Colorado Division of Wildlife

Boreal Toad Research Progress Report

2007-2010

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September 2011

Disease monitoring in boreal toad populations

Introduction

Boreal toads (formerly *Bufo boreas boreas*, now *Anaxyrus boreas*), once common in the mountains of Colorado (Burt 1933; Burger and Bragg 1947; Blair 1951), began mysteriously declining beginning in the 1970s (Corn et al. 1989; Carey 1993). A Recovery Team was formed in 1994 to proactively respond to significant declines in boreal toad distributions in the Southern Rocky Mountains. These declines resulted in an "Endangered" listing by Colorado and New Mexico and a "Status 2" species designation in Wyoming. Moreover, the boreal toad was considered "warranted but precluded" for federal listing under the Endangered Species Act (ESA) from 1995 until 2005, when the species was removed from consideration (USFWS 2005) as a result of the finding that the Southern Rocky Mountain Population (SRMP) of *A. boreas* did not constitute a species, subspecies, or distinct population segment under the ESA. Even so, *A. boreas* retains its' Colorado endangered status. Recent publications on the genetics of the group (Goebel et al. 2009, Switzer 2010) may generate additional discussion at the Federal level over the uniqueness of the SRMP. However, both recent publications suggest that the SRMP is more closely related to toads in Utah, southern Idaho, and Nevada than previously thought.

The Boreal Toad Recovery Team currently operates generally under the guidance of a Recovery Plan and Conservation Agreement (Loeffler 2001). This document, a revision of the 1997 original, is in need of additional revision to keep content applicable to ongoing activities and to document progress.

Since 1995, a broad range of research has been completed, by numerous members of the Recovery Team, as outlined in the Conservation Plan and Agreement. Highlights of this research include the investigation of UV radiation impacts, statewide genetic analyses, heavy metal toxicology, habitat use and movement, early life history ecology, predators, long term population monitoring, immunology, pathology, disease testing methodologies, and other topics. This research constitutes great progress toward understanding boreal toad biology and the circumstances which resulted in population declines.

In the late 1990s, researchers first described the chytrid fungus *Batrachochytrium dendrobatidis* (hereafter *Bd*) that causes the amphibian disease chytridiomycosis, infecting frogs in areas experiencing amphibian population declines in Central America and Australia (Berger et al. 1998; Berger et al. 1999; Longcore et al. 1999). In 1999, a decline in the Henderson/Urad boreal toad population in Clear Creek County was attributed to this newly described chytrid (Jones 2000; Livo 2000; Milius 1999). Subsequent pathological work by Dr. Allan Pessier showed that chytrid fungus was present at this locality as early as 1995. The fungus has now been identified in boreal toads from a number of populations in Colorado and evidence suggests the pathogen was in Colorado during the declines in the late 1970's and early 1980's (Carey et al. 1999). Of six hypotheses proposed as explanations for the worldwide amphibian crisis (Collins and Storfer 2003), chytridiomycosis as an emerging infectious disease seems to be the most compelling explanation for *B. boreas* declines in the Southern Rocky Mountains. Others that may be playing a role in Colorado are land use changes and perhaps contaminants and global change phenomena including UV and climate elements.

The CDOW contracted with researchers at the University of Maine at Orono in 2000 to develop a polymerase chain reaction (PCR) test for *Bd* (Annis et al. 2004), making it possible to conduct long-term monitoring of boreal toad breeding sites. The chytrid fungus infects the skin of an amphibian, so samples from amphibians may be obtained by swabbing the skin, after which the animal may be released.

The majority of known, active, reasonably accessible breeding populations of boreal toads in Colorado have been monitored since 2003. The data collected also allow the monitoring of the spread of the fungus within Colorado. Eventually the data may be subject to occupancy analyses that will allow formal estimation of the rate of spread and, possibly, the rate of "local extinction" of the fungus. However, at present most experts doubt the possibility of local extinction of *B. dendrobatidis*.

Currently it is very difficult to sample the environment apart from amphibians for the chytrid fungus, although it has been accomplished using water filtration (Walker 2007; Kirshtein 2007). Attempts to isolate Bd from organisms other than amphibians or from non-living, keratin-containing elements of organisms have been attempted (Wixson and Rogers 2009); these have been almost universally unsuccessful. To say the least, no truly reliable testing avenues have been found to date. This issue is important because many potential toad translocation sites contain no amphibians – making them attractive but very uncertain at the same time. Refinements and improvements in environmental sampling remain an acute need in research on *B. dendrobatidis*.

Methods

Breeding site monitoring – Current practices for obtaining *Bd* samples are detailed in Livo 2004 (available at http://wildlife.state.co.us/NR/rdonlyres/710BBC95-2DCF-4CF9-8443-D4561DBC3B69/0/PCRsampling2004.pdf).
Briefly, the preferred (least invasive) technique was used for the vast majority of tests reported here. This consists of stroking a glue-free cotton swab over the abdomen of the amphibian 20 times and swabbing the rear feet and webbing a total of five additional strokes. For amphibians < 20 mm snout-vent length, the rear feet are not swabbed, rather the abdomen is swabbed 25 strokes. Samples acquired for CDOW's ongoing breeding site monitoring were submitted to a private lab for analysis. Most breeding site swabs were obtained by CDOW biologists or biologists from Colorado Natural Heritage Program (CNHP) under a contract with CDOW. Other contributors included USFS and BLM personnel.

At each site, investigators endeavored to obtain samples from 20 individuals if available. Samples were often obtained over multiple dates, introducing the possibility that some individual toads may have been re-sampled. The data were used to gauge the disease status of the breeding sites and, if positive, the prevalence of infection. The latter metric was calculated as a simple binomial proportion, and is not a true measure of the prevalence of infection especially in those cases where samples were obtained over multiple dates.

Bd investigations – I monitored two known positive sites in the Kannah Creek drainage on Grand Mesa for prevalence and seasonality of *Bd* infection. One of the sites was in the former translocation area at the location known as Pond 4 (Rogers 2004). Each year from 2007

through 2010 chorus frogs *Pseudacris triseriata* were sampled on 3 – 5 occasions at these sites. The goal on each occasion was to sample 20 frogs except in 2010 when the target was 15 frogs.

In 2008 and 2009 I also used these sites to assess the effectiveness of a filtration method for capturing *Bd* zoospores from natural waters (Kirshtein et al. 2007). Replicate water filtration samples were collected at each site on four occasions in 2008 and three occasions in 2009. The DNA extraction from each filtrate was subjected to multiple quantitative PCR reactions, allowing analysis of the results in an occupancy framework. Chorus frog *P. triseriata Bd* swabs (n=20) were collected at each water filtration site on each occasion.

In 2009 modifications were made to the filtration technique in an attempt to enhance efficiency. All samples were pre-filtered through 20-µm mesh Pecap screen, thus filtering out some of the larger suspended matter that tended to quickly plug the 0.22-µm Sterivex filters used to collect the filtrates. Controlled tests in the lab on known zoospore concentrations were conducted to test whether the pre-filter operation reduced the recapture of zoospores. In addition to pre-filtering, field samples were collected in pairs and one of each pair was preserved with 33% of the amount of Lysis buffer as normal – thus effectively tripling the density of any zoospores in the filtrate that was submitted to the lab for analysis.

Frog swab samples were collected in 2010 as well (n=15 each site and occasion). All samples were submitted to the same lab for evaluation of the presence of *Bd* DNA by non-quantitative PCR.

In 2010 water filtration was employed at a prospective translocation site in Gunnison County. In all respects the site appeared to be a suitable toad site, however it lacked other amphibians and so its *Bd* status was a mystery. In conjunction with the water filtration, we also released two radio-equipped adult male toads from surplus brood animals at the CDOW Native Aquatic Species Restoration Facility. These animals were tracked down and swabbed weekly from early July through mid August. The swab samples were tested by the standard PCR test to ascertain whether they had become infected with *Bd*.

Results and Discussion

Breeding site monitoring – Many breeding sites were monitored each year from 2003 to the present, but not all breeding sites were monitored, nor was each site monitored in every year. Moreover, new sites have been discovered over the years and were added to the monitoring plan. Considerable data have been amassed on the status of known boreal toad breeding sites in Colorado (Table 1). A couple of observations are notable. A site that becomes positive typically becomes very quickly a site that exhibits high prevalence, usually followed by difficulty in finding toads to swab. This is consistent with the known high pathogenicity of *Bd* to boreal toads. However, on at least two occasions, single positive swabs failed to result in this scenario (see LR06 and LR08). Given boreal toad susceptibility to *Bd* it is most likely that the positive tests obtained at these sites represent sample contamination.

The mechanisms of site-to-site transmission remain uncertain. It may be possible for toads to carry *Bd* from one site to another. Ongoing research indicates that toads do occasionally

visit different breeding sites in different years (Lambert and Gaughan 2008, Lambert 2010). There are also instances of sites becoming enzootic for *Bd* for which we are aware of no near-by breeding sites that could be the source of a carrier animal, e.g. GU05 Upper Taylor River. That site became positive in 2008 with very low prevalence, and the results were confirmed in 2009 with very high prevalence. There are no other known breeding sites closer than five miles. However, there was once a breeding population about six miles downstream of GU05 near the Dorchester Campground (B. Lambert, CNHP, personal communication). The mysterious nature of *Bd* movement argues for continued vigilance and strict adherence to disinfection protocols on the part of field workers.

All of the known breeding sites comprising the metapopulation in Chaffee County remain negative for *Bd*, however there is a recently discovered (2007) site on the Gunnison County side of Cottonwood Pass (GU06, Cow Creek) that produced *Bd*-positive results in 2007 on all four animals that were swabbed. This situation is a cause of some concern, especially since CNHP contractors discovered the site and monitored it in 2008 and 2009.

Bd investigations – Estimates of *Bd* prevalence in the chorus frog population at two sites in the upper Kannah Creek drainage on Grand Mesa demonstrate seasonality in *Bd* infection (Figure 1). Infection rates among chorus frogs generally peak during the late breeding season and just after breeding concludes, a logical result in light of extensive animal to animal contact during breeding. Infection rates tended to be higher in 2007 and peaked earlier than other years, which is congruent with a low snowpack year. Field visits in 2007 were initiated just after Memorial Day; in the other years the sites were still snow-covered for 2-3 additional weeks.

Water filtration testing from these sites provided some surprising results. Although the two ponds are demonstrably positive sites, water filtration in 2008 initially yielded only five positive filtrates combined from the 40 filtrates collected (five filtrates from each pond for four consecutive weeks beginning June 24). The lab retested one weak positive sample three times to confirm the result and reported another positive result, but also two negative results. This prompted a discussion of what might happen if additional samples were re-tested. Indeed, seven additional samples that had previously been reported as negative were classified as positive after additional PCR reactions were run. A few samples were tested as many as 13 times with various techniques including gene releaser. However, none of the positive samples yielded a positive reaction every time.

These events precipitated an analysis of the data using the occupancy framework (MacKenzie et al. 2002), which accommodates imperfect detection of the organism being sought, clearly the case in these samples. Modeling the methods used indicated that the use of the regular PCR preparation was the most effective. Those samples treated with gene releaser actually gave a lower probability of detection, possibly as a result of dilution of the product by the gene releaser itself. Although the PCR test is very sensitive and not generally regarded as one which results in false negatives, in this case a high number of false negatives were encountered, suggesting that there are very few copies of the target DNA in the filtrates.

Further analysis of the data using the regular PCR reaction and incorporating four separate tests on each sample, showed that the probability of detecting *Bd* in a filter (given the

Table 1. Breeding site monitoring conducted from 2003 – 2010. Sample size denoted by 'n'; prevalence (Prev) is the proportion of samples that returned a positive PCR test result for *Bd*. Only sites that were monitored in at least one year are included.

Year	2003		2004		2005		2006		2007		2008		2009		2010	
Site	n	Prev	n	Prev	n	Prev	n	Prev	n	Prev	n	Prev	n	Prev	n	Prev
CC02									17	0.53	23	0.58	5	0.60	3	
CC04					20	0.25	5	1.00			1	1.00				
CC06													6	0.00	6	0.00
CC07							4	0.00								
CF01			8	0.00	20	0.00	20	0.00	20	0.00	20	0.00	20	0.00	20	0.00
CF02					20	0.00	20	0.00	21	0.00	20	0.00	20	0.00	20	0.00
CF03			10	0.00	20	0.00	20	0.00	20	0.00	20	0.00	15	0.00	17	0.00
CF04			11	0.00	15	0.00	20	0.00	20	0.00	20	0.00	20	0.00	20	0.00
CF05					3	0.00	1	0.00	5	0.00	5	0.00	3	0.00	1	0.00
CF06			2	0.00	5	0.00	10	0.00	2	0.00	13	0.00				
CF07			6	0.00	11	0.00	7	0.00	20	0.00	10	0.00	15	0.00	18	0.00
CF08			9	0.00	20	0.00	20	0.00	20	0.00	21	0.00	18	0.00	20	0.00
CF09			6	0.00	20	0.00	20	0.00	7	0.00	20	0.00	9	0.00	20	0.00
CF10	12	0.00			11	0.00	14	0.00	18	0.00	15	0.00	16	0.00	11	0.00
CF12			4	0.00	16	0.00	20	0.00	21	0.00	20	0.00	20	0.00	20	0.00
CF13			4	0.00	4	0.00	6	0.00	12	0.00						
CF15			1	0.00	4	0.00	3	0.00								
CF16							4	0.00	13	0.00	20	0.00	4	0.00		
CF17											17	0.00	20	0.00	20	0.00
CF18											2	0.00	5	0.00		
CF19											1	0.00				
EA01	2	0.00														
EA02			3	0.00	20	0.00	14	0.00	19	0.00	19	0.00	4	0.00		
EA03			8	0.00	9	0.00	6	0.00	11	0.00	14	0.00	6	0.00		
EA04							13	0.00	4	0.00	6	0.00	7	0.00	4	0.00
EA05															10	0.00
GR02	7	1.00 [a]							9	0.67						
GR04													1	0.00		
GR05							3	0.67					1	0.00	2	0.50
GR07													3	0.00		
GR08															1	0.00
GU01					4	0.00	18	0.00	20	0.00	21	0.00	20	0.00	20	0.00
GU02													1	0.00	1	0.00
GU03					2	0.00	1	0.00	7	0.00	13	0.00	19	0.00	20	0.00

Year	2003		2004		2005		2006		2007		2008		2009		2010	
Site	n	Prev	n	Prev	n	Prev	n	Prev	n	Prev	n	Prev	n	Prev	n	Prev
GU04	8	0.00			22	0.00	20	0.80	15	0.93	2	0.50				
GU05					5	0.00	16	0.00	16	0.00	20	0.05	21	0.86		
GU06									4	0.75	3	0.00				
HI01							7	0.00			5	0.40				
JA01									1[b]	1.00						
JA02													2	1.00		
JA03			16	0.13	10	0.70									9	0.66
LA01													2	0.00	2	0.00
LR01					2	1.00										
LR03	12	0.00														
LR06			1	0.00			11	0.00	12	0.08[c]	20	0.00	20	0.00	20	0.00
LR07							10	0.00			20	0.00	20	0.00	20	0.00
LR08					4	0.00	8	0.00	30	0.03[c]	15	0.00	9	0.00		
ME01									20	0.20	18	0.67	30	0.30	17	0.41
MI01											12	0.83				
PA01			2	0.00			1	0.00								
PA02							1	0.00					2	0.00	3	0.00
PI02	4	0.00	3	0.00	8	0.00	20	0.00	11	0.00	20	0.00	24	0.00	20	0.00
PI03							1	0.00	3	0.00	2	0.00	5	0.00	17	0.58
PI05					2	0.00			14	0.21	2	1.00	2	0.00	3	0.33
PI06							4	0.00	4	0.00						
PI07															4	0.00
PI08															6	0.00
RO04	5	1.00			25	0.40			10	0.90	20	0.70				
RO05	12	0.00	8	0.00	20	0.00			20	0.00	20	0.00			21	0.00
RO06	25	0.00	6	0.17	21	0.86									2	0.00
SU03	2	1.00														
SU04	3	0.00	4	0.00	6	0.00			1	0.00	2	0.00				
SU05					2	0.00					3	0.00				
SU06													2	0.00		

a: The site test positive in 2003 or before, but n and prevalence are unknown.

b: The animal swabbed at this site was a chorus frog *Pseudacris triseriata.*

c: The one animal that tested positive was likely a spurious result, as evidenced by the lack of spread of the pathogen over the succeeding two years.

presence of *Bd*) in any single test was just 0.497. Three tests would be needed on each filter to achieve a probability of detecting *Bd* in a positive filter greater than 0.85 (Figure 2).

The second evaluation of the filtration technique in 2009 included models that allowed the comparison of the amounts of lysis added to the filter. A lysis volume effect was evident in three of the top four models (as ranked by AICc, Burnham and Anderson 2002). Parameter estimates from the top model, allowing only lysis volume to affect probability of detection, showed that the usual 0.9 mL of lysis provided better probability of detection ($p = 0.251$) than the reduced 0.3 mL ($p = 0.079$, Figure 3). At first glance this seems counterintuitive since the hypothesis was that a lower lysis volume would equate to a higher concentration of target DNA copies in the filter. However, perhaps the lower lysis volume was insufficient to properly lyse and preserve the DNA present in the filter. Sadly, the probability of detection using the standard analysis that equated to the 2008 analysis was much lower than in 2008 (Figure 3), a result for which I have no ready explanation.

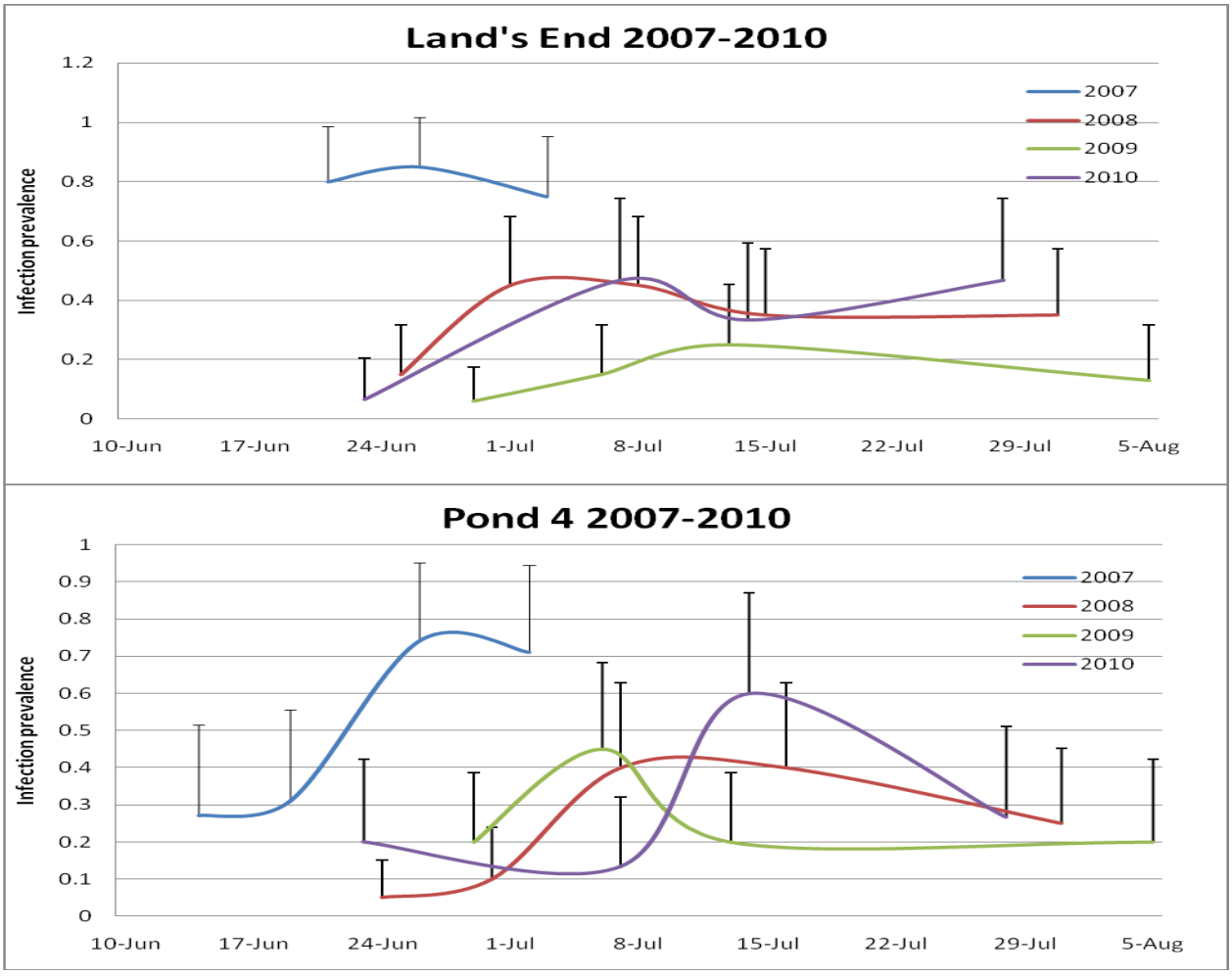

Figure 1. Estimates of infection prevalence among chorus frogs at Land's End pond (top panel, a closed basin) and Pond 4 (bottom panel, a flow-through pond) on Grand Mesa from 2007 through 2010. The displayed point estimates and upper 95% confidence limits are based on the binomial proportion method. Lines are "smoothed".

Once the probability of detecting *Bd* in a filter has been determined, it is of course of paramount interest to look at the probability of detecting *Bd* in a natural water. Therefore we turn attention from examining a filter through a series of PCR reactions to examining a pond through repeated filtrates. I did this by collapsing the data sets to look at the overall result on a given filter. If the filter tested positive at least once in the four reactions, it was considered a site

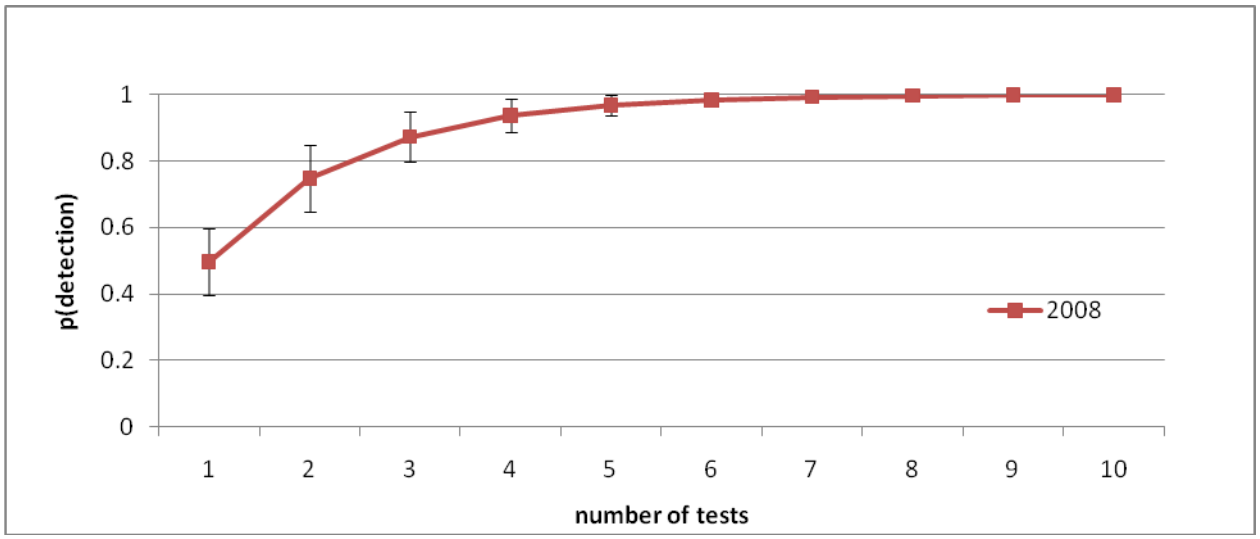

Figure 2. The probability of detection of *Bd* in a single test of a water filtrate was only ~0.5 based on the best supported model from the 2008 data. Additional tests of the same filtrate increase the probability of detection as depicted here. Error bars are ± 1SE.

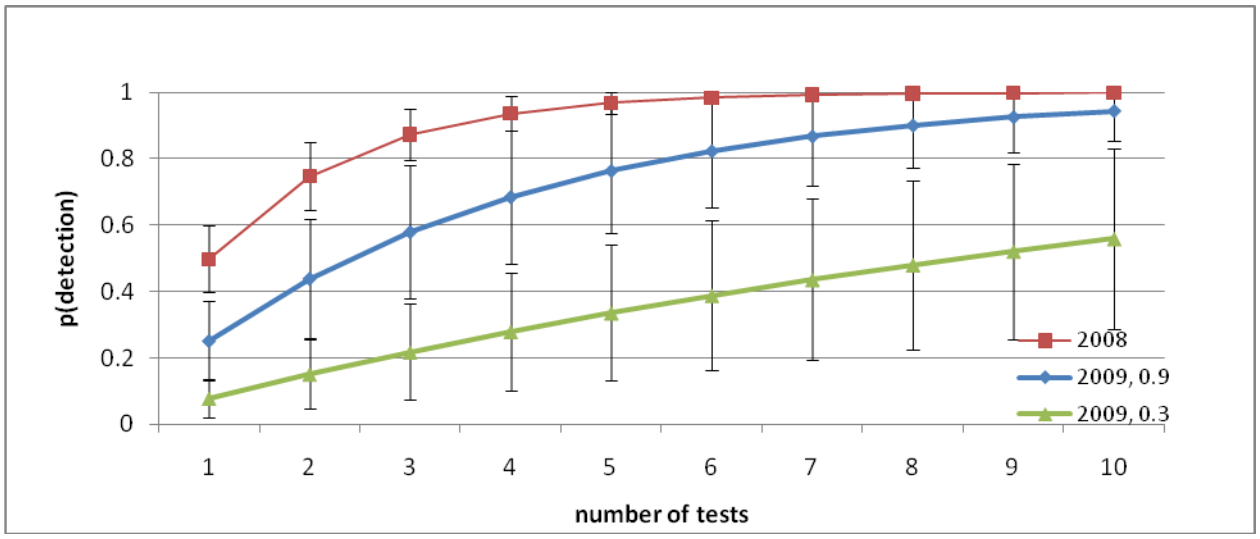

Figure 3. The probability of detection of *Bd* in a single test of a water filtrate in 2009, using 0.9 mL lysis buffer, was only ~0.25 based on the best supported model. Additional tests of the same filtrate increase the probability of detection as depicted here. For comparison, the 2008 values and the 2009 values for reduced (0.3 mL) lysis are shown. Error bars are ± 1SE.

visit yielding a valid detection of our organism of interest. Once again, results varied between the years. In 2008 the probability of detecting *Bd* in a pond through a single filter, given that *Bd* was present in the pond, was 0.36. In 2009 the probability was 0.25. Additional filters, as with additional tests of a filter, increase the probability of detection on a given occasion (Figure 4).

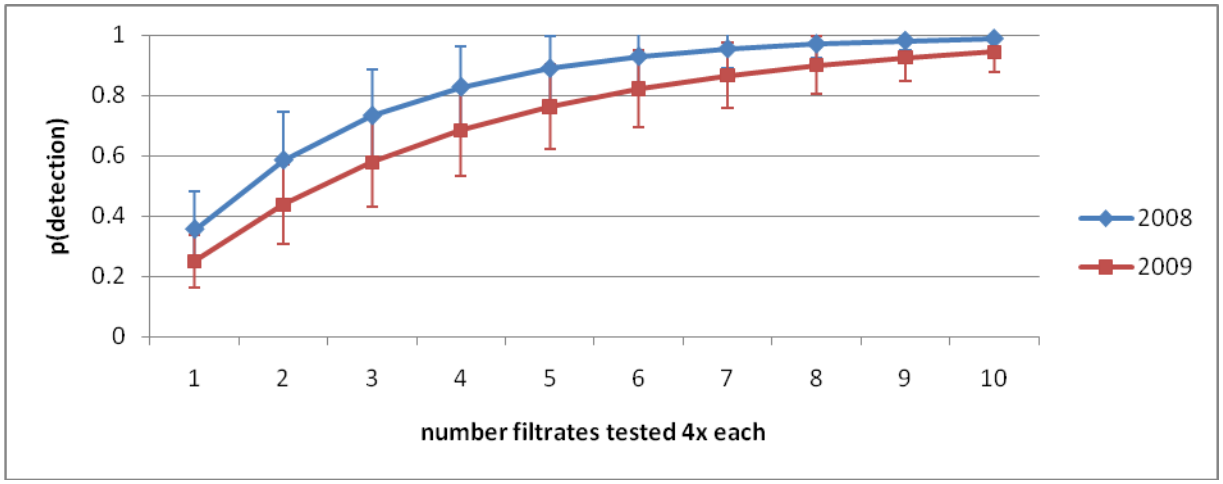

Figure 4. The probability of detection of *Bd* in a pond rises as additional filtrates are examined. This figure is based on quadruplicate PCR reactions on every filtrate collected. Error bars are ± 1SE.

These results were applied in a practical way in 2010 when the proposed translocation site in Gunnison County was monitored via water filtration and the two sentinel toads equipped with radio transmitters. Four filtrates were collected on three different occasions from each of four different ponds distributed throughout the proposed translocation site. Using the more conservative 2009 modeling estimates (p = 0.25 of detecting *Bd* in a single filter), the probability of detecting *Bd* in a single pond over the course of the three occasions would be calculated as $p = 1-(1-0.25)^3 = 0.578$. Considering the site as a whole, the probability of detecting *Bd* at the site over the course of the summer would be calculated as $p = 1-(1-0.25)^{12} = 0.968$.

Therefore, despite the fact that the probability of detecting *Bd* in a single filter is far less than 1, even though tested four times, enough tests were conducted to allow the conclusion with a high degree of certainty that the site does not presently harbor *Bd*. This conclusion was also supported by the sentinel toad swabs. Although one of the toads slipped out of the radio belt, we obtained 11 swabs from the toads between July 1 and August 19. All of them returned negative test results via the PCR reaction.

Conclusions and Recommendations

Water filtration can be used to reasonably ascertain the *Bd* status of a proposed translocation site. However, to achieve the level of certainty required is quite expensive since even the low detection probabilities observed in 2009 required four separate PCR reactions on a

given filtrate, and a number of filtrates must be tested as well. The cost to test the 12 samples collected in 2010 was about $485.00, in addition to the supplies required to collect the samples.

Given that there remains some uncomfortable uncertainty about the true probability of detection in water filtrates due to the differing results in 2008 and 2009, a viable alternative to water filtration is the use of sentinel toads with radios. Boreal toads are known to be susceptible to *Bd* infection, so if it is present at a site sentinel animals should acquire it within weeks of release. The CDOW presently has an ample supply of older radios that can be refurbished for about $60.00 each, and over the years there will likely always be a few toads at the NASRF that are expendable due to age or other factors. While this strategy is also expensive (the cost to test the 11 swabs in 2010 was about $320.00, and weekly technician trips were involved), it more reliable than water filtration. It should be noted that either refurbishing radios or ordering new ones requires considerable lead time. Radios needed by early June must be ordered by early January. Sentinel animals will provide the best information possible if they are released late in the breeding season at a given site. The probability of them encountering *Bd* diminishes as the summer season progresses.

If water filtration is used, it would be wise to include some measure of sentinel toad effort as well if suitable toads are available for release.

Photographic identification of boreal toads *Bufo boreas* (*Anaxyrus boreas*) and development of a computer program for identifying toads based on photos

Introduction

Photographic identification of individual animals has been demonstrated in a number of vertebrate animals, e.g. gray seals *Halichoerus grypus* (Karlsson et al. 2005), African penguins *Spheniscus demersus* (Burghardt et al. 2004), whale sharks *Rhincodon typus* (Arzoumanian et al. 2005), red-spotted newts *Notophthalmus viridescens viridescens* (Davis and Grayson 2007), marbled salamanders *Ambystoma opacum* (Gamble et al. 2007), and yellow-bellied toads *Bombina variegata* (Barundun and Reyer 1998). Boreal toads exhibit unique blotch patterns in the ventral region, making this species a candidate for individual identification through pattern recognition techniques as well.

Inserting passive integrated transponder (PIT) tags in wild toads in Colorado has proven effective in allowing researchers to individually identify adult animals (Muths 2003, Scherer et al. 2005, Muths et al. 2010). However, only animals exceeding a minimum weight are deemed suitable for such tags, and an extensive tagging trial among the CDOW's captive broodstock demonstrated that PIT tags were very poorly retained among those captive animals (Kevin Rogers, Colorado Division of Wildlife, personal communication). If photographs of boreal toads are reliable in allowing subsequent identification of individual animals it would be possible to potentially identify younger animals as well as the hatchery brood animals. The identification of younger animals in field studies opens possibilities for the study of additional population or life history parameters.

The early development of the belly pattern among boreal toads and whether the pattern is faithful and distinguishable as the animal grows were significant questions to be addressed. I also wished to determine whether a computer algorithm could be applied or developed to assist in matching photographs. Therefore the goals of this study were to develop methods to use photographs for record-keeping among the CDOW captive brood stock, determine whether patterns were stable throughout life, and explore the use of computer-aided identification.

Methods

All broodstock toads housed at NASRF were photographed in 2007 using a digital camera. As background, a small white board was used. Rulers were affixed to the whiteboard so that approximate snout-vent lengths of the photographed animals can be obtained. Each animal's identification number was written on the white board, and the first photograph of each animal was taken from a distance that revealed both the belly pattern and the identification number. This insured that the two items of interest were positively tied together. A second photograph of each toad was a close-up of the belly to allow greater resolution of the belly pattern.

All toads at NASRF belong to uniquely identified lots. These lots usually contain sibling animals but in a very few instances contain toads capture in the wild as adults and so have unknown relationships. Each animal was assigned a unique identification number by appending an individual number to the lot number, thus ensuring consistency over time and precluding the possibility of accidental duplicate individual identification numbers. The best photograph of

each toad was placed on a Powerpoint slide with up to five others, and identification numbers were added to each thumbnail photo. These pages of thumbnails were printed, laminated, and sent to NASRF for use with the captive population there.

Questions regarding the stability of the belly pattern, especially among young toads, were addressed during the latter half of 2007 and extending through July 2008. Forty 2007 year-class toads were transferred to Cheyenne Mountain Zoo in September 2007. They were individually photographed and assigned identification numbers in September. Thereafter, each toad was measured (snout-vent length), weighed, and re-photographed in October, November, December, January, February, April, and July. On each occasion, zoo personnel visually matched the photos against the original set obtained in September 2007. Later, three CDOW technicians matched the initial September photographs against varying sets of later photographs.

To develop software to compare and match photographs of toads, we collaborated with Carlos Anderson, PhD student at Michigan State University. Mr. Anderson was supplied with toad photographs to develop an algorithm, and later supplied with photographs of different toads to test the algorithm.

We have also supplied paired photographs to David Pilliod (USGS, Boise, Idaho) to use with the software Identifrog, an alternative digital identification program also under continuing development.

Results and Discussion

In 2007 we demonstrated that photographs of belly patterns were useful at NASRF for identifying individual toads. With the laminated photos of a single lot spread out on a counter or taped to a cabinet, and numbered tubs lined up on the counter, a culturist can usually separate a lot of 12-15 toads into their individual containers in 3-4 minutes. With a very few lots that exhibit fairly non-descript belly patterns it takes a bit longer, but to date we have never failed to be able to distinguish the toads from one another. This result gave assurance that accurate records of parentage can be maintained for future captive-bred brood animals at NASRF despite the dismal PIT tag retention displayed in our captive brood population. Therefore, each spring the toads that will remain at NASRF from the previous year class are now photographed and assigned identification numbers, and the laminated copies produced for use at the hatchery. It is a system that has continued to work well in the hatchery context.

The determination of the stability of belly pattern over the first year of life was also successful. Average toad size upon the first photographs in September was 33 mm and 3.5 gm; and average toad size at the end of the trial the following July was 53 mm and 10.9 gm. Cheyenne Mountain Zoo personnel who conducted the photography sessions were universally successful in matching toad photographs from differing occasions. Three different CDOW technicians matched a total of 5 sets with the original set. Only one toad was mis-identified, and that was a case of incorrect recording of results rather than a true misidentification because the two toads in question were markedly different. In some cases similar toads were not successfully discriminated until all the more obvious matches were constructed. These results suggest that

belly patterns will remain stable as the toads grow, and generally they become more distinct as the pigmented spots fill in (Figure 5).

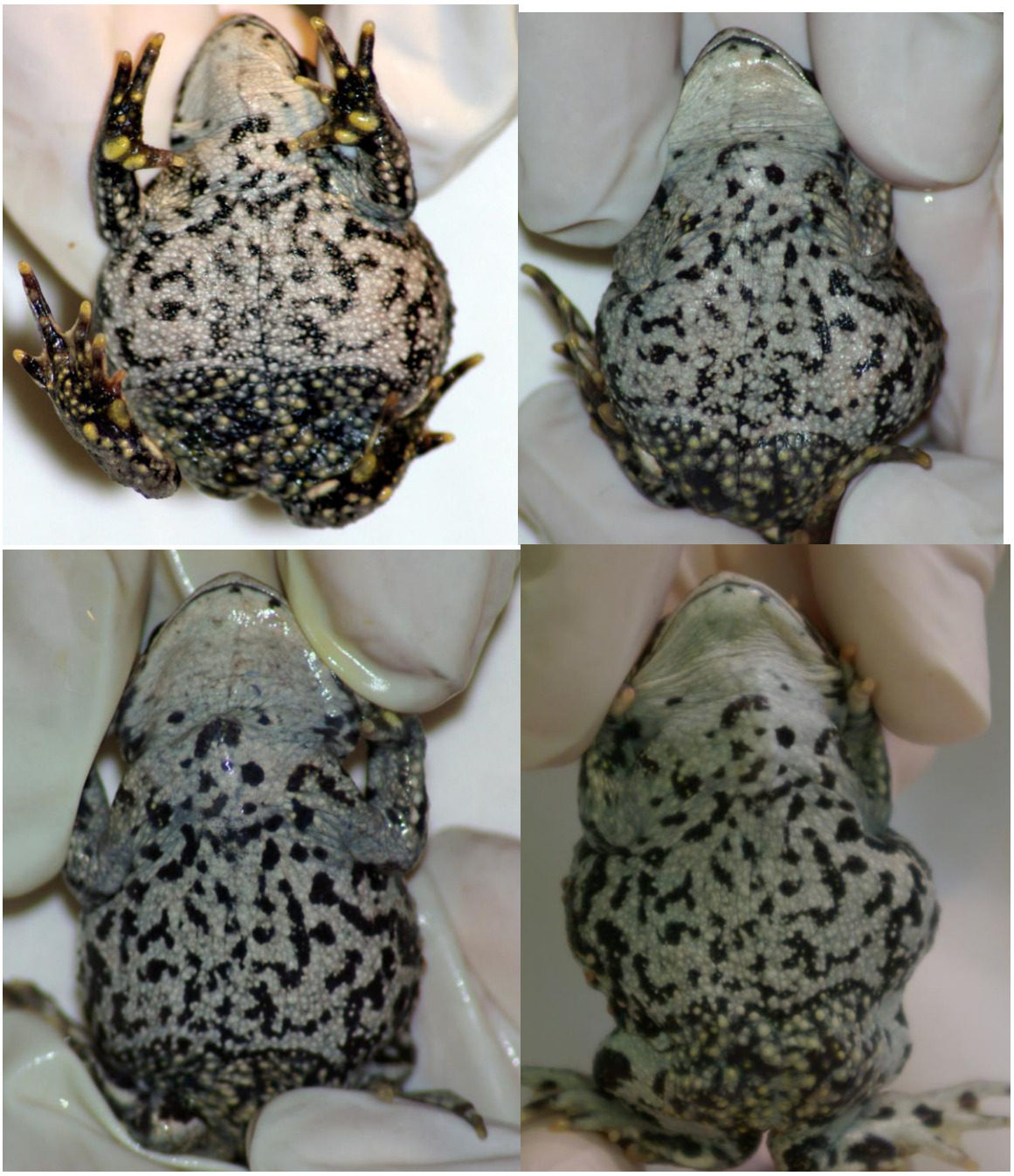

Figure 5. Four photos taken of the same toad at Cheyenne Mountain Zoo over 10 months. Clockwise from top left: September 2007, February 2008, April 2008, July 2008.

Conclusions and Recommendations

Although the software was demonstrated to be quite accurate using hatchery toad photographs, it consistently crashed on some state computers, and it also required extensive computer time to run the matching algorithm with so many toad photos in the NASRF database. A further complication results from the program being written on a 32-bit platform whereas those older computers are being replaced with 64-bit machines. Consequently the program currently has limited utility.

However, the accuracy of photographs for individual identification was clearly demonstrated, and the Native Species Hatchery continues to use such photos with excellent results. It is recommended that hatchery staff continue to use individual identifications with supporting photos to track animals and breeding history at NASRF. Each brood lot kept should be photographed as early as it is reasonably possible to obtain individual identifications of the animals in the event of inadvertent lot mixing.

Hatchery production and breeding research

Introduction

Reproduction in captive amphibians is notoriously difficult and often unsuccessful (Browne and Zippel 2008). However, the plight of amphibians around the world has generated renewed interest in research in this arena apart from commonly cultured animals such as *Xenopus spp*. Recognizing that consistent breeding success with the captive broodstock at NASRF is critical to the stabilization of the status of the boreal toad in Colorado, Wyoming and New Mexico, we invited collaboration in 2008 with Dr. Andy Kouba of Memphis Zoo. Dr. Kouba has worked extensively with Wyoming toad *A. baxteri* and has conducted research on hormonal stimulation of breeding in anurans. He initially visited NASRF in July 2007 and was asked to return in spring 2008 to train NASRF personnel in intra-peritoneal injection technique as well as the artificial collection of boreal toad sperm. Largely following his recommendations for hormone volume and timing, 2008 proved to be a successful year, with NASRF producing tadpoles for both New Mexico and Rocky Mountain National Park.

In late fall 2008, the trailer formerly serving as an isolation unit at the Fish Research Hatchery in Bellvue was moved to NASRF. It was retrofitted for use as a breeding research facility and will allow CDOW to conduct rigorous experimentation on boreal toad breeding apart from production responsibilities at NASRF. It now contains six toad tanks for holding groups of toads together, and 30 aquaria that can be used to hold individual pairs in amplexus. Holding toad pairs in separate aquaria will allow estimation of mean number of eggs per clutch for each treatment, as well as hatching success.

This new facility will allow CDOW to make good use of the many adult toads living at NASRF as well as refine husbandry and hormone practices to further increase the success and productivity of the captive breeding program. Among the questions we hope to address with breeding experiments are the effect of differing lengths of hibernation, differing hormone treatment regimes, cooler and more fluctuating water temperatures during breeding, and possibly the effects of diet.

The first experiment executed in the facility was designed to answer whether a shorter hibernation period improved egg quantity or quality. If a less-than-ideal captive diet is compromising breeding success and egg quality, the shorter hibernation season may result in more, better eggs, since the adults will be consuming less bodily resources through the shorter hibernation.

Methods - Experiment 1

Six lots of brood toads were randomly selected (with minimum lot size and adequate age as constraints) in fall 2008 and separated into two groups of three lots. Within each lot, individual toads were randomly assigned to regular or short hibernation length. Regular

hibernation began in mid-December 2008 and short hibernation at the beginning of March 2009. None of the females had been used in breeding attempts in 2008. Some had never been induced to breed, although all toads used in these trials belonged to year classes 2000, 2001, and 2002.

The groups were removed from hibernation in successive weeks during late April. Males and females were placed in tanks according to hibernation length and interbreeding suitability (non-sibling males and females) the day following removal from hibernation chambers. The following day we commenced hormone injections to stimulate breeding activity. All hormonal injections were intra-peritoneal; the females were injected in the morning and the males in the late afternoon or early evening. Usually about 9 hours elapsed between the injections. All toads received Luteinizing Hormone Releasing Hormone analogue (LHRHa) dosing of 0.2 µg/gm body weight. Females received human Chorionic Gonadotrophin (hCG) dosing of 10 IU/gm body weight and males received 6 IU/gm body weight. These doses were based upon recommendations by Dr. Andy Kouba of the Memphis Zoo to the Wyoming toad recovery program.

No effort was made to get specific pairs together, but as males grasped females the pairs were removed from the common tank to a randomly pre-selected aquarium in the research trailer. This was done in order to track clutch success. The aquaria were adjusted to hold 2-3 cm of water, with the entire bottom of the aquarium covered.

Success during the first week of trials was disappointing, so during the second week slight adjustments were made to the hormone injection regime. The females received a hormone cocktail in the morning, as in the first week, but also received an injection of LHRHa only in the evening after the males had received the cocktail. In addition, some of the toads from the first week of trials that appeared gravid were paired with males and given additional injections during the second week.

Water temperatures were monitored in representative tanks and aquaria, and a barometric pressure logger was also deployed in the research trailer. Ice was added to the tanks periodically to simulate diel temperature fluctuations that might be experienced in nature, in part because this method was tried *ad hoc* by hatchery staff in 2008, a successful year.

Clutches that were laid were photographed in detail so that eggs could be enumerated more conveniently. Later, tadpoles were physically counted so that percent hatch could be determined.

Results and Discussion - Experiment 1

Water temperatures during the experiment (Figure 1) were within the range observed during peak breeding periods in the wild (Figure 2). However, temperatures experienced at NASRF tended toward the high end of the temperature range when peak breeding activity in the wild has been observed. Adding ice periodically to cool the temperatures had little lasting effect

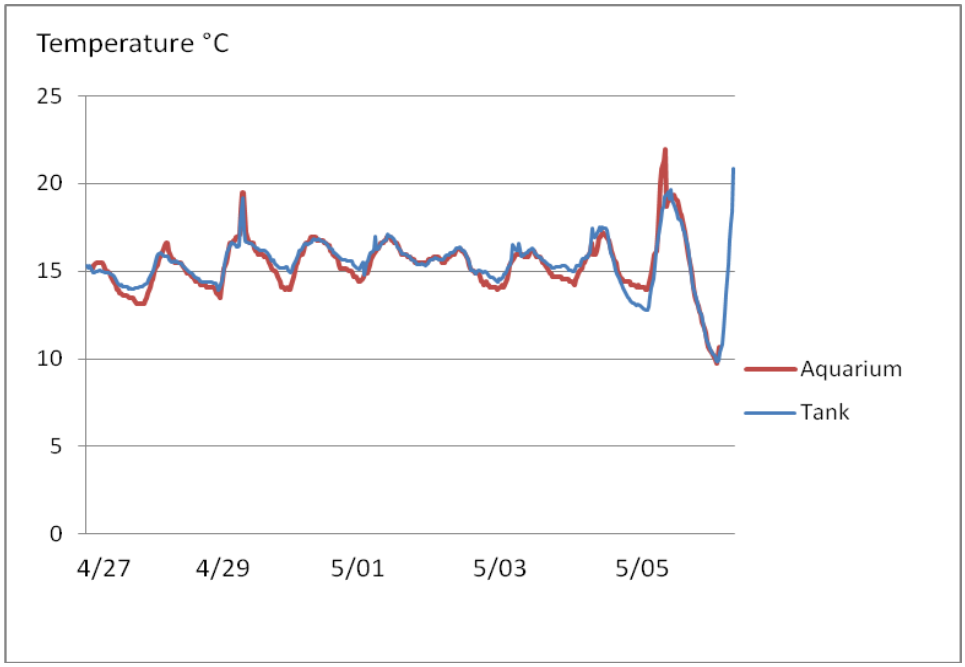

Figure 1. Temperature (°C) monitored twice hourly in a representative holding tank and a representative breeding aquarium during week two of the 2009 breeding experiment.

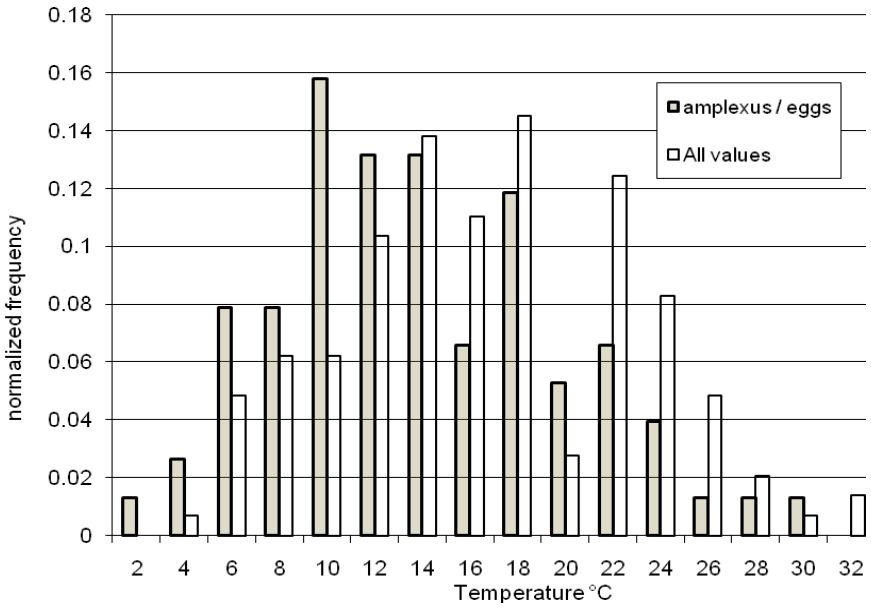

Figure 2. Relative frequency (normalized) of breeding behavior at known and regularly monitored boreal toad breeding sites during May and June of several years. The “amplexus/eggs” series shows spot temperatures taken on survey occasions when survey personnel observed toads in amplexus or recently laid clutches. The series “All values” shows all spot temperatures taken on May / June breeding site visits.

on water temperature in the tanks. Whether adding ice, and the resultant temperature fluctuations, affected toad behavior is difficult to assess because no controls were used. It did not induce breeding behavior to any significant degree, despite anecdotal observations by hatchery staff that such ice additions the previous year had appeared to have that effect.

The response of toads during both weeks of trials was disappointing. The additional hormone injection administered to females during the second week had little or no effect, so responses were pooled over the two weeks for each hibernation length. More females engaged in amplexus from the March hibernation group than from the December group (50% vs. 26%), but five December females laid eggs whereas just three March females laid eggs. Some of the females that laid eggs did so without benefit of amplexus.

The mean number of eggs produced from December-hibernated females was 1387 (SE 371) and from March-hibernated females 870 (SE 573). Just 9.4% (SE 5.1) of "December" eggs hatched and 0.32% (SE 0.323) of "March" eggs. The best clutch only hatched 27.85%. Such results clearly indicate that further work must be done to elucidate what factors are involved with successful breeding in the captive population.

These results are particularly puzzling, since the hormone injections and the December hibernation length matched the parameters of the 2008 production breeding at NASRF, which was a very successful event. Hibernation entry, hibernation length and conditions, treatment upon exit from hibernation, newly acquired hormone from the same provider as in 2008, and treatment during the breeding trials were all designed to replicate the successful 2008 season, leaving the length of hibernation as the only variable to examine.

The initiation of amplexus among pairs and the commencement of egg deposition were plotted on the barometric pressure monitor results, as nearly as they could be determined from periodic visits to the holding facilities. No apparent relationship between these events and the prevailing barometric pressure emerged with the limited data set achieved during this experiment. That is, there was no tendency observed for toads to lay eggs disproportionately during either rising or falling barometer conditions (Figure 3).

One factor that likely plays a role in varying hatchery success is the frequency with which females are prompted to breed. In nature, it is clear that boreal toad females do not breed every year. While Carey (1976) suggested females may breed every other year, CDOW investigators have found that females may wait two or more years to return to breeding sites (Mark Jones, Colorado Division of Wildlife, personal communication. More recently, Muths et al. (2010) used capture-recapture models to show that females are obligate non-breeders the year after a breeding year, and that there is just a 36% chance that such a female will breed the year following the obligate non-breeding year.

In contrast, until 2009 little consideration was given to this factor at NASRF, when it was decided that, ideally, females would only be bred in alternating years. Even so, this philosophy is

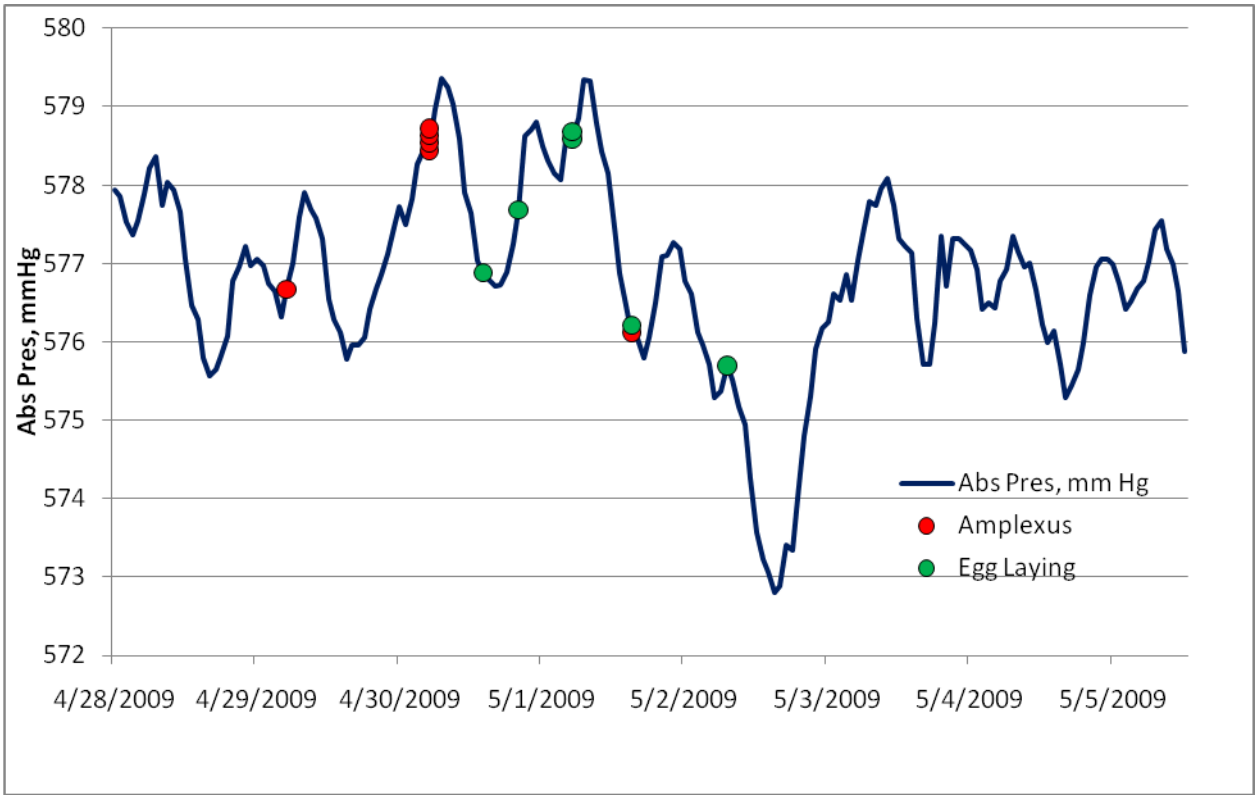

Figure 3. Barometric pressure measured in the research facility (uncorrected) with corresponding observations of amplexing behavior and egg laying, as noted upon periodic visits to the facility, 2009.

subservient to the pressure to produce eggs for ongoing translocation projects and if the selected females aren't successful it is common to add to the breeding effort females that were supposed to be non-breeders. This raises concerns about the quality of the product as well as potential harmful effects on the female. Although we have not produced evidence of harm or decreased egg quality, the mixed success at the hatchery prompted a change to attempt breeding only half the females from a given brood stock during any year. (NASRF staff empirically note they seem to have a good breeding year "every other year").

Recommendations for future experimental work include the execution of a formal dose-response hormone treatment using suitable toads (not having bred the previous year). The proposed experiment does carry some risk, as building a dose-response curve will require hormone doses much higher than currently used. Even so, at this point there are animals at NASRF deemed expendable and the time is right to try. As it has been the last few years, if any changes are made they are made in small increments based on the lack of mortality and lack of breeding response in previous years. Perhaps there is a higher hormone dose level that will both be safe and more likely to induce egg-laying behavior.

Additionally, there may be benefit in exploring lower level "priming doses" of hormones, either in advance of actual breeding by a week or two, or even prior to placing the toads in hibernation. Discussions have also occurred about possibly feeding the toads for several days to a week after pulling from hibernation and before breeding. Roth et al. (2010) had the best success with females that were not hibernated at all, but the non-hibernation is confounded with increase weight. Would heavier females that were also hibernated perform even better?

Table 2. Numbers of hormone-injected females engaging in amplexus and measures of clutch success from breeding experiment 1, investigating the effects of hibernation length.

Hibernation	Amplexus	Clutches	Egg mean	Tadpole mean	% Hatch
December	5 / 19	5[a]	1387	176	9.41
March	8 / 19	3	870	2	0.32

a: Two of these clutches were produced with no amplexus observed.

Methods - Experiment 2

Experiment 2 occurred in spring 2010 and was designed to examine response of female toads to various doses of hormone. Prior to the 2010 breeding experiment we conducted an exercise to reduce the size of brood lots housed at NASRF in order to create space for additional brood lot diversity. Consequently animals that were removed from the brood stock (about 45 females and 70 males) were available for breeding experimentation. Individuals were randomly selected from this group of animals for inclusion in the experimental population. Toads were then randomly assigned to groups to be bred immediately upon withdrawal from hibernation (within 2 days, typical of previous operations at NASRF) or fed *ad libitum* with crickets and worms for one week prior to breeding. Within those two groups, female toads were randomly assigned one of three levels of Luteinizing Hormone Releasing Hormone analogue (LHRHa) hormone treatment, resulting in eight female toads per treatment. Corresponding males (n = 8 or 10 per treatment) were randomly assigned also, but all males received the same hormone dose. Three of the 48 female toads used in this experiment were part of the 2009 experiment; the others had not been used recently in breeding attempts.

The two groups were removed from hibernation in late April. Males and females from the group to be bred immediately were placed in tanks according to hormone treatment regimen and interbreeding suitability (non-sibling males and females) the day following removal from hibernation chambers. The following day we commenced hormone injections to stimulate breeding activity. Hormone injections were given using the same methods and timing as in 2009. Female toads received LHRH doses of 0.2, 0.8, or 1.6 µg/gm body weight. All males received LHRHa at 0.2 µg/gm body weight. Females received human Chorionic Gonadotrophin (hCG)

doses of 10 IU/gm body weight and males received 6 IU/gm body weight. The same treatments were repeated during the second week of trials using the animals that had been fed.

No effort was made to get specific pairs together, but as males grasped females the pairs were removed from the common tank to a randomly pre-selected aquarium in the research trailer. This was done in order to track clutch success. The aquaria were adjusted to hold 2-3 cm of water, with the entire bottom of the aquarium covered. Amplexing pairs were left in aquaria until eggs were laid, or pairs separated and remained separated for more than a few hours. Some pairs remained in amplexus without laying eggs for a full week; these were separated at the end of the trial and placed back with their respective groups.

I used PROC GENMOD (SAS Institute) to analyze the response of female toads in amplexing behavior and clutch production using models that incorporated feeding regimen, hormone dose, and female weight. I use PROC GLM (SAS Institute) to examine similar models using the number of eggs produced as the response variable.

As in 2009, water temperatures were monitored in representative tanks and aquaria, and a barometric pressure logger was also deployed in the research trailer. However, no attempts were made in 2010 to manipulate water temperature by adding ice.

Results and Discussion - Experiment 2

Once again, water temperatures during the experiment (Figure 4) tended toward the high end of the temperature range when peak breeding activity in natural breeding populations has been observed (Figure 2). Some extreme temperature data points displayed in Figure 4 are the result of the temperature sensor being jostled by toad behavior and thus reading air temperature rather than water temperature.

Toad response varied among the treatments, but was again disappointing overall with regard to egg production. This metric is obviously the one that really matters in view of the need to produce animals for translocation or repatriation in the wild. Unfortunately, only five of the 48 females involved in this experiment laid eggs (Table 3). As in 2009, no pattern was revealed when examining the barometric pressure at the time amplexus or egg laying was first noted for pairs of toads (Figure 5). Although this evaluation is somewhat subjective since investigators intentionally refrained from entering the trailer too often (in order to not disturb breeding behavior unnecessarily), the periods of rising and falling barometric pressure were generally long enough in duration that it was clear there was no correlation. Amplexus was observed in rising, falling, and relatively stable barometric conditions. The dense clusters of amplexus observations occurred in the hours following hormone injections.

The data from this experiment revealed one thing clearly – in every analysis conducted female weight was a significant predictor of success. Heavier females were more likely to engage in amplexus (chi-square = 27.11, df = 1, p = 0.0001) and to lay eggs (chi-square = 10.18,

df = 1, p = 0.0014). Female weight also was also a significant predictor of the number of eggs laid, despite the fact that just five clutches were realized (F = 10.0, df = 1, p = 0.0029). In this

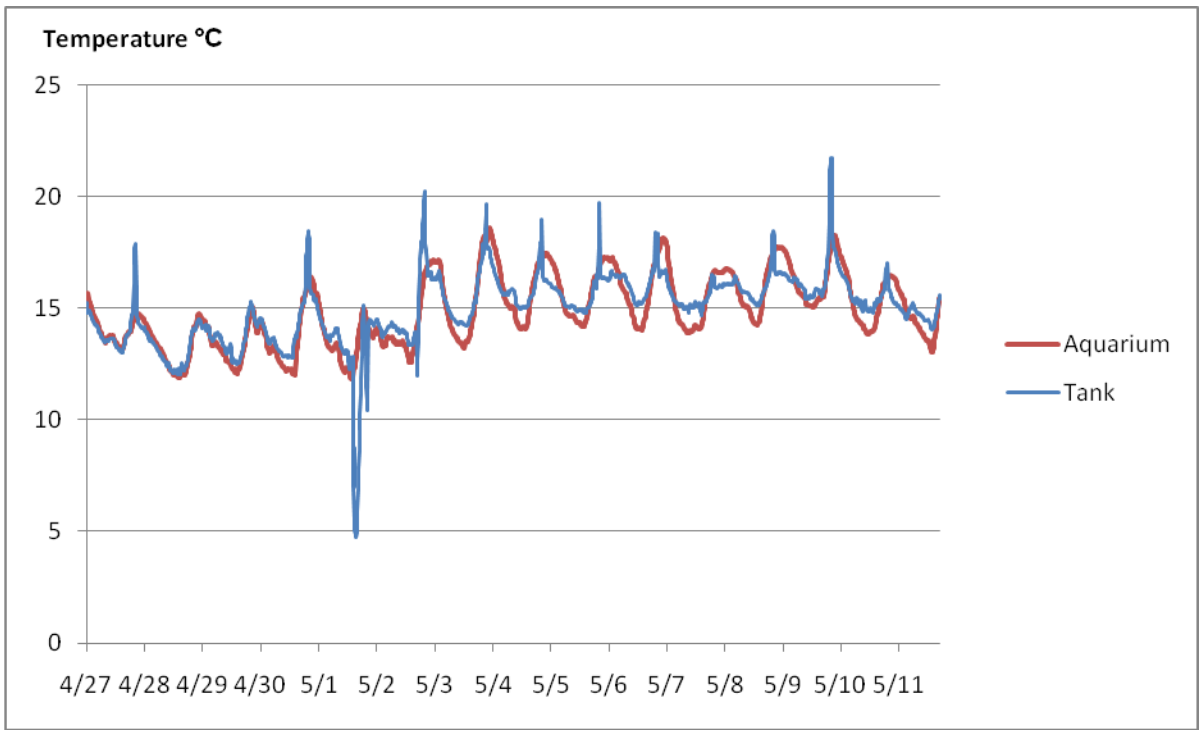

Figure 4. Temperature (°C) monitored four (aquarium) or five (tank) times hourly in a representative holding tank and a representative breeding aquarium throughout the duration of the 2010 breeding experiment.

Table 3. Numbers of hormone-injected females engaging in amplexus and measures of clutch success from breeding experiment 2. There were eight female toads in each treatment.

LHRHa dose	Amplexus	Clutches	Egg mean	Tadpole mean	% Hatch
	Not fed, bred immediately out of hibernation				
0.2 µg/gm	1	1	1361	2	0.15
0.8 µg/gm	3	2	2616	1219	46.6
1.6 µg/gm	7	0	---	---	---
	Fed one week before breeding				
0.2 µg/gm	3	1	980	0	0.00
0.8 µg/gm	6	1	1086	446	41.1
1.6 µg/gm	2	0	---	---	---

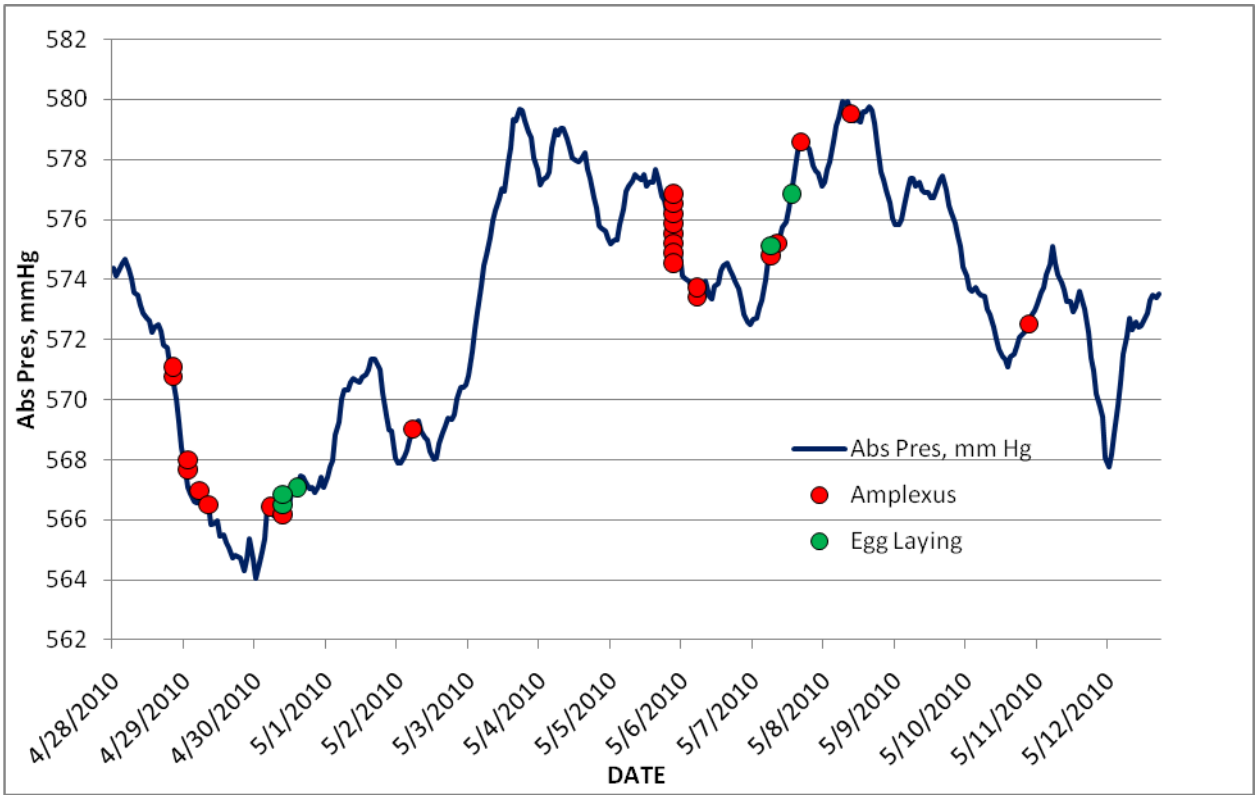

Figure 5. Barometric pressure measured in the research facility (uncorrected) with corresponding observations of amplexing behavior and egg laying, as noted upon periodic visits to the facility, 2010.

experiment, females that laid eggs averaged 44.76 gm (SE 1.828) compared to an average weight of 33.93 gm (SE 1.116) for those that failed to lay eggs.

The issue of female weight in the brood stock held at NASRF appears to be a significant one. Roth et al. (2010) also found that egg-producing females were significantly heavier on average than their non-producing counterparts. Consequently they argued that the best strategy for maximizing egg numbers was to forego hibernating females, but instead attempt to improve their body condition by continued feeding. I note that they started breeding experiments with females as young as age 2, with little success until age 4. They did not breed females for their research older than age 5.

A mature wild female commonly weighs in excess of 70 gm, whereas our successful toads involved in the 2010 experiment averaged less than 45 gm. Moreover, our successful females in 2010 were only very slightly larger than the non-breeding females of Roth et al. (2010), which averaged 43.6 gm. Additionally, there were a number of females involved in the 2010 experiment that weighed in excess of 40 gm (up to 48 gm) and yet did not provide eggs.

Conclusions and Recommendations

These facts suggest that females presently housed at NASRF are undersized for the purposes of egg production. Unfortunately, little can be done about the size of mature toads presently at our facility. As the captive breeding program at NASRF moves forward, it is recommended that every effort be expended to maximize growth of newly acquired broodstock animals, whether produced in captivity or obtained from the wild, over the first few years of life. Although some steps have been taken in that direction in recent years (e.g., not hibernating during the first winter of life and recent changes in feeding programs to include food items other than crickets), it is likely that a significant problem lies in the density of animals kept in a single tank during early life. It is entirely possible that eventual adult size is being partially limited by space considerations. Therefore, I recommend that perhaps no more than nine or ten animals be held for brood from any sibling group, down from the current 15 or more. Although this may create a few situations where less than ideal sex ratios are obtained in the mature toads, it is a risk we ought to take in trade for the larger toad weights that are likely to result.

Aerial surveys for toad habitat

It has been suggested that the ideal toad breeding habitat is one that holds water throughout the summer but dries up in the fall. The advantage for toads in such a situation would be sufficient time for metamorphosis, but a deterrent to tiger salamander *Ambystoma tigrinum*, since salamander larvae are known to be a boreal toad tadpole predator (Mark Jones, CDOW, personal communication).

In 2007 the CDOW purchased Ikonos 4-meter color-infrared satellite imagery covering 85 km^2 of Eagle County near known boreal toad breeding sites. This imagery was captured in late September 2005, and was paired with mid-July National Agriculture Imagery Program (NAIP) 1-meter color aerial photography. The CDOW GIS group processed both images to identify areas that appeared wet in July, then either wet or dry in September. All sites that fit these criteria and were less than 11,500 feet elevation were identified.

During the 2007 field season ground visits were made to as many of the sites as possible. Some were excluded from visitation on the basis of information from Forest Service personnel regarding the suitability of the area for amphibians, and a few were not visited because of remoteness or surrounding topography.

Although several of the sites were determined to be suitable boreal toad habitat after ground visits, no new boreal toad breeding sites were located. Some of the sites that appeared to be good habitat from the aerial images were only marginally suitable – some suffering from lack of water and others from poor basin configuration characteristics such as lack of shallows or solar exposure.

The technique did show some promise for identifying potential areas to visit, but most of these same areas might have been chosen by a biologist using a combination of topographic maps or software and Google Earth views. There might be utility in reviving the project if the CDOW in-house camera equipment reaches a point of being equipped to its full potential. Then CDOW could target certain areas and not be constrained to areas where good satellite images are commercially available.

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