Baseline Population Inventory of Amphibians on the Mountain Longleaf National Wildlife Refuge and Screening for the Amphibian Disease *Batrachochytrium dendrobatidis*

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Introduction:

Amphibians are facing worldwide population declines, range contractions, and species extinction. Within the last 30 years, over 200 species have become extinct and close to one-third of the world's amphibians are imperiled (IUCN, 2010). A recent trend analysis indicates that amphibian decline may be even more widespread and severe than previously realized and includes species for which there has been little conservation concern or assessment focus in the past (Adams *et al*. 2013). Factors such as invasive species, disease, changes in land use, climate change effects and the interactions of these factors all form current hypotheses that attempt to explain this dilemma (McCallum, 2007). This is alarming considering that the Southeast contains the highest level of amphibian diversity in the United States. It is imperative that we obtain and maintain current information on amphibian communities inhabiting our public lands so that we can adaptively manage resources for their long-term survival.

Although a number of causes appear related to amphibian declines in recent years, one of the leading factors is the infectious disease known as chytridiomycosis. This etiologic agent is an invasive chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*) that affects a variety of amphibian species. *Bd* infects the keratinized epidermal layers of the skin that disrupts vital functions such as respiration and osmoregulation. The infective stage of *Bd* is a flagellated aquatic zoospore, which relies on a moist or aquatic environment for survival apart from the host. *Bd* thrives best at cooler temperatures (below 23°C), and presence of the pathogen is therefore likely impacted by other associated environmental variables such as altitude, seasonality or climatic fluctuations, and water flow. Epizootic outbreaks of chytridiomycosis have been associated with high morbidity and mortality levels, subsequently causing amphibian population declines and extirpations.

From July of 2012 to June of 2013, we conducted baseline inventories for amphibians and sampled for the disease *Batrachochytrium dendrobatidis* on the Mountain Longleaf National Wildlife Refuge. Our objectives were to 1) quantify amphibian community structure within isolated communities identified as ecologically significant by the Refuge Habitat Management Plan (USFWS, 2005), 2) determine presence/absence of *Batrachochytrium dendrobatidis* in the amphibian communities inhabiting headwater springs, seeps and other refuge aquatic habitats, and 3) determine the prevalence of abnormalities in frog populations on the Refuge. These results add to our understanding of amphibian distribution on Mountain Longleaf NWR as well as on adjacent private, State of Alabama and USDA Forest Service managed lands.

Study Area Description:

The Mountain Longleaf National Wildlife Refuge is situated within the Alabama Valley and Ridge physiographic region on lands formerly known as Fort McClellan in Anniston, Calhoun County, Alabama. The climate of the region consists of long, humid, and warm summers with short mild winters. Average minimum/maximum temperatures for the Anniston area range from 10˚C and 23˚C, respectively and yearly rainfall averages ca. 131 cm (Southeast Regional Climate Center). Hydrologically, it is associated with the Coosa-Tallapoosa River Basin and contained within the Middle Coosa Subbasin. The Choccolocco Mountain range forms a major surface water divide on the refuge which contains the headwaters for five subwatersheds (Figure 3). East and south of this divide, surface water drains into tributaries of Choccolocco Creek and then into the Coosa River. To the west of the mountain, surface water flows into Cane Creek and tributaries of the Little Tallahatchee Creek before entering the Coosa River.

Methods and Materials:

We determined species presence/absence across the Refuge's landscape during the timeframe of July 2012 to June 2013. A suite of techniques were employed which included automated frog call data loggers, terrestrial and aquatic searches, active listening, cover boards, and arboreal PVC refugia.

Figure 1: Froglogger attached to a pine tree in riparian zone pool habitat at site six.

Frog Call Data Loggers: Four automated frog call data loggers (Figure 1) were deployed at four locations to monitor for calling anurans in March of 2013 and removed in June of 2013 (Figure 3; Table 1). Data loggers were programed to record each evening for five minutes per episode at 1800 and 2200 hours. Recordings were analyzed utilizing playback identification to determine species presence/absence of calling male frogs (Dodd, 2003).

Terrestrial and Aquatic Searches: We conducted terrestrial and aquatic searches at all locations (Figure 3; Table 1) by looking under rocks, leaves and woody debris and by using dip-nets and seines in ponds and streams (Figure 2). Active listening for calling male anurans was also utilized and noted when calls were encountered. Captured animals were identified to species and released at point of capture.

Figure 2: Looking for marbled salamanders at site one in December 2012 before the vernal pond filled with water.

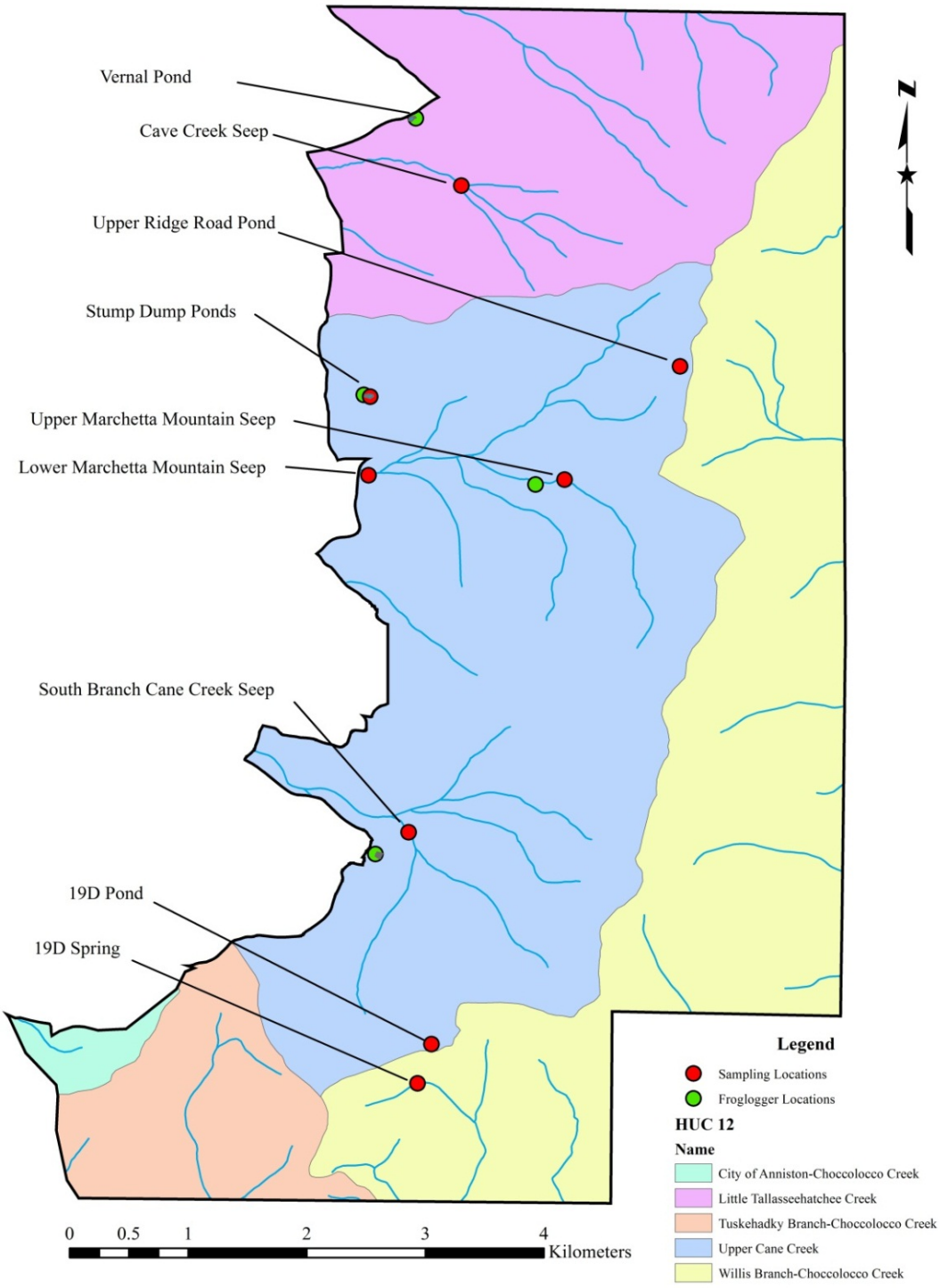

Figure 3. Subwatersheds and sampling locations on Mountain Longleaf NWR.

Table1: Survey Site Elevation and Locations.

Site Number/Name	Elevation	Coordinates
Site 1: Vernal Pond near Cave Creek Seep	296 m	33.740559˚ N -85.757879˚ W
Site 2: Cave Creek Seep	306 m	33.735395˚ N -85.754406˚ W
Site 3: Upper Ridge Road Pond	511 m	33.721670˚ N -85.737797˚ W
Site 4: Stump Dump Ponds	328 m	33.719498˚ N -85.761767˚ W
Site 5: Upper Marcheta Mountain Seep	312 m	33.713094˚ N -85.746585˚ W
Site 6: Upper Marcheta Mountain Pine Bog	308 m	33.712695˚ N -85.748812˚ W
Site 7: Lower Marcheta Mountain Seep	298 m	33.713456˚ N -85.761480˚ W
Site 8: South Branch Cane Creek Seep	293 m	33.686281˚ N -85.758441˚ W
Site 9: South Branch Cane Creek Storage Yard Pits	317 m	33.684621˚ N -85.760931˚ W
Site 10: 19D Pond	531 m	33.670131˚ N -85.756706˚ W
Site 11: 19D Spring	461 m	33.667233˚ N -85.757919˚ W

Coverboards: Artificial cover was created from non-treated plywood cut to the dimensions of 60 cm X 60 cm. A total of 24 boards were arrayed in groups of four (Figure 4) a few centimeters from each other along a linear transect 10 meters apart (Dodd, 2003). They were emplaced at four locations in July of 2012 to allow them to age properly and provide secure hiding places before we began checking them in September of 2012 and March and June 2013. Artificial covers were deployed at Cave Creek Seep, Upper Marcheta Mountain Seep, Lower Marcheta Mountain Seep and the South Branch of Cave Creek Seep (Figure 3; Table 1).

Figure 4: Portion of coverboard transect at site seven.

Figure 5: PVC refugia for Hylid treefrogs at site five.

Arboreal PVC Refugia: We sampled sites using polyvinylchloride (PVC) for the detection of Hylid tree frog presence. PVC refugia were deployed near pool breeding sites within streamside riparian zone habitats. Refugia were constructed from white PVC pipe (5.25 cm inside diameter X 80 cm long) that were fitted with a T fitting on the top and a cap on the bottom end that allowed them to hold water (Figure 5). All pipes were hung from trees with aluminum nails with the top being at a height of two meters and were positioned within shaded areas in the riparian zone not more than 20 meters from stream bank (Boughton *et al*, 2000; Smith *et al*, 2006). A total of 18 PVC refugia were arrayed in a linear transect 30 to 40 meters apart at four different locations. We emplaced all refugia during July of 2012 and checked them during our September 2012 and July 2013 efforts for occupancy. Arboreal PVC refugia were deployed at Cave Creek Seep, Upper Marcheta Mountain Seep, Lower Marcheta Mountain Seep and the South Branch of Cave Creek Seep (Figure 3; Table 1).

Batrachochytrium dendrobatidis (*Bd*) Screening:

In December 2012 (sites 4, 5, and 7) and June 2013 (sites 1, 4, and 9) amphibians were swabbed using individual, sterilized cotton swabs for the presence of *Bd* (Figure 3; Table 1). During the December effort adult individuals were swabbed separately at site 4 (n=2), site 5 (n=6) and site 7 (n=6). During the June effort we collected larvae and then selected a subsample of individuals; these samples were combined onto one swab in the field. After swabbing specimens the swab was broken ~3 cm from the tip and placed into a sterile screw cap microfuge tube and then frozen in the field in a cryogenic Dewar containing liquid nitrogen. DNA was extracted from the swabs by adding 50 µl of PrepMan® Ultra Sample Preparation Reagent (Life Technologies Corporation) and 50 mg of Zirconium/silica beads (Biospec: Cat. # 11079105z). Samples were homogenized for 10 minutes, followed by incubation for 10 minutes at 100˚C and then allowed to cool for 5 minutes to ambient temperature, followed by centrifugation for 10 minutes at 10 000 G. A 15 µL sample of the derived DNA supernatant was used in our quantitative PCR (qPCR) protocols along with primers (5'-CCTTGATATAATACAGTGTGCCATATGTC-3' and 5' TCGGTTCTCTAGGCAACAGTTT-3') that targeted a region encompassing a coding region (5.8S ribosomal RNA gene) and an adjacent non-coding Internal Transcribed Spacer (ITS1) region. Assays were performed using the probe MGB2 5'-CGAGTCGAACAAAAT-3' following Boyle *et al* (2004). Quantitative PCR conditions consisted of initial denaturation at 95˚C for 10 min, followed by 10 seconds at 95˚C and 60 seconds at 60˚C for 50 cycles. *Bd* Spore counts were done using a Petroff-Hausser counting chamber followed by DNA extraction utilizing the same procedure used for field samples for use as our positive control. Positive controls consisted of DNA equivalent to approximately 100, 10, and 1 zoospores and a negative control consisting of 15 uL of 10 mM Tris that were run concurrently with field samples.

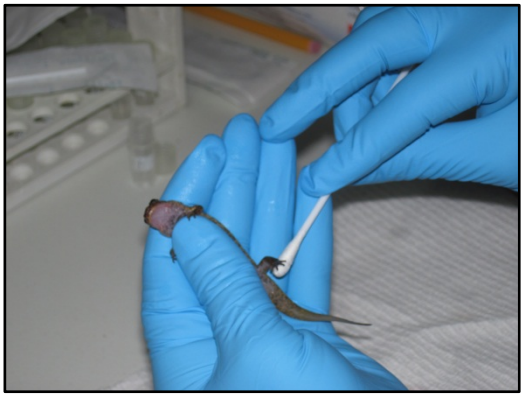

Figure 6: Swabbing a salamander for *Bd*.

Results:

A total of 11 sites were examined at different degrees of analysis utilizing a combination of techniques to obtain a baseline indication of amphibian occupancy on the Refuge (Table 1). We encountered 15 species of anurans and ten species of caudata during our inventory of the Mountain Longleaf NWR (Table 2). We tested for *Bd* at three sites during December 2012 and at three sites in June 2013. No malformed amphibians were found and all samples tested negative for the presence of *Bd*.

Figure 7: Pickerel Frog captured at site two.

Table 2: Amphibian species encountered on Mountain Longleaf NWR. X=an encounter event and the superscript number represents how the animal was captured or detected. 1=captured during aquatic and terrestrial searches; 2=active male calling during survey trip; 3=collected under coverboards; 4=male recorded calling by Froglogger. Species having an superscript asterisk indicates a potential new record for the refuge.

	Sites										
Scientific Name	1	2	3	4	5	6	7	8	9	10	11
Acris crepitans *				X^{4}					X^{4}		
Bufo americanus	X^{4}		X^{1}	$X^{2,4}$		X^{4}			$X^{1,4}$		
Bufo fowleri				X^{4}					X^{4}		
Gastrophryne carolinensis	X^{4}			X^{4}		X^{4}			X^{4}		
Hyla cinerea *				X^{4}							
Hyla chrysoscelis *	X^{4}	X^{2}		X^{4}		X^{4}			X^{4}		
Hyla gratiosa *				$X^{1,4}$					X^{4}		
Pseudacris brachyphona	X^{4}	X^{1}		X^{4}		X^{4}			X^{4}		
Pseudacris crucifer *	X^{4}		X^{1}	$X^{2,4}$		X^{4}	X^{2}		$X^{2,4}$		
Pseudacris feriarum	X^{4}			X^{4}		X^{4}	$X^{1,2}$		$X^{1,2,4}$		
Rana catesbeiana				$X^{1,4}$					X^{4}		
Rana clamitans	X^{2}	X^{1}	X^{2}	$X^{1,4}$		X^{4}			X^{4}		
Rana palustris		X^{1}		X^{4}					X^{4}		
Rana sphenocephala				$X^{1,2}$					$X^{1,4}$		
Scaphiopus holbrookii *	X^{4}										
Ambystoma maculatum *	X^{1}	X^{1}	X^{1}	X^{1}							
Ambystoma opacum *	X^{1}										
Desmognathus conanti					X^{1}						X^{1}
Eurycea cirrigera *		X^{1}			X^{1}		X^{3}				X^{1}
Eurycea guttolineata *		X^{1}									
Gyrinophilus porphyriticus					X^{1}						
Plethodon glutinosus		$X^{1,3}$					X^{3}	X^{3}			
Plethodon websteri							X^{3}			X^{1}	
Pseudotriton ruber					X^{1}						
Notophthalmus viridescens							X^{1}				

Discussion:

The earliest amphibian collections occurred in the vicinity of Mountain Longleaf National Wildlife Refuge in August of 1919 on Camp McClellan (Dunn, 1920). Dunn encountered ten species of amphibians gathered from unknown sites. The low number of species captured may be expected under the climatic conditions found in the relatively hot, dry summer months of northeast Alabama and certainly do not do justice to the diversity of amphibians that have since been described.

The most comprehensive study of the herpetofauna in the area (Cline and Adams, 1997) was an examination of (former) Fort McClellan which included lands that are now under the stewardship of the U.S. Fish & Wildlife Service. A total of sixteen species of frogs and toads and twelve species of salamanders were reported within the Fort's administrative boundary. The 19D pond was the most intensive site studied on lands currently known as the Mountain Longleaf NWR (Figure 8). We visited this natural spring-fed pond in July 2012 and found predatory fishes (sunfishes, e.g., bass and bream). These fish species can be voracious predators of amphibian eggs and larvae, and can eat adult amphibians as well. The impact on amphibian populations can be severe, and based upon this discovery we did not return to this site.

Figure 8: 19D Pond (site ten).

A list of amphibians that potentially occur in the vicinity of the Mountain Longleaf NWR is listed in Appendix 1. This list is based on studies by Mount (1964), Mount and Folkerts (1968), Rubenstein (1969), Mount (1975), Redmond (1975), Redmond and Mount (1976), Cline and Adams (1997), Highton (1995 & 1997), Rogers (2002), Edmundson (2009), Graham *et al.* (2012), and Macek (2012). It is highly probable that amphibians captured in these studies would be found within the Refuge boundaries. In fact, we found most of the species on this list and documented new amphibian captures not previously reported on the Refuge (Table 2). Given the short time period of this study, we do not know if the remaining non-encountered species avoided detection/capture, or whether they were not there to encounter. Longer term studies examining amphibian communities should address that question.

We had great results in detecting adult male anurans utilizing the Frogloggers. These were relatively easy to set up, were deployed during March to June 2013 and detected many species calling at each of the four sites. The recorders were set to log twice a day at 1800 hrs and 2200 hrs (for 5 minute time periods) which meant that we accumulated 40 minutes of tape a day. The time it took to review all tapes from the deployment period of 88 days took approximately 72 hrs. We did not examine the calling intensity of individual species in this study instead we focused on the determination of presence/absence based on calling males. The time spent listening to all of the recordings was extremely laborious and future analysis might be lessened if reliable software could be utilized to identify calls. While it is possible that we may have

missed detection of some less audible species during intense calling periods by species such as the spring peeper or Cope's gray treefrog, we detected these species through other repetitive sampling methods. It is also noted that utilizing a quality set of headphones is imperative and will dramatically increase the listener's ability to acquire less audible calls.

Our use of cover boards produced several salamander captures in December 2012 and March 2013 but did not result in any encounters during our June 2013 effort. Conversely, our strategy of using arboreal PVC refugia in riparian zone breeding habitats resulted in zero encounters of arboreal Hylid anurans. While these capture methods were successful year-round in other studies (Boughton *et al*, 2000; Dodd 2003; Smith *et al*, 2006), these methods did not work as well as we had hoped. Many amphibians make use of distinctly different habitats during different times of the year and will move between habitat types for reproduction, feeding, or hibernation. However, during hot, dry summer months, amphibians may limit their activity to avoid energy expenditure during unfavorable environmental conditions and thus kept away from our artificial refugia.

The stump-dump ponds (Figure 9) along Bain's Gap Road and the pits on the old Army fuel storage yard (former range 24a) on the south branch of Cane Creek produced high levels of anuran diversity (Table 2). These two sets of water bodies are both man-made and are good candidates for long-term monitoring. Adding new man-made ponds or restoring wetland habitat on the Refuge would create additional habitat for amphibians and can provide for a richer diversity of amphibian fauna.

Figure 9: One of the ponds located at the Stump Dump (site four).

We did not detect *Bd* or malformed frogs in our surveys. Previous surveys of *Bd* in the southeastern United States have demonstrated widespread occurrence and a broad host range in terms of the species that can potentially be infected, including both pond-breeding and stream-associated amphibians (Rothermel et al. 2008, Timpe et al. 2008). Because of funding and time constraints, we consider this a preliminary search for *Bd*. Additional amphibian health surveys may prove useful in the early detection of this disease and can be important in maintaining amphibian populations on the Refuge.

Management Implications:

Public lands often contain ecological infrastructure that no longer exists in surrounding areas that may be related to access restrictions or possibly by lack of exploitation by natural resource managers. Also, by comparing species richness between protected and unprotected sites we are also often able to predict the effectiveness of land managers' in the conservation of biodiversity. The unique nature of the Longleaf Pine Ecosystem is that fire is a frequent and required element to maintain stands. The array of seeps and shrub bogs and manmade ponds across the Refuge allows for a unique assemblage of forest dwelling amphibians to persist through probable recolonization directly following a fire event.

This report is the first step in inventory and monitoring amphibian populations on the Mountain Longleaf National Wildlife Refuge. Amphibians, as a taxa, could be considered a priory set of species for biological planning. Land-use management can be balanced with amphibian diversity and conservation and the Refuge can serve as a vital function in preserving amphibians from environmental toxicity and urban development.

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Appendix 1. Species that occur in or around the Mountain Longleaf NWR based upon perusal of previous studies (see text) in the vicinity of the Refuge.

Common Name	Scientific Name
Northern Cricket Frog	*Acris crepitans*
Southern Cricket Frog	*Acris gryllus gryllus*
American Toad	*Bufo americanus*
Fowler's Toad	*Bufo fowleri*
Eastern Narrow-mouthed Toad	*Gastrophryne carolinensis*
Green Treefrog	*Hyla cinerea*
Cope's Gray Treefrog	*Hyla chrysoscelis*
Barking Treefrog	*Hyla gratiosa*
Squirrel Treefrog	*Hyla squirella*
Mountain Chorus Frog	*Pseudacris brachyphona*
Spring Peeper	*Pseudacris crucifer*
Upland Chorus Frog	*Pseudacris feriarum*
American Bullfrog	*Rana catesbeiana*
Green Frog	*Rana clamitans*
Pickerel Frog	*Rana palustris*
Southern Leopard Frog	*Rana sphenocephala*
Wood Frog	*Rana sylvatica*
Eastern Spadefoot Toad	*Scaphiopus holbrookii*
Spotted Salamander	*Ambystoma maculatum*
Marbled Salamander	*Ambystoma opacum*
Eastern Tiger Salamander	*Ambystoma tigrinum*
Seepage Salamander	*Desmognathus aeneus*
Spotted Dusky Salamander	*Desmognathus conanti*
Seal Salamander	*Desmognathus monticola*
Southern Two-lined Salamander	*Eurycea cirrigera*
Three-lined Salamander	*Eurycea guttolineata*
Northern Spring Salamander	*Gyrinophilus porphyriticus*
Four-toed Salamander	*Hemidactylium scutatum*
Northern Slimy Salamander	*Plethodon glutinosus*
Webster’s Salamander	*Plethodon websteri*
Mud Salamander	*Pseudotriton montanus*
Northern Red Salamander	*Pseudotriton ruber*
Eastern Newt	*Notophthalmus viridescens*