ROCKY MOUNTAIN AMPHIBIAN PROJECT:

STUDY DESIGN FOR LONG-TERM MONITORING OF AMPHIBIANS IN THE ROCKY MOUNTAIN REGION

Collaborators:

Wyoming Natural Diversity Database
Wyoming Game and Fish Department
Colorado Natural Heritage Program
Medicine Bow National Forest
Routt National Forest
Bridger-Teton National Forest

INTRODUCTION

Amphibians are sensitive to environmental change and many populations in the intermountain west have been shown to have declined in the past decade. The Boreal Toad, for example, has declined in distribution and abundance throughout its range in the past 20 years, is listed as endangered by Colorado and New Mexico, and is a Species of Greatest Conservation Need (SGCN) in Wyoming. Western populations of the Northern Leopard Frog have also experienced recent widespread declines (CFNE et al. 2006, USFWS 2011). Chytrid fungus is believed to be a major cause of world-wide amphibian declines and extinctions and has been implicated in the decline of several amphibian species in Wyoming and Colorado. Other known threats to Rocky Mountain amphibians include pesticides, herbicides, environmental pollutants, invasive species, introduced fish, UV radiation, and habitat loss and fragmentation.

Climate change and changes in habitat due to the recent mountain pine beetle outbreak also pose significant potential threats to amphibians in the Rocky Mountains (Jorgensen 1986, Reading 2007, McMenamin et al. 2008, Griffiths et al. 2010). More concerning is that these threats may work synergistically against amphibian populations. Substantial evidence links climate change with amphibian declines due to chytrid fungus (Pounds 2006, Rohr et al. 2008, Rohr and Raffel 2010, etc.), suggesting that interactions between these threats could lead to significant losses in amphibian biodiversity in the Rocky Mountain region. Monitoring of amphibian populations is critical to identifying problems and allowing management practices to be adjusted in a timely manner. In order to appropriately manage habitat, resource managers require a better understanding of how these potential threats are impacting the status and trends of local amphibian populations.

The Wyoming Natural Diversity Database (WYNDD), Wyoming Game and Fish Department (WGFD), Colorado Natural Heritage Program (CNHP), Medicine Bow and Routt National Forests (MBRNF), and Bridger-Teton National Forest (BTNF) worked together to develop a long-term amphibian monitoring program for the region. The resulting monitoring program considers guidelines set forth by the Amphibian Research and Monitoring Initiative (ARMI) and resembles

the mid-level occupancy-based modeling proposed by the U.S. Geological Survey (Corn et al. 2005), with protocols optimized for long-term implementation in national forests in the Rocky Mountain region. The purpose of the monitoring program is to allow resource managers to monitor trends in amphibian populations in the Rocky Mountain region. The final study design for long-term amphibian monitoring outlined below is based on results from a 2011 pilot study and subsequent power analysis on the MBRNF. To ensure successful long-term monitoring of amphibian populations at the multi-state level, the proposed monitoring plan is designed to facilitate a collaborative effort between multiple partners.

GOALS & OBJECTIVES

The overall goal of this project is to design and implement a sustainable, long-term amphibian monitoring program in the Rocky Mountain region to track amphibian populations. Sustainability of the monitoring plan ultimately depends upon a realistic study design capable of detecting changes in populations and able to be implemented through partnerships with multiple entities. Specific objectives of this monitoring plan were to:

1. Coordinate with land and wildlife management agencies to generate further interest and partnerships for amphibian monitoring.
2. Develop a feasible and effective study design that can accommodate participation from multiple agencies through consultation with biologists from the USFS and state wildlife management agencies, and with statisticians.
3. Conduct annual amphibian training and coordination sessions for all surveyors and collaborators.
4. Collect epithelial swab samples from amphibian species in order to test for chytrid fungus at all survey catchments.
5. Collect data for survey- and site-specific habitat variables.
6. Determine annual species-specific occupancy estimates.
7. Collaborate with other agencies and organizations to generate commitment and train local biologists and interested parties to conduct this monitoring in future years, thereby spreading the workload and decreasing dependence on targeted annual funding.
8. Establish a central data repository where all partners can submit and obtain monitoring data.

TECHNICAL APPROACH

Study Area

The amphibian monitoring program initially will take place on the Medicine Bow National Forest and Bridger-Teton National Forest in Wyoming and the Routt National Forest in

Colorado. The national forests occur in the Rocky Mountains and encompass valleys, meadows, wetlands, conifer forests, and alpine areas in portions of at least 8 major mountain ranges. Lodgepole pines (*Pinus contorta*) in many of these mountain ranges have been heavily impacted by mountain pine beetle outbreaks. Surveys will occur in amphibian habitat within lodgepole pine, mixed conifer, and subalpine forest types. Amphibian habitat in the national forests includes wet meadows, bogs, beaver ponds, springs and backwaters or slow moving areas along mountain streams.

Survey Methods

Survey methods are similar to guidelines set forth by ARMI and permit occupancy-based modeling of amphibian populations (Corn et al. 2005). Protocols have been optimized for long-term implementation by various land managers in this region. Following methods adapted from amphibian monitoring protocols used by Yellowstone, Grand Teton, and Glacier National Parks, we will survey all aquatic sites within a survey area (hereafter *catchment*). For the purpose of this study, a *site* is defined as a unique aquatic feature (wetland, pond, wet meadow, bog, stream reach, etc.) within a catchment. A *catchment* is the survey area encompassing a cluster of sites in a given location (Figure 1). Surveying multiple sites within a catchment not only increases the likelihood of detecting a species if it is present in the catchment, but also accommodates annual variability in the persistence of wetlands and/or the use of a specific wetland by amphibians. For this study, a catchment is the primary sample unit for occupancy analyses; however, site-level analyses within catchments can be conducted to investigate observed changes in occupancy within catchments.

Survey efforts will be conducted during the breeding season (May-July depending on elevation and weather conditions) when species are most detectable and will consist of visual encounter surveys of all amphibian habitat within a catchment. Evidence of breeding (egg masses, larvae, metamorphs) as well as the presence of any adults and juveniles will be noted for each species at each site. Surveys primarily will be conducted by 2 observers working independently at each site (dual-observer method) to allow for estimation of detection probabilities for each species. Each survey is conducted independently with no discussion of findings or peer correction of datasheets after survey is complete. The standard dual-observer method has both surveyors each survey around the perimeter of a water body until they meet at the opposite end, wait for

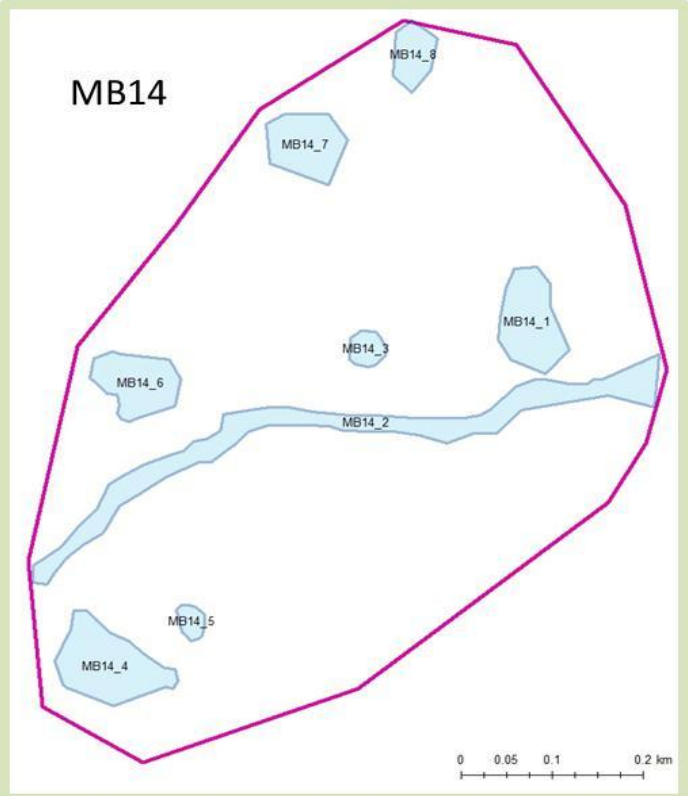

Figure 1. Example catchment (MB14) outlined in red with wetland survey sites in blue.

a short period of time (approximately 10 minutes) and then switch sides and complete the survey.

For better detection of tadpoles, surveyors will dipnet every 5-10m or in patches of good habitat for amphibian larvae (quiet inlets/backwater areas or patches of emergent vegetation, with each dipnet event consisting of at least five sweeps with the net. Tadpoles will be identified to genus using a tadpole key. If tadpoles cannot be identified in the field, 1-2 individuals should be collected and preserved in vials containing ethanol and sent to WYNDD, WGFD, or CNHP for identification. For unidentifiable animals or egg masses, photographs should be taken and sent to WYNDD, WGFD, or CNHP for identification. A subset of frogs and toads detected will be swabbed for chytrid fungus (*Batrachochytrium dendrobatidis*) following procedures outlined by Livo (2003; Appendix A). Chytrid samples will be sent to a lab for PCR testing. All survey and sampling gear will be decontaminated between drainages and between isolated sites within drainages to prevent the spread of chytrid fungus among sample locations (Appendix A).

Site Selection

Sites selection for the final long-term monitoring study design ultimately considered power analysis results, realistic timeframes and capabilities of survey crews, level of financial support, and accessibility of survey areas. Results from power analyses of data collected during the 2011 pilot study suggest that power to detect a statistically significant change in occupancy is likely limited to the more common species (e.g. Boreal Chorus Frog (*Pseudacris maculata*)). However, the proposed study design does allow land managers to track trends in occupancy and extinction rates of all amphibians on the forest, alerting managers to any potential problems so that more detailed investigations into the cause of the problem can be initiated. Results from power analyses further informed study design development by indicating that, given realistic funding levels, in order to improve our power to detect changes in amphibian populations it would be necessary to narrow our scope of inference from all potential amphibian habitat within the forests to areas most likely to be suitable for amphibians (higher densities of wetlands, streams, and/or wet meadows).

Results from the 2011 pilot study also informed the size of the area to be surveyed. Because accessing survey areas can be time consuming and costly in remote mountain regions, we used the pilot data to determine the number of sites within a catchment that could be surveyed in 1 visit to that catchment (1 day). During the pilot study, survey crews were able to survey an average of 6 sites (range = 2-12 sites) within a catchment in a day, resulting in a median catchment size of about 35 hectares.

Based on the above information, we recommend a study design with 32 catchments of approximately 35 ha on the MBRNF and 36 catchments on the BTNF which should be surveyed twice during the amphibian breeding season (late-May to late-July depending on elevation). The ultimate size of a catchment depended on the density of wetland sites or water bodies within the catchment and catchments typically contain at least 4 unique wetlands or water bodies.

Catchments were selected from areas in the MBRNF and BTNF most likely to provide amphibian habitat. To do this, we developed an index of the likelihood that an area contained amphibian habitat based primarily on the amount of wetland edge habitat within the surrounding 35 ha area of any given point in the study area. Areas with large amounts of wetland edge habitat were considered most likely to provide habitat for breeding amphibians. Areas with little wetland edge habitat were considered less suitable for breeding amphibians. Vegetation data were not considered in habitat analyses because the narrow extent of riparian and wetland vegetation surrounding aquatic habitat are not well represented in existing GIS layers. We used ArcGIS to conduct all amphibian habitat analyses.

Habitat Analyses

Water datasets for Wyoming and Colorado consisted of wetland polygons from the National Wetlands Inventory (NWI) and stream data from the National Hydrography Dataset (NHD). The wetland polygons were converted to linear features representing wetland perimeter, the area most likely to contain amphibian breeding habitat. Stream data were used in habitat analyses on the MBRNF, but resulted in too many areas chosen that had fast moving streams but no amphibian breeding habitat (ponds, wet meadows, etc.) and, thus, were later excluded from the monitoring program. Therefore, streams were not included in habitat analyses for the BTNF. Wetland edge layers were converted to a raster layer and focal statistics were used to calculate the proportion of cells in a 35ha area (335 m circular radius) around each raster cell.

Random Sampling

We used ArcGIS to randomly sample survey catchments across the MBRNF and BTNF. The selection process was weighted based on ease of access, with areas < 3 km from a road given higher probability of selection than areas farther than 3km from road (80:20). Because a large amount of the BTNF is roadless (wilderness), we also randomly sampled catchments within 1km of a trail in designated wilderness areas on the BTNF. In order to ensure that sampling was balanced across an elevational gradient, we stratified sampling across 3 elevation classes (low, medium, and high) on each national forest. Elevation classes were sampled in proportion to their representation on the national forests.

We randomly selected the desired number of catchments on each forest, plus a number of alternate catchments. Alternate catchments were used to replace primary catchments if access to primary catchments was restricted or habitat in primary catchments was deemed unsuitable for amphibians. We then digitized at least 4 survey sites encompassing all presumed amphibian habitat within each primary catchment (see Figure 2 for example).

Figure 2. Example of catchment (outlined in red) with survey sites digitized to encompass different wetland features.

Data Management and Analysis

Following surveys, data will be entered into an amphibian monitoring database designed and maintained by the University of Wyoming. All collaborators will have access to the data. For this study, as with the Yellowstone monitoring program, the catchment is the primary sample unit for occupancy analyses; however, site-level analyses within catchments can be conducted to investigate observed changes in occupancy within catchments. Following surveys, catchment occupancy will be modeled for each species. This will result in estimates of the proportion of all catchments occupied, the probability that a particular catchment is occupied, and detection probability (with associated confidence intervals). These estimates will be obtained for the presence of each species and the presence of breeding. This technique will allow monitoring of changes over time by tracking the proportion of catchments occupied and other population parameters (e.g., colonization/extinction rates, proportion of catchments where breeding is evident).

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APPENDIX A

Chytrid Fungus Decontamination

&

Collection Protocols

Detection of (*Batrachochytrium dendrobatidis*), the Chytrid Fungus Associated with Global Amphibian Declines, in Montana Amphibians

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In order to identify potential causes of declines in the northern leopard frog (*Rana pipiens*) and western toad (*Bufo boreas*) which have been noted since the 1980s and assess the risk posed to other amphibian species whose status is uncertain, we submitted 98 tissue samples gathered from 8 amphibian species across Montana for PCR based identification of the chytrid fungus (*Batrachochytrium dendrobatidis*). This chytrid fungus has been associated with declines, extirpations, and losses of numerous amphibian populations and entire species around the globe over the last 2 decades. Tissue samples from 30 museum voucher specimens of 3 species collected in the Flathead Valley in the 1970s, prior to amphibian declines in the area, were all negative for *B. dendrobatidis*. However, 4 species and 26 of 68 tissue samples gathered during inventory work across the state since 1998 tested positive for *B. dendrobatidis*. In light of its association with other amphibian declines, *B. dendrobatidis*, acting alone or synergistically with other stressors, is a potential cause of the declines observed and should be regarded as an ongoing threat to Montana amphibians. In order to prevent additional spread of this fungal pathogen personnel working in either lentic or lotic systems should thoroughly rinse and decontaminate all equipment with 10% bleach between (1) any sites where dead, dying, or ill amphibians are encountered, (2) sites located in different local watersheds or definitive clusters of sites, (3) all breeding sites of sensitive species separated by more than 1 kilometer.

Fungal and Viral Pathogen Decontamination Procedures and Useful References on Fungal Pathogens

When to Decontaminate

1. After any site where dead, dying, or ill animals are encountered
2. Between sites located in different watersheds
3. Between individual sites that are surveyed when traveling distances greater than 5 kilometers or between definitive clusters of sites.
4. Between all breeding sites of sensitive species that are surveyed and separated by more than 1 kilometer.

What to Decontaminate

1. Boots
2. Dipnets
3. Socks
4. Fingernails
5. Any other body parts, clothing, or other equipment that was exposed to waters or mud.

Washing and Decontamination Procedures (separate issues)

1. Washing - Once surveys are completed at a site or watershed scrub and rinse all equipment to remove any lingering mud. In general it is a good idea to do this between all sites if possible.
2. Decontamination - Prepare a mixture of 10% bleach by putting 4 ounces of bleach (half cup) in one gallon of clean water in a waterproof tub or bucket that can be carried in your vehicle between watersheds or sites. Use a fresh bottle of bleach each field season for this. Also in order to ensure that concentrations remain around 10%, a new bleach mixture should be made on a regular basis. If the solution of disinfectant becomes cloudy or brown with mud, silt, and vegetation, it should be discarded and a fresh solution made. Diluted bleach solutions should also be discarded after decontaminating equipment from any site where dead, dying, or ill animals are encountered. When discarding used bleach pour it out at least 30-40 meters away from water.
3. After rinsing equipment dip and thoroughly scrub individual items in the container of 10% bleach. An alternative approach for remote sites and where carrying a tub of bleach is impractical is to spray rinsed equipment with a concentrated (25-30%) bleach solution out of a large spray bottle and then let equipment dry between sites.
4. Do not rinse bleached equipment between sites. Instead allow the bleach to remain on the equipment to ensure that all fungal pathogens are killed. Most bleach will evaporate between sites so the amount of bleach introduced at the next site should be quickly diluted.

Handling Ill or Dying Animals

1. When handling ill or dying animals at a site use fresh rubber gloves for each animal to ensure that you are not transferring pathogens between individual animals.
2. Place individual animals in individual zip lock bags and keep them on ice continuously prior to shipping them to a pathologist for analysis.

Methods for obtaining *Batrachochytrium dendrobatidis* (Bd) samples for PCR testing

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General considerations

In terms of PCR samples, bleach and flame destroy DNA, while alcohol preserves DNA. Collection of samples for PCR testing requires that equipment used to collect samples not inhibit DNA detection while also not contaminating the current sample with DNA from a previous sample. In addition, equipment should be decontaminated so that it does not spread Bd (or other pathogens) from one animal to another.

The PCR sample collection methods described here have been used for obtaining samples from Colorado amphibians for Bd (*Batrachochytrium dendrobatidis*) detection. These methods should apply generally to collection of samples for Bd testing from amphibians.

Steps for obtaining samples

Collect animals

Animals should be collected with clean, decontaminated equipment, individually handled with fresh disposable gloves, and placed in individual containers prior to obtaining the samples. Although using Purell or other hand decontamination solutions may prevent the spread of live Bd from one animal to another, it is likely to allow contamination of samples with Bd DNA (in other words, if you handle a Bd-negative animal after handling a Bd-positive animal, the PCR samples you obtain may both appear to be positive for Bd).

Do not place multiple animals in the same container prior to sampling. In this situation, a single infected animal could infect others, and PCR tests could have inflated numbers of positive test results.

Equipment (such as individual containers for holding animals) can be cleaned and bleached so that they can be reused. However, this equipment must be rinsed well and allowed to dry prior to reuse so that there is no residual bleach (note that even parts per million bleach in/on/around a sample could possibly destroy all of the DNA in a sample over the course of a few weeks.)

Obtaining samples

Skin swabs are the preferred method of collecting samples from live individuals as the same individual can be tested repeatedly over time.

Obtain the PCR sample before doing other procedures with the animal (for example, before weighing, checking PIT tags, and so on). Samples require the following equipment:

- Swabs: use cotton swabs on 2mm-diameter wood without adhesive (such as Puritan Cotton-Tipped Applicators, #VWR 10806-005, or equivalent) cut to lengths (ca. 3-cm) that fit into 2-ml tubes.

- 2-ml screw-cap tubes containing 1 ml of 70 percent ethanol (2.0 ml screw cap tube with cap/500 per bag, VWR catalog # 20170-217, or equivalent)

To obtain the sample, hold the animal (using fresh gloves) in one hand, and gently but firmly swab the ventral surface 25 times; for large animals, you may swab the ventral surface 20 times and the feet and webbing 5 times.

FIGURE 1. Swabbing ventral surface of amphibian.

Place the swab (cotton side down) in the tube. Secure the lid and place in a rack or other container so that the tube remains upright. (Leakage from one tube with BD may get on other tubes and result in contamination of your samples.)

FIGURE 2. Insert swab or stick into tube with sample at bottom of tube.

Other skin tissues (such as toe clip samples or samples of ventral skin from dead animals) may also be collected for PCR testing. Use fine scissors to obtain the tissue. Between each sample, clean the scissors with an ethanol-soaked swab or tissue, and then hold the blades over an open flame to destroy any DNA from the previous sample. Place each sample in a 2 ml tube containing 1 ml of 70 percent ethanol.

FIGURE 3. Cleaning scissor blades with alcohol.

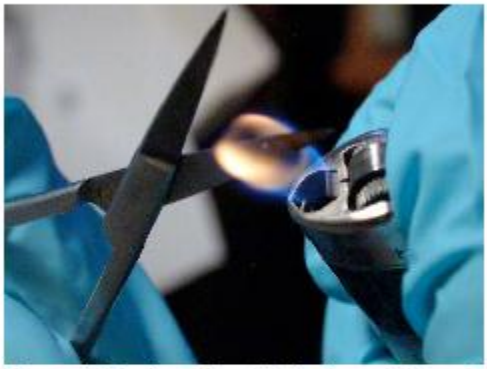
FIGURE 4. Passing scissor blades through flame to destroy residual DNA.

Toe clipping: If you collect toe clips from live individuals, use fine scissors to amputate the toe tip. When selecting a toe to amputate, you should avoid especially important digits such as the thumb, to avoid having an undue effect on the ability of the animal to feed, reproduce, and so on. Toe clips have rates of false-negative results similar to skin scrapes, but may have more potential for false-positive results through contamination. To obtain a toe clip, cut off the toe tip with the scissors. If bone protrudes from the wound, trim the bone further back (preferably to a joint) so that skin covers the wound, then dab a drop of Vet-bond or other sealant on the wound. In past studies, I selected the right rear toe, and continued to encounter individuals with this digit missing 2 to 3 years after the initial sampling.