'-DDD	o,p'-DDE	o,p'-DDT	OCDD
KNA01	< 0.00378	< 0.00378	< 0.00378	< 0.00378	< 0.00378	< 0.00378	< 0.00378	< 0.00378	< 0.00378	< 0.00378	< 0.00378	< 0.00378	< 0.00010
KNA02	< 0.00013	< 0.00013	< 0.00013	< 0.00013	0.00272	< 0.00013	0.00153	< 0.00013	0.01075	< 0.00013	0.00331	< 0.00013	0.00003
KNA03	< 0.00444	< 0.00444	< 0.00444	< 0.00444	< 0.00444	< 0.00444	< 0.00444	< 0.00444	< 0.00444	< 0.00444	< 0.00444	< 0.00444	< 0.00007
KNA08	< 0.00560	< 0.00560	< 0.00560	< 0.00560	< 0.00560	< 0.00560	< 0.00560	< 0.00560	< 0.00560	< 0.00560	< 0.00560	< 0.00560	< 0.00007
KNA08	< 0.00042	< 0.00042	0.00136	< 0.00042	0.00810	< 0.00042	0.00611	0.01031	0.00845	0.00131	< 0.00042	< 0.00042	0.00012
KNA12	< 0.00006	< 0.00006	0.00034	< 0.00006	0.00093	0.00042	0.00035	0.00082	< 0.00006	0.00120	0.00110	0.00006	0.00002
KNA14	< 0.00048	< 0.00048	0.00094	0.00054	0.00251	< 0.00048	0.00395	< 0.00048	< 0.00048	< 0.00048	< 0.00048	0.00090	< 0.00010
KNA21	< 0.00019	< 0.00019	0.00052	< 0.00019	0.00280	0.00077	0.00356	0.00105	0.00569	0.00045	0.00300	0.00036	0.00011
KNA31	< 0.00336	< 0.00336	< 0.00336	< 0.00336	< 0.00336	< 0.00336	< 0.00336	< 0.00336	< 0.00336	< 0.00336	< 0.00336	< 0.00336	< 0.00005
KNA46	< 0.00539	< 0.00539	< 0.00539	< 0.00539	< 0.00539	< 0.00539	< 0.00539	< 0.00539	< 0.00539	< 0.00539	< 0.00539	< 0.00539	< 0.00006
KNA47	< 0.00032	< 0.00032	< 0.00032	0.00193	0.00452	0.00118	0.00656	0.00338	0.00513	< 0.00032	< 0.00032	0.00159	< 0.00006
KNA51	< 0.00372	< 0.00372	< 0.00372	< 0.00372	< 0.00372	< 0.00372	< 0.00372	< 0.00372	< 0.00372	< 0.00372	0.00644	0.00391	< 0.00006
KNA54	< 0.00018	< 0.00018	0.00054	< 0.00018	0.00379	< 0.00018	0.00267	0.00509	< 0.00018	< 0.00018	< 0.00018	< 0.00018	< 0.00004
KNA55	0.00147	< 0.00024	0.00127	< 0.00024	0.00291	< 0.00024	0.00368	0.00098	< 0.00024	< 0.00024	< 0.00024	< 0.00024	< 0.00005
KNA56	< 0.00029	< 0.00029	0.00083	0.00069	0.00481	< 0.00029	0.00141	0.00141	< 0.00029	< 0.00029	< 0.00029	0.00050	< 0.00006
KNA60	0.00126	< 0.00036	0.01605	0.01285	0.01150	< 0.00036	0.05970	0.00962	< 0.00036	< 0.00036	< 0.00036	0.01423	< 0.00007
KNA62	< 0.00453	< 0.00453	< 0.00453	< 0.00453	< 0.00453	< 0.00453	< 0.00453	< 0.00453	< 0.00453	< 0.00453	< 0.00453	< 0.00453	< 0.00007
KNA62	< 0.00024	< 0.00024	0.00803	< 0.00024	0.00124	< 0.00024	0.00700	0.00319	< 0.00024	< 0.00024	< 0.00024	0.00145	< 0.00005
KNA90	< 0.00007	< 0.00007	0.00038	0.00020	0.00022	< 0.00007	0.00013	< 0.00007	< 0.00007	0.00023	0.00041	0.00067	0.00002
KNA95	< 0.00020	< 0.00020	< 0.00020	< 0.00020	< 0.00020	< 0.00020	0.00324	< 0.00020	< 0.00020	0.00031	0.00216	< 0.00020	0.00056
KNA97	< 0.00306	< 0.00306	< 0.00306	< 0.00306	< 0.00306	< 0.00306	< 0.00306	< 0.00306	< 0.00306	< 0.00306	< 0.00306	< 0.00306	< 0.00004
KNA111	< 0.00012	< 0.00012	0.00050	0.00015	< 0.00012	0.00217	0.00276	0.00085	< 0.00012	0.00029	< 0.00012	0.00022	< 0.00002
KNA141	< 0.00013	< 0.00013	0.00058	0.00013	0.00050	0.00114	0.00211	0.00068	0.00270	< 0.00013	< 0.00013	0.00024	< 0.00003

All results in mg kg^{-1} dry weight

Table 4. Organochlorines in study site sediment.

Site	oxychlordane	p,p'-DDD	p,p'-DDE	p,p'-DDT	PCB-TOTAL	pentachloro-anisole	trans-nonachlor
KNA01	< 0.00378	< 0.00378	< 0.00378	0.00382	0.10714	< 0.00378	< 0.00378
KNA02	< 0.00013	< 0.00013	< 0.00013	0.00488	0.63158	0.00042	0.00197
KNA03	< 0.00444	< 0.00444	< 0.00444	0.00807	0.08922	< 0.00444	< 0.00444
KNA08	< 0.00560	< 0.00560	< 0.00560	< 0.00560	0.47619	< 0.00560	< 0.00560
KNA08	< 0.00042	< 0.00042	< 0.00042	0.00553	0.46114	< 0.00042	0.00180
KNA12	< 0.00006	0.00045	0.00022	0.00266	0.09303	0.00073	0.00262
KNA14	< 0.00048	< 0.00048	< 0.00048	0.00944	0.54702	0.00194	0.00931
KNA21	< 0.00019	< 0.00019	0.00069	0.00225	0.53215	0.00206	0.00140
KNA31	< 0.00336	< 0.00336	< 0.00336	< 0.00336	0.18037	< 0.00336	< 0.00336
KNA46	< 0.00539	< 0.00539	< 0.00539	0.00931	0.13306	< 0.00539	< 0.00539
KNA47	0.00363	0.00181	0.00056	0.00800	0.52318	0.00097	0.01154
KNA51	< 0.00372	< 0.00372	< 0.00372	0.00446	0.32362	0.00440	< 0.00372
KNA54	< 0.00018	0.00171	0.00097	0.00247	0.35103	0.00033	0.00287
KNA55	< 0.00024	0.00867	0.00160	0.00315	0.37730	0.00037	0.00583
KNA56	< 0.00029	< 0.00029	< 0.00029	0.00313	0.20694	< 0.00029	0.00038
KNA60	< 0.00036	0.03076	0.01129	0.03070	4.58939	0.00164	0.03756
KNA62	< 0.00453	< 0.00453	< 0.00453	< 0.00453	0.07612	< 0.00453	< 0.00453
KNA62	0.00497	0.00275	0.00211	0.00515	0.49661	< 0.00024	0.00183
KNA90	< 0.00007	< 0.00007	< 0.00007	0.00035	0.05694	0.00009	< 0.00007
KNA95	< 0.00020	< 0.00020	0.00023	0.00171	0.09091	0.00036	0.00087
KNA97	< 0.00306	< 0.00306	< 0.00306	< 0.00306	0.09195	< 0.00306	< 0.00306
KNA111	< 0.00012	< 0.00012	0.00165	0.00352	0.19829	0.00296	0.00194
KNA141	< 0.00013	< 0.00013	0.00060	0.00131	0.27536	0.00260	0.00304

All results in mg kg^{-1} dry weight

Table 5. Inorganic contaminants in sediment.

SiteID	Ag	Al	As	B	Ba	Be	Ca	Cd	Co	Cr	Cu	Fe	Hg	K	Mg	Mn	Mo	Na	Ni
KNA01	0.53	9610	5.14	6	62.8	0.1	7280	0.177	3.14	9.21	21.7	7710	0.069	439	1390	187	1.58	1350	8.53
KNA02	< 0.192	11300	3.44	< 1.92	103	0.166	3650	0.238	3.56	10.5	14	9860	0.0536	283	1400	121	< 0.96	NA	11
KNA03	< 0.198	12000	4.75	6.04	281	0.184	5650	0.238	4.3	12.9	21	7980	0.052	492	1780	185	< 0.99	666	15
KNA08	< 0.198	2320	1.2	5.53	52.2	< 0.0496	4640	0.0564	1.19	5.53	4.43	3600	< 0.0248	1980	2090	265	< 0.99	332	5.38
KNA08	< 0.193	9200	2.25	< 1.93	55.1	0.125	4060	0.0927	3.68	13.4	11.5	7540	0.0913	446	2640	136	< 0.97		11.7
KNA12	0.543	17200	6.08	< 1.98	224	0.328	2740	0.224	5.76	25.3	20.5	16400	0.0909	1050	3720	335	< 0.99		18.9
KNA14	< 0.193	5340	1.81	< 1.93	68.6	0.102	3620	0.0671	2.44	5.79	8.43	4250	0.0498	227	982	182	< 0.97		5.67
KNA21	< 0.189	7780	2.15	< 1.89	229	0.133	2330	0.178	1.9	10.4	13.9	5290	0.0709	381	1100	83	< 0.94		6.9
KNA31	< 0.198	4830	5.1	5.81	84.9	0.0599	5030	0.0916	2.13	5.42	12.4	6060	0.044	319	1210	177	< 0.99	448	6.81
KNA46	< 0.199	8600	2.78	5.84	106	0.159	16300	0.179	2.91	11.1	14.4	7200	0.0524	525	1900	200	< 0.99	622	10.3
KNA47	< 0.192	9640	3.6	< 1.92	103	0.205	3970	0.281	4.08	14.3	13	9530	0.0618	510	2080	264	< 0.96		11.8
KNA51	0.472	3420	89.3	5.12	31.3	0.0654	8660	0.224	2.05	4.23	13.3	3340	0.0356	759	800	77.4	2.17	440	5.71
KNA54	< 0.197	5190	90.3	3.77	18.6	0.186	11800	0.42	1.27	9.14	31.2	3670	0.0586	404	998	37.3	5.8		7.38
KNA55	< 0.196	5250	22.7	5.53	14.3	0.0822	10300	0.212	2.31	4.91	17.4	2380	0.0558	251	806	40.5	4.38		7.13
KNA56	< 0.193	7040	61.2	3.32	45.4	0.151	11900	0.684	3.86	9.77	20.5	6730	0.039	346	1720	77.2	3.56		9.75
KNA60	< 0.197	7530	1.36	< 1.97	59.2	0.144	3510	0.0949	1.78	8.31	15	3470	0.0668	553	1010	179	< 0.98		5.06
KNA62	0.425	8790	3.46	4.12	60.7	0.131	4700	0.13	4.65	11.7	15.8	5900	0.0405	409	1880	107	< 1	488	12.3
KNA62	< 0.191	12500	6.18	< 1.91	123	0.259	5650	0.292	5.49	19.7	29.3	13400	0.0395	1070	3310	303	< 0.96		19.2
KNA90	< 0.192	24700	3.91	< 1.92	90.7	0.337	3900	0.158	6.12	20.6	26.5	13900	0.043	917	3880	222	< 0.96		17.9
KNA95	< 0.188	12200	7.22	3.88	96	0.233	11500	0.0861	6.36	23.2	18.8	18500	0.0627	1140	5070	504	< 0.94		15.2
KNA97	< 0.199	8260	16.7	20.4	83.4	0.161	12200	0.154	4.09	21.7	14.4	21100	0.0275	1050	3580	360	2.65	385	12.8
KNA111	< 0.194	13300	3.39	< 1.94	165	0.247	7780	0.209	6.31	18.4	20.9	13000	0.0946	602	3010	278	1.27		13.5
KNA141	< 0.193	10300	1.41	< 1.93	65.9	0.135	2510	0.0692	1.25	9.35	15.1	3290	0.0363	252	791	80.5	< 0.96		7.95

All results in mg kg^{-1} dry weight

Table 5. Inorganic contaminants in sediment.

SiteID	P	Pb	S	Se	Si	Sr	Ti	V	Zn
KNA01	738	4.24	2520	0.447	738	61.1	374	29.7	36.3
KNA02	589	2.22	2440	0.555		32.1	252	32.7	41.9
KNA03	489	5.38	3120	0.892	729	57.9	355	37.1	52.6
KNA08	537	1.39	1200	0.071	176	30.7	62.1	5.93	406
KNA08	526	2.77	2720	0.398		38.3	474	24.8	58.9
KNA12	837	7.76	453	0.284		25.4	748	45	71.4
KNA14	487	1.97	1880	0.348		31.7	132	16.8	20.3
KNA21	805	5.01	2120	0.468		21.4	300	29.2	31.5
KNA31	678	2.78	2490	0.368	423	49.5	136	15.1	28.7
KNA46	716	3.56	1550	0.286	688	68.2	242	21.5	41.4
KNA47	608	3.29	1310	0.313		31.2	355	28.1	34
KNA51	806	2.67	4800	1.13	541	40	78	9.61	28
KNA54	795	1.92	8240	7.48		41.7	171	13.2	22
KNA55	600	1.72	6660	2.92		39.6	170	13.7	48.2
KNA56	541	2.61	9700	3.86		39.8	210	16.9	36.2
KNA60	1360	2.66	2110	0.353		27.8	90.7	15.8	40.4
KNA62	512	3.2	3470	0.431	634	49.2	348	27.9	61.7
KNA62	755	7.31	2430	0.517		38.4	366	40.9	90.5
KNA90	620	8.99	1590	0.468		34.4	967	53.3	86.2
KNA95	894	5.82	965	0.23		53.9	797	39.7	42
KNA97	1050	24	2400	0.618	564	63.7	325	29	61.2
KNA111	1350	7.99	2610	1.38		54.9	173	47.2	36.7
KNA141	524	4.84	2030	0.5		20.5	311	23.4	20

All results in mg kg^{-1} dry weight

Table 6. Inorganics in study site water.

SiteID	Al	As	Ba	Ca	Cd	Cr	Cu	Fe	K	Mg	Mn	Mo	Na	P	Pb	S	Se	Si	Sr	Ti	V	Zn
KNA01	160	0.6	16	6040	< 0.02	< 5	< 5	1100	780	1950	24	< 10	4000	< 50	1.2	300	0.1	900	36	< 5	1	5
KNA02	784	2.6	25	3940	0.02	< 5	< 5	2330	3090	1450	78	< 10	4000	< 50	0.2	500	0.2	600	27	18	10	7
KNA03	90	0.4	18	1190	0.02	< 5	< 5	191	830	570	10	< 10	< 2000	< 50	0.1	100	< 0.1	100	9.3	< 5	< 1	8
KNA08	352	0.5	11	2770	0.02	< 5	< 5	518	1490	1050	44	< 10	< 2000	< 50	0.3	400	< 0.1	900	14	< 5	1	32
KNA12	334	3.5	39	2000	0.04	< 5	< 5	1810	3430	820	10	< 10	< 2000	110	0.4	200	0.2	300	17	15	< 10	< 5
KNA14	132	0.3	3	980	< 0.02	< 5	< 5	427	720	550	20	< 10	< 2000	< 50	< 0.1	< 100	0.1	200	7.2	< 5	< 10	< 5
KNA21	209	0.6	42	1160	< 0.02	< 5	< 5	114	620	600	26	< 10	< 2000	< 50	0.1	100	< 0.1	< 100	9.6	< 5	< 10	7
KNA31	207	1.8	22	3350	< 0.02	< 5	< 5	1320	820	1410	160	< 10	3000	< 50	0.2	< 100	< 0.1	4100	24	< 5	1	39
KNA46	< 50	0.4	2	1040	< 0.02	< 5	< 5	30	950	550	4	< 10	< 2000	< 50	0.7	< 100	< 0.1	100	5.8	< 5	< 1	< 5
KNA47	< 50	0.4	4	1230	< 0.02	< 5	< 5	166	740	600	3	< 10	< 2000	< 50	< 0.1	< 100	< 0.1	100	8.9	< 5	< 10	< 5
KNA51	70	9.1	6	8880	0.07	< 5	< 5	345	540	710	22	< 10	< 2000	70	0.1	1400	< 0.1	400	33	< 5	< 1	6
KNA54	< 50	28	5	27200	0.03	< 5	< 5	50	1030	1260	< 2	< 10	2000	< 50	< 0.1	7600	0.2	600	80	< 5	< 10	< 5
KNA55	< 50	8.4	5	25500	< 0.02	< 5	< 5	60	630	1140	20	< 10	< 2000	< 50	0.1	4200	0.2	3300	68	< 5	< 10	< 5
KNA56	< 50	8.4	13	26500	0.02	< 5	< 5	80	360	1290	< 2	< 10	< 2000	< 50	0.1	9300	0.2	2200	72	< 5	< 10	< 5
KNA60	60	0.5	10	3430	0.04	< 5	< 5	426	2000	860	137	< 10	< 2000	60	< 0.1	100	< 0.1	300	19	< 5	< 10	10
KNA62	70	0.5	1	3590	< 0.02	< 5	< 5	198	150	1040	6	< 10	2000	< 50	0.1	100	< 0.1	3000	23	< 5	< 1	24
KNA62	< 50	0.5	4	4930	< 0.02	< 5	< 5	60	770	1170	19	< 10	< 2000	< 50	< 0.1	200	0.1	200	25	< 5	< 10	< 5
KNA90	129	1	13	5210	0.83	< 5	< 5	60	970	2580	28	< 10	17000	60	0.1	100	< 0.1	< 100	45	< 5	< 10	42
KNA95	< 50	1	7	9600	< 0.02	< 5	< 5	200	480	1250	45	20	2000	100	< 0.1	100	0.2	4700	31	< 5	< 10	10
KNA97	< 50	4.1	18	29500	< 0.02	< 5	< 5	2970	840	3100	128	< 10	3000	< 50	0.3	300	< 0.1	7300	118	< 5	< 1	< 5
KNA111	2870	4.8	52	6590	0.16	6	11	8070	5700	2530	337	< 10	3000	993	1.8	600	0.3	6100	38	50	10	20
KNA141	634	1.6	21	3910	0.07	< 5	< 5	3310	4430	1470	273	< 10	2000	106	0.4	400	< 0.1	900	22	17	< 10	13

All results in ug l^{-1}