Contaminants Report Number: R6/726C/12

DEPARTMENT OF THE INTERIOR
U.S. FISH AND WILDLIFE SERVICE
REGION 6

Assessment of Atrazine Effects and the pathogenic chytrid fungus (*Batrachochytrium dendrobatidis*) **to the Endangered Wyoming Toad (*Bufo baxteri*) and the Chorus Frog (*Pseudacris triseriata*) at Mortenson National Wildlife Refuge, Wyoming**

By

Kimberly Dickerson

in cooperation with

Dr. Carol Helbing, University of Victoria, Canada
and Dr. Mark Jordan, Indiana University-Purdue University, Indiana

USFWS photo

FFS# 6N58
DEQ Project ID: 200860001.1
June 2013

Abstract

The Wyoming toad (*Anaxyrus baxteri*, formerly *Bufo baxteri*) population in the Laramie Plains of Wyoming declined dramatically in the 1970s and was listed as an endangered species in 1984. The Federal Register Notice Listing Package included habitat loss, pesticide applications, and disease as potential reasons for the decline and as threats to the recovery of the toad. Several investigations were conducted to determine why reintroduction efforts continue to have limited success but none have indicated serious concern to date. However, the pathogenic chytrid fungus (*Batrachochytrium dendrobatidis*, *Bd*), which has decimated amphibian populations worldwide, was found at the primary reintroduction site at Mortenson Lake National Wildlife Refuge (NWR). Additionally, research has indicated that the widely used herbicide atrazine can adversely affect amphibians, including suppressing immune functions and increasing their susceptibility to disease (e.g *Bd*). Therefore, we conducted this study to determine if Wyoming toads and chorus frogs are being exposed to atrazine and if so, are sublethal effects occurring, including their susceptibility to *Bd*.

In the fall of 2004, we collected water samples to establish if atrazine was present at the refuge. In 2004, atrazine metabolites were detected in water at the refuge. In 2006, we deployed Polar Organic Chemical Integrative Samplers (POCIS) to capture any pulses of atrazine that may be carried by the Laramie River to the refuge. Atrazine as the parent product was detected in the Laramie River upstream of the refuge but not at the refuge itself. In 2008, we conducted our sampling during the spring, when atrazine is typically applied to obtain a more effective understanding of how much atrazine may enter the refuge when amphibians are the most sensitive (reproduction, metamorphosis) to the effects of the herbicide. Unlike previous years, there were no detections of atrazine and its metabolites in POCIS extracts. We also sampled algae, the food source of tadpoles, but no atrazine was detected.

To substantiate our results from 2008 and ensure that amphibians were not being exposed to atrazine during the most sensitive life stages, we redeployed POCIS and took water and algae samples from the refuge and a reference site in 2009. In addition, we placed chorus frog and Wyoming toad tadpoles *in-situ* at the refuge and chorus frog tadpoles *in-situ* at a reference site. Wyoming toad tadpoles kept at the breeding laboratory were used as reference animals as they could not be placed at the reference site. No atrazine, atrazine metabolites, or other pesticides and herbicides were detected in POCIS extracts and inorganic compounds in water samples were either low or below detection

In-situ animals were collected once the tadpoles metamorphed. None of the metamorphs showed any observable abnormalities. A portion of each species from the refuge and the reference site was analyzed for the presence of atrazine and its metabolites. None of the metamorphs had any detectable concentrations. Livers were dissected from the other portion of metamorphs of each species and evaluated for gene expression of the thyroid hormone receptor β, estrogen receptor α, vitellogenin (VTG), and cytochrome P450 family 19 (CYP19) The carcasses were sent for sexing and testing for the presence of *Bd*. Gene expression in liver samples showed that Wyoming toad

metamorphs from Mortenson Lake NWR had reduced gene expression when compared to reference metamorphs. There was no difference in gene expression in chorus frog metamorph livers from Mortenson Lake NWR when compared to reference animals.

Screening for Bd on the carcasses indicated expected results. Chorus frogs from Vedauwoo were negative indicating that this area remains free of the infection. Wyoming toads from Red Buttes were also negative, a requirement of the captive breeding facility. The presence of *Bd* in both chorus frogs and Wyoming toad metamorphs at Mortenson Lake NWR was confirmed. The significant reduction in gene expression from Wyoming toad metamorphs at Mortenson Lake is due to any number of stressors, which the presence of *Bd* at Mortenson Lake may be contributing, but it is unlikely that this is from the exposure to atrazine. The Wyoming toad may be more sensitive to the disease than other native toad or frog species due to the lack of recruitment and low genetic diversity in the Wyoming toad population at Mortenson Lake explaining historical declines or because of the low genetic diversity in the current toad population. The chorus frog may be less sensitive, may act as a carrier, or may remain at Mortenson simply because of recolonization from populations at surrounding lakes.

Recovery of the Wyoming toad will need to focus on disease management and, as identified in the draft recovery plan, include developing survival assurance colonies, halting the spread of *Bd*, treating amphibians for *Bd*, and developing strategies to decrease *Bd* pathogenicity and host susceptibility in infected populations. It is probable that this disease management will also benefit the chorus frog and prevent further declines in its population.

Keywords: FFS# 6N58, DEQ Project ID: 200860001.1, congressional district 1, Mortenson Lake National Wildlife Refuge, Wyoming toad, chorus frog, *Bufo baxteri*, *Pseudacris triseriata*, atrazine, gene expression, chytrid fungus, *Bactrachochytrium dendrobatidis*

TABLE OF CONTENTS

Figures

Tables

Appendices

List of Acronyms and Abbreviations

Agencies
USEPA – U.S. Environmental Protection Agency
USFWS – U.S. Fish and Wildlife Service
USFS – U.S. Forest Service

Locations
ACF – Analytical Control Facility
EST - Environmental Sampling Technologies, Inc.
MSCL - Mississippi State Chemical Laboratory
NWR – National Wildlife Refuge

Measurements
g - gram
mL - milliliter
ppb – part per billion
SVL - snout to vent length
µg/L – microgram per liter
ww – wet weight
≥ – equal to or greater than

Chemicals
Al - aluminum
Ba - barium
cDNA – complementary DNA
CYP19 - cytochrome P450 family 19
DNA/RNA - Deoxyribonucleic acid / Ribonucleic acid
Fe - iron
Mg - magnesium
Mn - manganese
mRNA – messenger RNA
OCs – organochlorine compounds
OPs – organophosphate chemicals
PCBs – polychlorinated biphenyls
Se - selenium
Sr – strontium
T_3 – thyroid hormone triiodothyronine
T_4 – thyroid hormone thyroxine
V - vanadium
VTG - vitellogenin
Z - zinc

Miscellaneous terms

HPDE – high density polyethylene
IRED - Interim Reregistration Eligibility Decision
POCIS - Polar Organic Chemical Integrative Samplers
QA/QC – Quality Assurance/Quality Control
SOP – standard operating procedure
TWA – time weighted average

Use of trade names does not imply endorsement by the U.S. Government.

INTRODUCTION

Atrazine, a triazine herbicide, is one of the most commonly applied herbicides in the United States (Solomon et al. 1996). It is used primarily to control annual broadleaf and grass weeds in corn and sorghum crops in the Midwest, but it is also used on landscape vegetation (Solomon *et al*. 1996). Atrazine can adversely affect mammals and fish and is classified as an endocrine disruptor (Tillitt *et al*. 1998), but until recently little information on the effects of atrazine to amphibians existed (Briggs 1992). Several studies (Reeder *et al*. 1998; Hayes et al. 2002a, 2002b, 2003, 2010; Tavera-Mendoza *et al*. 2002, Langlois *et al*. 2009) indicate that frogs exposed to atrazine during embryonic development alters differentiation in gonads, including testicular resorption and the formation of ovotestes (an organ containing both testicular and ovarian tissue).

In 2002, researchers from the University of California at Berkeley detected atrazine concentrations above 0.20 ppb in water from the North Platte River in Wyoming and found that 92% of male northern leopard frogs (*Lithobates* (formerly *Rana*) *pipiens*) collected (n=20) exhibited feminization (Hayes *et al*. 2002a, 2003). This frequency of feminization in frogs was higher than those frogs sampled at seven other sites in Utah, Nebraska, and Iowa (Hayes *et al*. 2002a, 2003). Such gonadal abnormalities observed in the male leopard frogs from the North Platte River site were similar in morphology to those induced by atrazine in leopard frogs in the laboratory (Hayes *et al*. 2002a, 2002b, 2003; Tavera-Mendoza *et al*. 2002). However, the source of the atrazine detected in water samples in Wyoming was not verified (Hayes *et al*. 2002a, 2003).

Similar to the North Platte River, the Laramie River originates in Colorado and feeds flows to Mortenson Lake National Wildlife Refuge (NWR) where the endangered Wyoming toad (*Anaxyrus baxteri* formerly *Bufo baxteri*) resides. Natural reproductive success and survival of Wyoming toads at the refuge continues to be poor since their reintroduction in 1995 by the U.S. Fish and Wildlife Service (USFWS). Atrazine was identified in the Wyoming Toad Federal Register Notice Listing Package as a threat to recovery of the Wyoming toad in the Laramie Basin (Code of Federal Register, Vol. 49, No. 11, Tuesday, January 1984, Pp. 1992-1994) initiating the

need for further investigation into atrazine concentrations present at Mortenson Lake NWR. Additionally, the chorus frog (*Pseudacris triseriata*) population at Mortenson Lake NWR has plummeted and northern leopard frogs have been extirpated from the entire Laramie Basin since 1985 (Baxter and Stone 1985) with no definitive cause identified.

We determined that atrazine is not used on federal or state land surrounding Mortenson Lake NWR or the Laramie River (Vernon LaFontaine USFS - Roosevelt Forest, Colorado, pers. comm. January, 30, 2003; Steve Kalowski, USFS - Medicine Bow Forest, Wyoming, pers. comm. January 31, 2003; Greg Eglin, USFS - Medicine Bow Fisheries, Wyoming, pers. comm. January 31, 2003). Atrazine is also not applied by Albany County Weed and Pest (Steve van Vleet, Weed & Pest, Wyoming, pers. comm. March 24, 2003). According to the Wyoming Department of Agriculture, the primary source of atrazine appears to be private non-crop (pastures and hay fields) land applications (Jim Bigelow, Wyoming Dept. of Agriculture, pers. comm. March 24, 2003; Mark Ferrell, Agricultural Extension Office, pers. comm. June 5, 2003). Because atrazine is a relatively mobile herbicide it can enter Mortenson Lake NWR via overland flow, air currents, rainfall, groundwater, and the Laramie River from distant sources and may be affecting the amphibian populations present at the refuge.

In addition to their mobility, triazines are classified as moderately persistent to persistent (Briggs 1992; Solomon *et al*. 1996) with a half-life of three to four months in the laboratory under aerobic conditions (USEPA 2006). Furthermore, atrazine is applied during the spring (late May and June) when Wyoming toads and chorus frogs are breeding and tadpoles are developing. During this period, metamorphosing amphibians are particularly vulnerable to the effects of chemicals that affect hormones and gene expression. In fact, metamorphosis is considered only second to embryogenesis in terms of sensitivity to chemically- and physically-induced damage (Murphy *et al*. 2000) as hormones regulate the structure of the reproductive organs during metamorphosis and there is intense gene expression during this time (Cooke 1981; Hayes 2000; Royte 2003). Therefore, one atrazine application may affect tadpole development (Royte 2003); and because of this, the U.S. Environmental Protection Agency (USEPA) has recently implemented new

precautions and measures for the protection of water when atrazine is used (as detailed in the Interim Reregistration Eligibility Decision (IRED) (http://www.epa.gov/oppsrrd1/reregistration/atrazine/).

High levels of atrazine can cause abnormalities in amphibians but direct toxicity is not a significant factor affecting amphibian populations (Allran and Karasov 2001). Instead, it is low atrazine concentrations that affect amphibians over time. For example, survivorship in larval spring peepers (*P. crucifer*), and several *Rana* species (some *Rana* spp. now *Lithobates*) was reduced by exposure to low concentrations of atrazine over a period of at least 30 days (Storrs and Kiesecker 2004). Low concentrations of atrazine can also delay metamorphosis and result in decreased weight, shorter snout to vent length (SVL), and lower hematocrits in tadpoles (Sullivan and Spence 2003).

A further reduction in tadpole growth can result from a decrease in food source. Atrazine causes reversible inhibition of photosynthesis in plants directly affecting phytoplankton, periphyton, and macrophytes in aquatic systems (DeNoyelles *et al*. 1982; Detenbeck *et al*. 1996; Solomon *et al*. 1996; Fairchild *et al*. 1998; Lockert *et al*. 2006). Additionally, atrazine can affect certain algal species (Butler *et al*. 1975; Huber 1993) at very small concentrations (10 to 20 ug/L dependent on exposure duration). Other algal species can uptake and bioconcentrate atrazine (Tang *et al*. 1998). In either case, a reduction in algae or a concentrated dose of atrazine in the primary food source of both Wyoming toad and chorus frog tadpoles could adversely affect their growth.

Results of other studies indicate that tadpole exposure to low and environmentally realistic concentrations ($\geq$ 2 ppb) of atrazine can alter gonadal differentiation and impair sexual development (Hayes *et al*. 2002a, 2002b, 2003; Sullivan and Spence 2003; Langlois *et al*. 2009). For example, Hayes *et al*. (2002a, 2002b, 2003) exposed African clawed frog larvae to atrazine at low concentrations (0.01 to 200 ppb) in the laboratory and examined gonadal histology and laryngeal size at metamorphosis. A smaller laryngeal size in exposed frogs affected their ability to call and consequently their breeding success. Atrazine $\geq$ 0.1 ppb induced hermaphroditism and

demasculinized larynges of exposed males; at 25 ppb testosterone levels decreased 10 times in the males. According to Hayes *et al*. (2002b), the effect levels reported in their study represent realistic exposures and suggest that other amphibian species exposed to atrazine in the wild could be at risk of impaired sexual development. It is likely that researchers may have not previously noticed the effects of atrazine on amphibians because the doses found to affect the gonadal tissues were small and the effects were internal. Therefore, exposed populations could decline and potentially become extinct before the developmental effects on individuals are identified (Hayes 2002b).

Furthermore, the presence of the pathogenic chytrid fungus (*Batrachochytrium dendrobatidis*, *Bd*) at Mortenson Lake NWR is contributing to the difficulty of recovering the Wyoming toad population. *Bd* lives in the keratinized skin tissue of metamorphosed and adult amphibians. Amphibians with the infection often exhibit sloughing skin, abnormal posture, lethargy, and the loss of righting reflex (Daszak *et al*. 1999). In heavy infections, the epidermis will be thickened impairing electrolyte transport resulting in cardiac arrest (Daszak *et al*. 1999; Voyles *et al*. 2009). In tadpoles, the fungus is found in the keratinized mouth parts (jaw sheaths and toothrows) and toe tips; but, because the skin of tadpoles lacks keratin, the infection is not lethal (Daszak *et al*. 1999). *Bd* likely contributed to the decline in the Wyoming toad populations and may have contributed to the extirpation of the northern leopard frog in the Laramie Plains area (USFWS 2012); but it is unclear at this time if the declining chorus frog population in the area is due to the fungus (Dr. A. Pessier, pers. comm. January 14, 2003).

It may also be possible that the amphibians at Mortenson Lake NWR are particularly susceptible to *Bd* infections because of a depressed immune system resulting from exposure to certain environmental contaminants including atrazine. Research on other amphibian species has shown that atrazine can negatively affect immune functions and increase the likelihood of disease or parasitic infections (Christin *et al*. 2003; Forson and Storfer 2006a, 2006b; Houck and Sessions 2006; Brodkin *et al*. 2007; Rohr *et al*. 2008; Rohr and McCoy 2009). With the potential exposure to atrazine and the presence of *Bd* at Mortenson Lake NWR, it is possible that during certain years

or during certain times of year, the immune systems of amphibians at the refuge may be more compromised than during other times.

Because previous investigations have not established a definitive cause for declining amphibian populations in the Laramie basin (Stone 1991; Ramirez 1992; Little *et al*. 2002; Dickerson *et al*. 2003), we conducted this study to (1) assess the presence of atrazine concentrations to which Wyoming toads and chorus frogs are being exposed to at Mortenson Lake NWR; (2) determine if adverse sublethal effects are occurring in these amphibians; and, (3) determine the prevalence of *Bd* in these amphibians.

METHODS

Study sites

The Laramie River is a main source of water to Mortenson Lake NWR where the endangered Wyoming toad resides. Therefore, the Laramie River was sampled at the headwaters in Colorado and the Colorado-Wyoming state border both above Mortenson Lake NWR, and at a downstream site (Monolith; Figure 1). Mortenson Lake was sampled as was a reference site (Vedauwoo) located in a mountainous area near the Laramie Basin. The reference site does not occur near any agricultural activities, does not have any other known contaminant influences, and the wetlands have strong populations of chorus and leopard frogs. Currently, the Vedauwoo area is believed to be free of *Bd* (Figure 1).

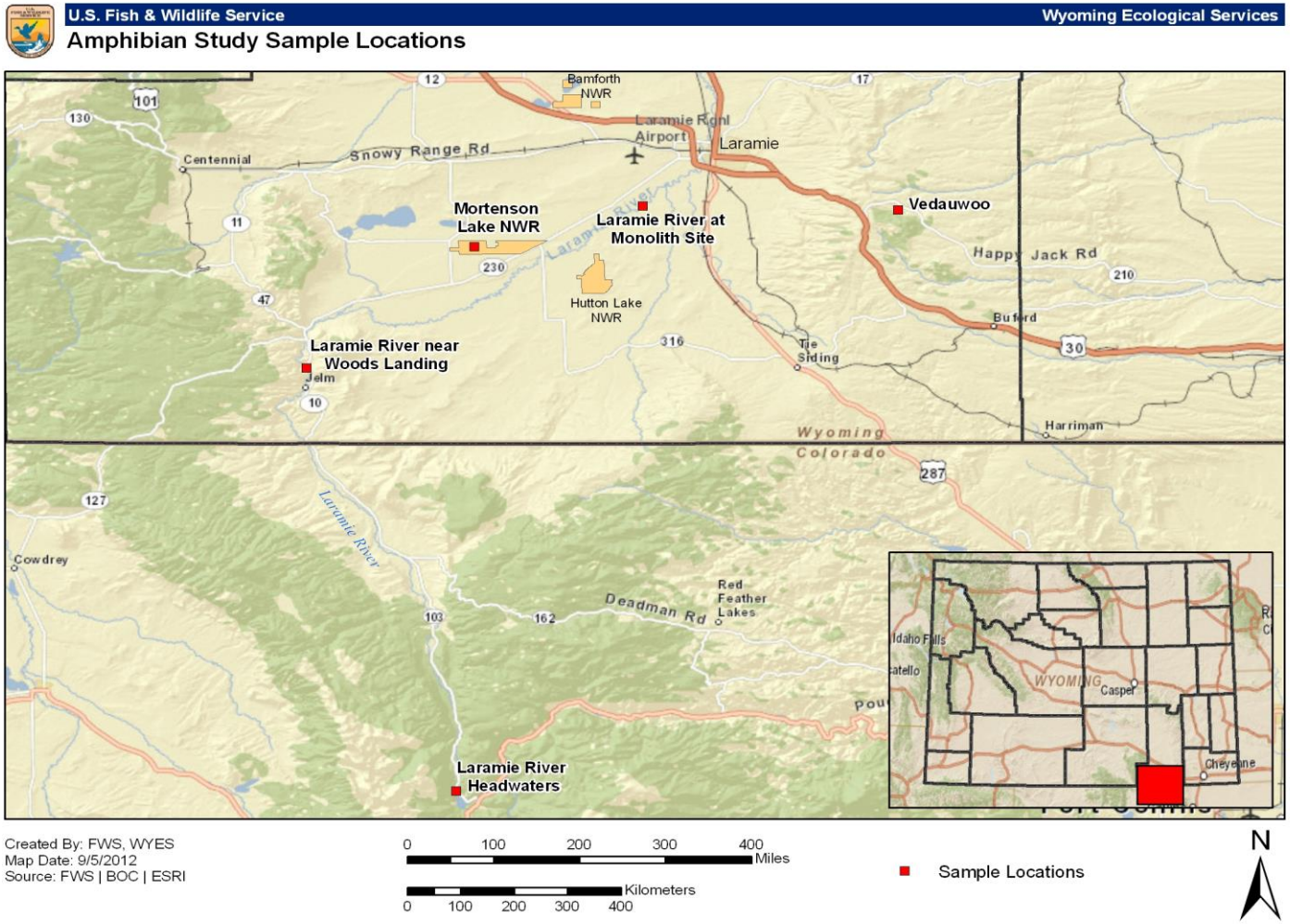

Figure 1. General sampling locations along the Laramie River including the headwaters, Colorado-Wyoming border, Mortenson Lake National Wildlife Refuge, and the Monolith access site and the reference site (Vedauwoo), Wyoming.

2004

Water samples were collected from the five sites on August 30, 2004, using chemically-cleaned 500 mL amber glass jars. Samples were placed on ice and submitted for atrazine and atrazine metabolites analysis to the Mississippi State Chemical Laboratory (MSCL) at Mississippi State University, Mississippi.

2006

To verify the presence of atrazine metabolites in water from the Laramie River, we deployed Polar Organic Chemical Integrative Samplers (POCIS), which are static water quality monitoring devices for hydrophilic organic contaminants that could be potentially endocrine disrupting or acutely toxic but that typically do not bioconcentrate (http//:www.EST-labs.com; Alvarez *et al*. 2007). Furthermore, POCIS allow for the measurement of contaminants over a period of time (time-weighted average (TWA)), rather than a discrete sample where an episodic event or spill could be missed (Alvarez *et al*. 2007; Alvarez 2010). POCIS devices were obtained from Environmental Sampling Technologies, Inc. (EST), St. Joseph, Missouri (http//:www.EST-labs.com) and included three individual POCIS membranes per canister per site. POCIS were deployed in the Laramie River at the Colorado-Wyoming state border and at the Monolith site, and a POCIS was deployed at Mortenson Lake. POCIS were left *in-situ* to monitor contaminants for 31 days (August 11 – Sept 11) after which the devices were collected and shipped to EST overnight for extraction of the membranes. The POCIS extract from each of the three membranes per canister at a site were composited into one sample for each site. The extracts were then shipped overnight to MSCL for triazine and atrazine metabolite analyses.

After receiving the analytical results of the extracts, we needed the analytical results to be on a per POCIS basis so analytical POCIS results were divided by three. We then calculated the time-weighted average water concentration as described in Alvarez (2010) and by David Alvarez (USGS, Missouri, pers. comm. February 5, 2013) using the equation:

$$C_w = (N)/(R_s t)$$

where C_w is the estimated chemical concentration in water, N is the amount of chemical per POCIS, R_s is the uptake sampling rate (0.24, a laboratory-derived number available at (http://www.cerc.usgs.gov/Branches.aspx?Branchld=8), and t is the number of days the POCIS was deployed (t = 31 d; Alvarez 2007). Because we had three POCIS at each site, we divided the POCIS analyte by three to get a concentration per POCIS for the equation

2008

A more in-depth investigation began in May 2008 when POCIS were deployed for 31 days (May-June) at the Laramie River at the Colorado/Wyoming border above Mortenson Lake NWR, Mortenson Lake, the Laramie River below Mortenson Lake NWR, and the reference site. The deployment period was aimed to coincide with amphibian breeding and egg-laying. The extracts from the POCIS devices were shipped to MSCL for analyses of atrazine and atrazine metabolites as in 2006. Algae was collected as a grab sample placed in amber glass jars, frozen, and sent to GPL Laboratories in Frederick, Maryland for analysis of atrazine.

2009

We deployed POCIS for 31 days (May and June) at the same four sites sampled in 2008. Extracts were shipped to MSCL for analyses of triazines and atrazine metabolites. Water grab samples were collected in HPDE chemically-clean bottles at Mortenson Lake and the reference site. These water samples were acidified to a pH<2 with nitric acid, chilled, and sent to Laboratory and Environmental Testing, Inc. in Columbia, Missouri, for metal analysis. Algae samples were collected by hand, placed in amber glass jars, frozen, and sent to MSCL for atrazine and atrazine metabolites, cyananzine, propazine, and simazine analyses. Additional algae samples were collected and sent to IEH-Warren Laboratory in Greely, Colorado, for nutritional analysis content to eliminate the possibility that any observed effects in tadpole health was not due to differences in the nutritional quality of food.

Wyoming toad tadpoles (Gosner stage <26)were received from the Red Buttes Laboratory captive breeding facility and placed in *in-situ* enclosures at Mortenson Lake. Chorus frog tadpoles (Gosner stage <26)collected from Mortenson Lake were placed in separate enclosures at Mortenson Lake. Enclosures consisted of a clear plastic tub, filled with site water along with site algae for food for the tadpoles, covered with netting to avoid predation, and secured to a post (Figure 2).

Figure 2. Tadpole *in-situ* enclosures at Mortenson Lake, Mortenson Lake National Wildlife Refuge, Wyoming.

Similarly, at the reference site, chorus frog tadpoles were collected from a pond at Vedauwoo, placed in enclosures, and fed site algae (Figure 3). Wyoming toads could not be placed at this site due to their endangered status and disease transmission concerns but data was collected on reference Wyoming toad tadpoles reared at the Red Buttes Laboratory captive breeding facility.

We monitored the tadpoles every other day in June for behavioral changes and gross abnormalities. As metamorphosis neared (Gosner 1960), we elevated a portion of the *in-situ* enclosures above the water level to prevent newly metamorphosed individuals from drowning and we monitored the tadpoles daily.

Figure 3. Tadpole *in-situ* enclosures at reference site (Vedauwoo), Wyoming.

Newly metamorphosed animals were collected daily. Individuals were weighed and examined for gross abnormalities. The SVL was measured and then the animals were placed in containers in coolers with wet ice until they fell into hibernation. At the USFWS field office the hibernating metamorphs were rapidly frozen to induce mortality

https://www.avma.org/KB/Policies/Documents/euthanasia.pdf). Five chorus frog metamorphs from Vedauwoo, five Wyoming toad metamorphs from Red Buttes Laboratory, and five metamorphs each of chorus frogs and Wyoming toads from Mortenson Lake NWR were placed into chemically-cleaned jars and shipped frozen overnight to MSCL for analysis of triazine herbicides, including atrazine and its metabolites.

Similarly, 10 Wyoming toad and 10 chorus frog metamorphs from Mortenson, and 10 Wyoming toad and seven chorus frog metamorphs as reference animals were placed into chemically-cleaned 2.0 mL LoBind® tubes filled with DNA/RNA Later solution. These animals were kept chilled and submitted to Dr. Aida Farag with the U.S. Geological Survey fisheries office in Jackson, Wyoming, for the extraction of the liver from the carcass. Livers were then forwarded in LoBind® Tubes with the DNA/RNA Later solution to Dr. Caren Helbing, University of Victoria, Canada, for cDNA array analysis using protocol in Appendix 1 (Helbing Laboratory SOP for tissue collection for gene expression analysis #005) and methods as described in report to the USFWS prepared by Drs. Nik Veldhoen and Caren Helbing 2011 (Appendix 2). Carcasses were fixed in 10% neutral buffered formalin and submitted to Dr. Mark Jordan at Purdue University for sex identification (necessary for cDNA array) and to determine if any metamorphs were hermaphroditic using the histology protocol found in Appendix 3 (methods as described in Dr. Jordan's sex identification report (2011) to the USFWS). Dr. Jordan also took skin samples from pelvic patch and hind limb sections, the areas most likely to show the highest *Bd* infection, to screen for the fungal infection (methods as described in Dr. Jordan's chytrid report (2011) to the USFWS found in Appendix 4).

Quality Assurance

All sample collection and chemical analyses were performed in accordance with the USFWS's quality assurance (QA) and quality control (QC) protocols as specified in the Analytical Control Facility's (ACF) Reference Manual and the USFWS's ACF approved methodologies (PACF 1990). All contract laboratories maintained by the ACF use a rigorous program of methods standardization and QA/QC assessment. Procedural blanks, duplicates, spiked samples, and

analysis of standard reference materials were used routinely with each batch of samples to evaluate and maintain QA/QC.

RESULTS and DISCUSSION

2004 and 2006

Atrazine, as the parent product, was not detected in the grab water sample from the Laramie River at the Wyoming-Colorado border in 2004, but the metabolites, desisopropyl atrazine (0.16 ppb) and desethyldesisopropyl atrazine (0.075 ppb) were detected at Mortenson Lake NWR, and desisopropyl atrazine (0.15 ppb) was detected in the Laramie River at the Monolith site downstream of Mortenson Lake NWR. Desethyldesisopropyl atrazine is a degradation product of atrazine and other triazine herbicides; whereas, desisopropyl atrazine is a degradation product of atrazine only, indicating the presence of atrazine in the system (USEPA 2006). Although not detected in any of the samples, desethyl atrazine is another breakdown product with a structure most similar to atrazine and is equal to atrazine in its ability to produce hormone imbalances (Hamilton *et al*. 2003).

According to Hayes *et al*. (2002a, 2002b), atrazine $\geq$0.1 ppb induced hermaphroditism and demasculinized the larynges of exposed African clawed frog male larvae in laboratory tests. Additionally in 2002, Hayes *et al*. (2002a, 2003) detected atrazine concentrations of 0.20 ug/Lppb in water from the North Platte River and found that 92% of male leopard frogs collected (n=20) in the river near Saratoga, Wyoming, exhibited feminization (Hayes *et al*. 2002a, 2003). However, because our water samples indicated only metabolites of atrazine it is unknown if the actual atrazine concentration in the river was great enough to adversely affect amphibians.

In 2006, we verified the presence of atrazine as the parent product with the detection of atrazine (1.2 ug/3 POCIS) in the POCIS extract from the Laramie River Colorado/Wyoming border site. The time weighted average (TWA) concentration in water for this site was 0.005 ug/L or 0.005 ppb. This concentration is below concentrations shown to induce hermaphroditism and/or sex

reversal in frogs (Hayes *et al*. 2002a, 2002b, 2003). Atrazine and/or its metabolites were not detected at any of the other sites in 2006.

2008

Because of the low flows in 2006 and only one detection of atrazine, we conducted our sampling during the spring in 2008, when atrazine is typically applied. Our expectation was to obtain a more complete understanding of how much atrazine may enter the refuge when amphibians are most sensitive (reproduction, metamorphosis) to the effects of the herbicide. Unlike previous years, there were no detections of triazines including atrazine and its metabolites in the POCIS extracts (Appendix 5). However, detection limits for atrazine from POCIS extracts are typically ug /POCIS or ng/POCIS. Unfortunately, laboratory error resulted in too high detection limits for 2008 and our results were reported in mg/POCIS. Flows were very high in the Laramie River during POCIS deployment so we could not be sure if the lack of atrazine detections was the result of high detection limits or because any atrazine concentration present declined exponentially in runoff as shown by Jurgensen and Hoagland (1990). Atrazine was not detected in algae samples from either Mortenson Lake NWR or Vedauwoo (all algal samples <0.33 ug/g wet weight). We did not determined if the particular species of algae we collected would bioconcentrate atrazine (Tang *et al*. 1998) and we did not quantify algal populations to determine if there was a reduction. If a pulsed exposure of atrazine occurred during spring runoff, the POCIS did not capture such an event during the 30 day time period these devices were deployed.

2009

None of the triazines, including atrazine and its metabolites was detected in POCIS extracts from the four sites (Appendix 6). Again however, we had analytical results with elevated detection limits. Inorganic compounds were either low or below detection limits in water samples collected from the reference site and at Mortenson Lake NWR (Table 1; Appendix 7).

Table 1. Concentrations of detected inorganics (mg/L wet weight) in water samples, Wyoming, 2009.

Location	Al	Ba	Fe	Mg	Mn	Se	Sr	V	Zn
Mortenson Lake	0.05	0.042	0.1	17.5	0.024	<0.0002	0.646	<0.001	0.006
Vedauwoo	0.39	0.0076	1.8	2.37	0.12	0.0002	0.0576	0.002	0.01

As in 2008, none of the triazines was detected in algae samples (Appendix 8). Because we were exposing tadpoles *in-situ*, nutritional content was also analyzed to determine if large differences existed between algae present at the reference site or the commercial formulation of algae fed to endangered Wyoming toad tadpoles at Red Buttes Laboratory and the alga present at Mortenson Lake NWR. The nutritional analysis revealed that the purchased algae were higher for all analyzed nutrients than either algal sample from the Vedauwoo or Mortenson Lake NWR. Nutritional components were comparable between these two latter sites (Appendix 9).

The original strategy was to place 75 captive-bred Wyoming toad tadpoles from Red Buttes Laboratory in enclosures at Mortenson Lake NWR and to capture 75 chorus frog tadpoles from Mortenson Lake NWR and placed them into identical. Similarly, 75 chorus frog tadpoles were to be captured from Vedauwoo and placed into enclosures on-site with 75 Wyoming toad tadpoles remaining at Red Buttes Laboratory to serve as reference animals. However, we were unable to capture enough chorus frog tadpoles after numerous site visits and survival of the Wyoming toad tadpoles from Red Buttes Laboratory was lower than expected.Furthermore, several tadpoles failed to metamorphose or did not survive metamorphosis so sample numbers were much lower than expected. The chorus frog tadpoles at Veduawoo took considerably longer to metamorphose than those at Mortenson, most likely due to cooler air and water temperatures. Captive Wyoming toads metamorphosed at similar rates to those at Mortenson. All animals that did metamorphose appeared healthy and no gross abnormalities were observed. Information on the date of metamorphosis, weight, and SVL of Wyoming toad and chorus frog metamorphs are in Appendix 10 and 11, respectively. Also, the original study plan specified the use of only male metamorph livers in the cDNA array analysis but due to low survival of both species we did not have enough

of either sex for this to be possible. Instead we conducted qPCR as discussed below. Additionally, it was not possible to identify the sex of the Wyoming toad metamorphs with certainty because gonadal development was not as advanced as that in the chorus frog metamorphs (see report in Appendix 3 and Appendices 12 and 13).

There were no detectable levels of triazine herbicides, including atrazine and its metabolites, in any of the chorus frog and Wyoming toad metamorphs from Mortenson Lake NWR or in any of the reference animals (Appendices 14-16). Information about atrazine concentrations in amphibian tissues is lacking but one study found that leopard frog metamorphs had atrazine concentrations about six times that of the water (Allran and Karasov 2000). The absence of detectable triazine herbicides in the amphibian tissues is consistent with the lack of detections found in the POCIS extracts.

Liver samples evaluated for gene expression of the thyroid hormone receptor β, estrogen receptor α, vitellogenin (VTG), and cytochrome P450 family 19 (CYP19) and compared to a control gene (ribosomal protein L8 or *rpL*8) showed that levels in Wyoming toad metamorphs from Mortenson Lake NWR were significantly reduced when compared to reference metamorphs (see Figure 1 in report to the USFWS prepared by Drs. Nik Veldhoen and Caren Helbing (2011) in Appendix 2). However, there were no differences in gene expression in chorus frog metamorph livers from Mortenson Lake NWR when compared to reference animals at Vedauwoo (Figure 1 in report to the USFWS prepared by Drs. Nik Veldhoen and Caren Helbing (2011) in Appendix 2).

The significant reduction in gene expression from Wyoming toad metamorphs at Mortenson Lake is unlikely related to the presence of atrazine but possibly may be the result of other stressor(s) such as the nutritional difference of the commercial algae formulation fed to the control toads throughout their development compared to that at Mortenson, the presence of *Bd* at Mortenson Lake, and/or the low genetic diversity in the Wyoming toad population (Martin *et al*. 2012) Also, because there was not a significant reduction in gene expression in chorus frogs at Mortenson, it is

possible that the Wyoming toad is a more sensitive to certain stressors (as suggested in report to the USFWS prepared by Drs. Nik Veldhoen and Caren Helbing (2011) in Appendix 2).

Results of the *Bd* screening led to the amphibians being grouped in one of three categories (Table 3; identified by Jordan's (2011) report to the USFWS in Appendix 4). The first included animals that tested positive for fungal zoosporangia and included epidermis sloughing. The second included suspect positive where there was a thickening of the epidermis, a characteristic of the presence of *Bd* infection, along with possible zoosporangia in the skin's basal layer. The last category was negative where none of the characteristics of a *Bd* infection was present.

Chorus frogs from Vedauwoo were negative for *Bd* suggesting that this area remains free of the infection. Wyoming toads from Red Buttes were also negative, a requirement of the captive breeding facility. The presence of *Bd* in both chorus frogs and Wyoming toad metamorphs at Mortenson Lake NWR was confirmed by the skin tissue screening, but this is expected, as chytrid has been present at the refuge for several years (i.e. *Bd* was documented in dead Wyoming toads collected from Mortenson Lake NWR in 2000 and 2001; USFWS 2012). Additionally, *Bd* was not formally described until 1999 but analysis of museum Wyoming toad specimens from the Laramie Basin since this time indicates that *Bd* was likely present as early as 1965 (USFWS 2012). Formal systematic surveys of live Wyoming toads at Mortenson began only in 2008, when toads were swabbed and tested specifically for *Bd*. Therefore, we have no definitive information as to how many Wyoming toads may have carried *Bd* during 2005 and 2006 when atrazine was present in the Laramie River system.

While the fungus is generally highly pathogenic to amphibians, hosts express a wide range of responses to infection as evident in the amphibians we sampled. This is due to variation among hosts and environmental conditions, but also to variation in the chytrid fungus itself (Retallick and Miera 2007). Certain amphibian species become infected and act as carriers of the fungus (e.g. *R. catesbeiana*), while others do not survive the infection (Lips 1999, Bradley *et al*. 2002, Retallick

et al. 2004, Garner *et al*. 2006, Lips *et al*. 2006). This may be the case at Mortenson (i.e. the chorus frog may act as a carrier while the Wyoming toad is more sensitive to the pathogen).

Table 2. Determination of *B*d infection in skin from the pelvic patch on the body and the hind limb in chorus frogs and Wyoming toads from Mortenson Lake NWR, 2009, as determined* by Jordan (2011).

Species	Site	Sample Identification	Body	Hindlimb
Chorus frog	Veduawoo	VWCFWB01	Negative	Negative
		VWCFWB02	Negative	Negative
		VWCFWB03	Negative	Negative
		VWCFWB04	Negative	No Specimen
		VWCFWB05	Negative	No Specimen
		VWCFWB06	Negative	Negative
		VWCFWB07	Negative	Negative
	Mortenson	MTCFWB01	**Suspect Positive**	No Specimen
		MTCFWB02	Negative	Negative
		MTCFWB03	Negative	Negative
		MTCFWB04	**Suspect Positive**	Negative
		MTCFWB05	**Positive**	Negative
		MTCFWB06	Negative	Negative
		MTCFWB07	Negative	**Suspect Positive**
		MTCFWB08	**Suspect Positive**	**Suspect Positive**
		MTCFWB09	Negative	Negative
		MTCFWB10	**Positive**	Negative
Wyoming toad	Mortenson	MTWTWB01	**Suspect Positive**	Negative
		MTWTWB02	**Suspect Positive**	Negative
		MTWTWB06	**Positive**	**Suspect Positive**
		MTWTWB07	Negative	**Suspect Positive**
		MTWTWB10	**Suspect Positive**	Negative
		MTWTWB11	Negative	**Suspect Positive**
		MTWTWB22	**Suspect Positive**	**Suspect Positive**
		MTWTWB23	Negative	Negative
		MTWTWB27	**Suspect Positive**	Negative
		MTWTWB28	**Positive**	Negative

*Positive diagnosis was assigned when fungal zoosporangia were observed and the epidermis showed areas of sloughing. Suspect positive diagnosis was assigned if there was a thickening of the epidermis with possible zoosporangia in its basal layers. Negative diagnosis was assigned if there was a lack of any of these features (Jordan 2011).

Additionally, unlike most fungal infections in amphibians, experimentally-induced infections with *Bd* show that the fungus is able to produce lethal disease in uniquely susceptible species without identifiable co-factors such as stress, immunosuppression, or high environmental organic loads (Nichols *et al*. 2001). In the presence of additional stressors, the Wyoming toad may be further

impacted. This also may help to explain why a chorus frog population remains present at Mortenson Lake, whereas the Wyoming toad is present only because of continued reintroduction efforts (USFWS 2012). Another possibility may be that the chorus frog population remains at Mortenson Lake simply because of recolonization from populations at surrounding lakes, whereas the Wyoming toad population is isolated.

MANAGEMENT RECOMMENDATIONS

Recovery of the Wyoming toad has been difficult due to a limited number of individuals contributing to the gene pool and the presence of *Bd* at Mortenson Lake NWR. The presence of atrazine in 2004 and 2006 may have added an additional stressor but even in the absence of atrazine, gene expression results indicate that the health of the Wyoming toad continues to be a challenge. Occasional water quality monitoring for atrazine in the springtime at the Wyoming/Colorado border and at the refuge is recommended. Deploying POCIS with a detection limit of ng, using of atrazine detection strips, or employing an Enzyme-Linked ImmunoSorbant Assay (ELISA) to determine the presence/absence of atrazine are methods considerations. However, the primary emphasis should be disease management. As identified in the draft recovery plan for the Wyoming toad (USFWS 2012), developing survival assurance colonies, halting the spread of Bd, treating amphibians for *Bd*, and developing strategies to decrease *Bd* pathogenicity and host susceptibility in infected populations (Woodhams *et al*. 2011) will be required to sustain the Wyoming toad. This disease management will also likely benefit the chorus frog and prevent further declines in its population as would determining if the chorus frog is a carrier of *Bd* and will continue to spread the disease to the Wyoming toad or if this species continues to decline at the refuge but is present due to natural recolonization from other sites.

Acknowledgements **-**

Funding for this project was provided by the Department of Interior's USFWS Department of Environmental Quality, Contaminants Investigation Program. Refuge manager, Ann Timberman, and members of the Wyoming toad recovery team provided support for conducting this project at Mortenson Lake National Wildlife. Medicine Bow Routt National U.S. Forest Service hydrologists, Larry Sandoval and Dave Gloss, provided chorus frog breeding location information and use of Vedauwoo as a reference site. USFWS Wyoming toad biologist, Jason Palmer, raised Wyoming toad tadpoles and provided technical and field assistance. University of Wyoming summer interns, Billy Reiter-Mardf and Megan Carlson, monitored tadpole and assisted with sample collections. Aida Farag, U.S. Geological Survey, dissected the amphibian livers for analysis. USFWS Wyoming toad recovery coordinator, Jan McKee, provided technical assistance and reviewed this manuscript. Both Matt Schwarz, USFWS environmental contaminants biologist, and Heidi Meador, a University of Wyoming intern with the Wyoming toad recovery program, reviewed this report and provided very helpful comments. Jim Lindstrom of the USFWS's Wyoming Ecological Field office developed the GIS map in this report.

LITERATURE CITED

Allran, J.W. and W.H. Karasov. 2000. Effects of atrazine and nitrates on northern leopard frog (*Rana pipiens*) larvae exposed in the laboratory from posthatch through metamorphosis. Environ. Toxicol. Chem. 19: 2850-2855.

Allran, J.W. and W.H. Karasov. 2001. Effects of atrazine on embryos, larvae, and adults of anuran amphibians. Environ. Toxicol. Chem. 20: 769-775.

Alvarez, D.A. 2010. Guidelines for the use of the semipermeable membrane device (SPMD) and the polar organic chemical integrative sampler (POCIS) in environmental monitoring studies. U.S. Geological Survey, Techniques and Methods 1-D4. Reston, VA. 28 pp.

Alvarez, D.A., J.N. Huckins, J.D. Petty, T. Jones-Lepp, F. Stuer-Lauridsen, D.T. Getting, J.P. Goddard, and A. Gravell. 2007. Tool for monitoring hydrophilic contaminants in water: polar

organic chemical integrative sampler (POCIS). *In*: R. Greenwood, G. Mills, and . Vrana, eds., *Comprehensive Analytical Chemistry 48*. Elsevier. United Kingdom. Pp. 171-197.

Baxter, G.T. and M.D. Stone. 1985. *Amphibians and Reptiles of Wyoming*. Wyoming Game and Fish Department. Cheyenne, WY. 137 pp.

Bradley, G. A., P. C. Rosen, M. J. Sredl, T. R. Jones, and J. E. Longcore. 2002. Chytridiomycosis in native Arizona frogs. J. Wildl. Diseas. 38: 206–212.

Briggs, S. A. and the staff of Rachel Carson Council. 1992. *Basic Guide to Pesticides: Their characteristics and hazards*. Taylor and Francis. Washington D.C. 283 pp.

Brodkin, M.A., H. Madhoun, M. Rameswaran, and I. Vatnick. 2007. Atrazine is an immune disruptor in adult northern leopard frogs (*Rana pipiens*). Environ. Toxicol. Chem. 26: 80-84.

Butler, G.L., T.R. Deason, and J.C. O'Kelley. 1975. The effect of atrazine, 2,4-D, methoxychlor, carbaryl, and diazinon on the growth of planktonic algae. Brit. Phycol. J. 10: 371-376.

Christin, M., A.D. Gendron, P. Brousseau, L Menard, D.J. Marcogliese, D. Cyr, S. Ruby, and M. Fournier. 2003. Effects of agricultural pesticides on the immune system of *Rana pipiens* and its resistance to parasitic infection. Environ. Toxicol. Chem. 22: 1127-1133.

Cooke, A.S. 1981. Tadpoles as indicators of harmful levels of pollution in the field. Environ. Pollut. 25: 123-133.

Daszak, P., L. Berger, A.A. Cunningham, A.D. Hyatt, D.E. Green, and R. Speare. 1999. Emerging infectious diseases and amphibian population declines. Emerg. Infect. Diseas. 5: 735-748.

DeNoyelles, F., W.D. Kettle, and D.E. Sinn. 1982. The responses of plankton communities in experimental ponds to atrazine, the most heavily used pesticide in the United States. Ecol. 63: 1285-1293.

Detenbeck, N.E., R. Hermanutz, K. Allen, and M.C. Swift. 1996. Fate and effects of the herbicides atrazine in flow-through wetland mesocosms. Environ. Toxicol. Chem. 15: 937-946.

Dickerson, K. M. Hooper, T. Huang, and M. Allen. 2003. Determination of pesticide aerial drift and associated effects to the endangered Wyoming toad (*Bufo baxteri*) at Mortenson and Hutton National Wildlife Refuges and potential reintroduction sites. U.S. Fish and Wildlife Contaminant Report No. R6/717C/02. Cheyenne, WY. 29 pp.

Fairchild, J.F., D.S. Ruessler, A.R. Carlson. 1998. Comparative sensitivity of five species of macrophytes and six species of algae to atrazine, metribuzin, alachlor, and metoachlor. Environ. Toxicol. Chem. 17: 1830-1834.

Forson, D.D. and A. Storfer. 2006a. Atrazine increases ranavirus susceptibility in the tiger salamander, *Ambystona tigrinum*. Ecol. Applic. 16: 2325-2332.

Forson, D.D. and A. Storfer. 2006b. Effects of atrazine iridovirus on survival and life-history traits of the long-toed salamander (*Ambystoma macrodacytlum*). Environ. Toxicol. Chem. 25: 168-173.

Garner, T.W.J., M.W. Perkins, P. Govindarajulu, D. Seglie, S. Walker, A.A. Cunningham, and M.C. Fisher. 2006. The emerging amphibian pathogen *Batrachochytrium dendrobatidis*, globally infects introduced populations of the North American bullfrog, *Rana catesbeiana*. Biol. Lett. 2: 455-459.

Gosner, K.L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetolog. 16: 183-190.

Hamilton, D.J., A. Ambrus, R.M. Dieterle, A.S. Felsot, C.A. Harris, P.T. Holland, A. Katayama, N. Kurihara, J. Linders, J. Unsworth, and S.S. Wong. 2003. Regulatory limits for pesticide residues in water. Pure Appl. Chem. 75: 1123-1155.

Hayes, T.B. 2000. Endocrine disruption in amphibians. *In*: D.W. Sparling, G. Linder, and C.A. Bishop, eds., *Ecotoxicology of Amphibians and Reptiles*. Society of Environmental Toxicology and Chemistry. Pensacola, FL. Pp. 573-593.

Hayes, T., K. Haston, M. Tsui, A. Hoang, C. Haeffele, and A. Vonk. 2002a. Feminization of male frogs in the wild. Nature. 419: 895-896.

Hayes, T.B., A. Collins, M. Lee, M. Mendoza, N. Noriega, A.A. Stuart, and A. Vonk. 2002b. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proc. Nat. Acad. Sci. 99: 5476-5480.

Hayes, T., K. Haston, M. Tsui, A. Hoang, C Haeffele, and A. Vonk. 2003. Atrazine-induced hermaphroditism at 0.1ppb in American leopard frogs (*Rana pipiens*): laboratory and field evidence. Environ. Health Perspect. 111: 568-575.

Hayes, T.B., V. Khoury, A. Narayan, M. Nazir, A. Park, T. Brown, L. Adame, E. Chan, D. Buchholz, T. Stueve, and S. Gallipeau. 2010. Atrazine induces complete feminization and chemical castration in male African clawed frogs (*Xenopus laevis*). Proc. Natur. Acad. Sci. 107: 4612-4617.

Houck, A. and S.K. Sessions. 2006. Could atrazine affect the immune system of the frog, *Rana pipiens*? Bios. 77: 107-112.

Huber, W. 1993. Ecotoxicological relevance of atrazine in aquatic systems. Environ. Toxicol. Chem. 12: 1865-1881.

Jurgensen, T.A. and K.D. Hoagland. 1990. Effects of short-term pulses of atrazine on attached algal communities in a small stream. Arch. Environ. Contam. Toxicol. 19: 617-623.

Langlois, V.S., A.C. Carew, B.D. Pauli, M.G Wade, G.M. Cooke, and V.L. Trudeau. 2009. Low levels of the herbicide atrazine alter sex ratios and reduce metamorphic success in *Rana pipien*s tadpoles raised in outdoor mesocosms. Environ. Health Perspect. 118: 552-557.

Lips, K.R., F. Brem, R. Brenes, J.D. Reeve, R.A. Alford, J. Voyles, C. Carey, L. Live, A.P. Pessier, and J.P. Collins. 2006. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. PNAS 103: 3165-3170.

Lips, K. R. 1999. Mass mortality and population declines of anurans at an upland site in western Panama. Conserv. Biol. 13: 117–125.

Little, E.E., R.D. Calfee, and K. Dickerson. 2002. Determination of impacts on the endangered Wyoming Toad (*Bufo baxteri*) at Mortenson National Wildlife Refuge from ammonium nitrate concentrations. U.S. Fish and Wildlife Contaminant Report No. R6/719C/02. Cheyenne, WY. 19 pp.

Lockert, C.K., K.D. Hoagland, and B.D. Siegfried. 2006. Comparative sensitivity of freshwater algae to atrazine. Bull. Environ. Toxicol. Contam. 76: 73-79.

Martin R.M., C.L. Keeler-Foster, K.G. Boykin, G. Zegers, and W.D. Wilson. 2012. Isolation and characterization of eight novel microsatellite loci in endangered Wyoming toad, *Bufo baxteri*. Conserv. Genet. Resour. 4: 347-349.

Murphy, J.E., C.A. Phillips, and V.R. Beasley. 2000. Aspects of amphibian ecology. *In*: D.W. Sparling, G. Linder, and C.A. Bishop, eds., *Ecotoxicology of Amphibians and Reptiles*. Society of Environmental Toxicology and Chemistry. Pensacola, FL. Pp. 141-178.

Nichols, D.K., Lamirande, E.W., Pessier, A.P. and Longcore J.E. 2001. Experimental transmission of cutaneous chytridiomycosis in dendrobatid frogs. J. Wildl. Diseas. 31: 1-11.

Patuxent Analytical Control Facility (PACF). 1990. Reference manual: U.S. Fish and Wildlife Service, Patuxent Analytical Control Facility, Laurel, Maryland.

Ramirez, Jr., P. 1992. Trace elements in water, sediments, and biota from wetlands in the Laramie Basin and their relationship to the recovery of the endangered Wyoming toad (*Bufo hemiophyrs baxteri*). U.S. Fish and Wildlife Contaminant Report No. R6/704C/92. Cheyenne, WY. 15 pp.

Reeder, A.L., G.L. Foley, D.K. Nichols, L.G. Hansen, B. Wikoff, S. Faeh, J. Eisold, M.B. Wheeler, R. Warner, J.E. Murphy, and V.R. Beasley. 1998. Forms and prevalence of intersexuality and effects of environmental contaminants on sexuality in cricket frogs (*Acris crepitans*). Environ. Health Perspect. 106: 261-266.

Retallick, R.W.R., H. McCalluum, and R. Speare. 2004. Endemic infection of the amphibian chytrid fungus in a frog community post-decline. PLoS Biol. 2: 1965-1971.

Retallick, R.W.R. and V. Miera. 2007. Strain differences in the amphibian chytrid *Bactrachochytrium dendrobatidis* and non-permanent, sub-lethal effects of infection. Diseas. Aquat. Organ. 75: 201-207.

Rohr, J.R., A.M. Schotthoefer, T.R. Raffel, H.J. Carrick, N. Halstead, J.T. Hoverman, C.M. Johnson, L.B. Johnson, C. Lieske, M.D. Piwoni, P.K. Schoff, V.R. Beasley. 2008. Agrochemicals increase trematode infections in a declining amphibian species. Nature. 455: 1235-1239.

Rohr, J.R. and K.A. McCoy. 2009. A qualitative meta-analysis reveals consistent effects of atrazine on freshwater fish and amphibians. Environ. Health Perspect. 118: 20-32.

Royte, E. February 2003. Transsexual frogs. Discover. Pp. 46-52.

Solomon, K.R., D.B. Baker, R.P. Richards, K.R. Dixon, S.J. Klaine, T.W. La Point, R.J. Kendall, C.P. Weisskopf, J.M. Giddings, J.P. Giesy, L.W. Hall, Jr., and W.M. Williams. 1996. Ecological risk assessment of atrazine in North American surface waters. Environ. Toxicol. Chem. 15: 31-76.

Stone, M.D. 1991. *Wyoming Toad Recovery Plan*. U.S. Fish and Wildlife Service. Denver, Colorado. 28 pp.

Storrs, S.I. and J.M Kiesecker. 2004. Survivorship patterns of larval amphibians exposed to low concentrations of atrazine. Environ. Health Perspect. 112: 1054-1057.

Sulllivan, K.B. and K.M. Spence. 2003. Effects of sublethal concentrations of atrazine and nitrate on metamorphosis of the African clawed frog. Environ. Toxicol. Chem. 22: 627-635.

Tang, J.X, K.D. Hoagland, and B.D. Siegfried. 1998. Uptake and bioconcentration of atrazine by selected freshwater algae. Environ. Toxicol. Chem. 17: 1085-1090.

Tavera-Mendoza, L., S. Ruby, P. Brousseau, M. Fournier, D. Cyr, and D. Marcogliese. 2002. Response of the amphibian tadpole (*Xenopus laevis*) to atrazine during sexual differentiation of the ovary. Environ. Toxicol. Chem. 21: 527-531.

Tillitt, D.E., K.R. Solomon, E.M. Mihaich, G. Cobb, L. Touart, and T.J. Kubiak. 1998. Role of exposure assessment in characterizing risks of endocrine-disrupting substances to wildlife. *In*: R. Kendall, R. Dickerson, J. Giesy, and W. Suk, eds., *Principles and Processes for Evaluating Endocrine Disruption in Wildlife*. Society of Environmental Toxicology and Chemistry. Pensacola, FL. Pp. 39-68.

U.S. Environmental Protection Agency. 2006. *Rerallgistration eligibility science chapter for atrazine: Environmental fate and effects chapter*. Washington D.C. 95 pp.

U.S. Fish and Wildlife Service (USFWS). 2012. Wyoming Toad (*Anaxyrus baxteri*) Draft Revised Recovery Plan 2012. Cheyenne, WY and Denver, CO.

Voyles, J., S. Young, L. Berger, C. Campbell, W.F. Voyles, A. Dinudom, D. Cook, R. Webb, R.A. Alford, L.F. Skerratt, and R. Speare. 2009. Pathogenesis of chytridmycosis, a cause of catastrophic amphibian declines. Sci. 236: 582-585.

Woodhams, Douglas C., Jaime Bosch, Cheryl J. Briggs, Scott Cashins, Leyla R. Davis, Antje Lauer, Erin Muths, Robert Puschendorf, Benedikt R. Schmidt, Brandon Sheafor and Jamie Voyles. 2011. Mitigating amphibian disease: strategies to maintain wild populations and control chytridiomycosis. Front. Zool. 8. http://www.frontiersinzoology.com/content/8/1/8

Appendices

Appendix 1. Standard operating procedures for tissue collection for gene expression analysis (#005 – Helbing Laboratory) as prepared by Dr. Nik Veldhoen and Dr. Caren Helbing, University of Victoria, Victoria, British Columbia, Canada.

Helbing Laboratory SOP Library

SOP # 005

Protocol Name: Tissue collection protocol for gene expression analyses

Purpose: Collection and preservation of tissue samples for use in downstream mRNA profiling analyses

Reagents Required:

RNA*later*® Tissue Preservative		
Ice or Ice packs		

Methods:

The following are recommendations for the collection and preservation of tissue samples for use in downstream mRNA profiling analyses and are based on the use of the tissue preservative solution, RNA*later* (Ambion Inc.; see below). Please incorporate this information into the logistics for field collection as well as in laboratory-based studies.

CAPTURED ANIMALS SHOULD BE PROCESSED QUICKLY AFTER DEATH FOR TISSUE SAMPLES. A delay in tissue collection and preservation can lead to degradation of the RNA complement (transcriptome) through post-mortem necrotic processes. Only one tissue-specific sample from a single individual should be placed in each screw-cap tube. Never combine either different tissues or different individuals in the same collection tube as cross contamination is assured. For consistency, each tissue type should be sampled from the same region across the animals examined (e.g. the same region of a liver lobe). A combination of scalpels, surgical scissors or biopsy punches can be used to isolate tissue samples. All implements should be rinsed well with distilled water or ethanol and wiped with kimwipes in between each tissue type collected and between each animal. Ideally, the use of disposable, single-use implements (such as disposable biopsy punches) is best; however, the costs for sufficient collection reagents can be prohibitive. ONCE ISOLATED, IT IS IMPORTANT TO PLACE EACH TISSUE SAMPLE IN THE TISSUE PRESERVATIVE TUBE IMMEDIATELY AND THEN STORE THE COLLECTED SAMPLES ON ICE (IN THE FIELD). In addition to the requisite screw-cap collection tubes (possibly pre-filled), RNA*later* preservative, dissection equipment, rinsing solution, and kimwipes, other items to include on field collections are a tissue collection inventory sheet to mark off the tissues collected

and record comments, extra unlabeled collection tubes containing tissue preservative as back-up, storage boxes for the collection tubes, and an ice cooler for sample storage.

With respect to the amount of tissue to collect and the volume of preservative solution required, please ensure that you have read the included protocol for the RNA*later* tissue preservative solution (Part C; Guidelines for use of RNA*later* solution). Label all tubes clearly with black permanent marker cross-referenced to a more detailed Excel spreadsheet. In general, very little tissue is required for genomics-based assessment (DNA array or real-time quantitative PCR) and a sufficient amount is collected to allow for repeated sampling if a failure occurs in downstream processing. For effective preservation, the depth of sampled tissue should be no greater than 0.5 cm and be one fifth or less of the volume of RNA*later* used. A suggested guide for tissue samples is approximately 3 mm in width by 10 mm in length placed in 1.3 ml of RNA*later* in a 1.5 ml screw-cap tube.
Small tadpoles such as from Pacific tree frog or chorus frog can be placed whole in 1.3 ml RNA*later* in a 1.5 ml screw-cap tube. after slitting the body cavity open to allow better penetration of the preservative into the body cavity. If the animals are less than 0.5 cm thick in the body, then slitting the body cavity is not necessary before placing in RNA*later*. Recent metamorphs should be placed in 1.6 ml of RNA*later* in a 2.0 ml screw cap cylindrical tube.
Recent Wyoming toad metamorphs should be preserved in at least 2.5 ml RNA*later* in an appropriate volume tube (see below about air space).
Collected egg and sperm samples should each be approximately 0.5 ml in volume placed in 3 ml of preservative solution.

BE CAREFUL NOT TO GROSSLY EXCEED THE RECOMMENDED TISSUE TO RNA*later* PRESERVATIVE RATIO AS THE TISSUE PRESERVATIVE WILL NOT WORK EFFECTIVELY. THE TISSUE SAMPLE MUST REMAIN FULLY IMMERSED IN PRESERVATIVE SOLUTION AT ALL TIMES.

DO NOT TO ALLOW A SIGNIFICANT AIR SPACE TO OCCUR IN THE COLLECTION TUBES AS THE POTENTIAL REMAINS FOR TISSUE TO COME OUT OF THE PRESERVATIVE SOLUTION DURING TRANSPORT AND STORAGE.

Once collected, place all tissue sample tubes in a storage box in a cooler with ice and/or ice packs until they can be later stored at 4°C in a fridge. For shipping, tissue samples immersed in preservative should be maintained at a cool temperature (on wet ice/ice packs). If subsequent downstream processing and analysis of the samples is to occur at a much later date (>one month) samples can be moved to -20°C storage AFTER an initial 24 hour maintenance at 4°C to allow sufficient penetration of the tissue by preservative solution. If the samples are put in -20°C, fill tube only 80% to allow for expansion when frozen otherwise tubes will burst.

For shipping, ensure that the samples are shipped early in the week to ensure that the samples are not stranded in transit over the weekend.

Last Updated: October 12, 2007 **Update Author:** Nik Veldhoen

Appendix 2. Report to the U.S. Fish and Wildlife Service: "Development of gene expression biomarkers and a quantitative real-time PCR assay for *Bufo baxteri* (Wyoming toad) and *Pseudacris triseriata* (chorus frog)" as prepared by Dr. Nik Veldhoen and Dr. Caren Helbing, University of Victoria, Victoria, British Columbia, Canada.

Development of Gene Expression Biomarkers and a Quantitative Real-time PCR Assay for *Bufo baxteri* (Wyoming toad) and *Pseudacris triseriata* (chorus frog)

Report prepared by:
Dr. Nik Veldhoen and Dr. Caren Helbing
Department of Biochemistry,
University of Victoria,
Suite 308A
Victoria, BC, Canada V8W 3P6

Report prepared for:
Kim Dickerson
U.S. Fish & Wildlife Service
5353 Yellowstone Road,
Cheyenne, Wyoming 82009

June 28, 2011

Purpose of the Research Program:

Water borne byproducts of human activity have the potential to find their way into riparian habitats and, by virtue of their bioactive properties, affect the development and fitness of wildlife species. A number of environmental chemical contaminants have been suggested to display endocrine disruptive activity. This is exemplified by the agricultural herbicide, atrazine (1-Chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine; CAS # 1912-24-9), of which the metabolites desethyldesisopropyl atrazine and desisopropyl atrazine can be found in measurable quantities in areas such as the Mortenson National Wildlife Refuge. Sexual development has been affected in select frog species through alteration of estrogen-dependent signalling pathways [1-5]. There is also some concern that thyroid hormone-mediated processes may be affected by this herbicide [6].

The present initiative focuses on the development of a quantitative real-time polymerase chain reaction (QPCR)-based assay that would evaluate the expression status of thyroid hormone-responsive and estrogen-responsive gene transcripts in the liver of Wyoming toad (*Bufo baxteri*) and

chorus frog (*Pseudacris triseriata*). The candidate genes, thyroid hormone receptor □, estrogen receptor □, vitellogenin (*VTG;* an important egg yolk protein) and cytochrome P450 family 19 (*CYP19*), also referred to as aromatase (important in the conversion of testosterone to estrogen), along with an invariant control gene, ribosomal protein L8 (*rpL8*), were targeted for cloning and development of the QPCR assay. The new molecular tools were then used to evaluate the gene expression status in Wyoming toad and chorus frog new metamorphs collected from locations with elevated levels of atrazine as well as from reference sites with low levels of the herbicide.

Methodology:

Total RNA was obtained from liver samples of the two amphibian species and used to produce cDNA. Sequences representing expressed regions of the *rpL8*, *TR*□, *ER*□, and *CYP19* genes were isolated by PCR from both frog species while *VTG* sequence was obtained from the chorus frog (Table 1). Several attempts were made to clone *VTG* cDNA sequence from Wyoming toad to no avail. Liver from an adult female was also used, subsequent to the many attempts from metamorph livers. This attempt was also unsuccessful.

The successfully cloned sequences had a high level of identity with other known frog sequences (Table 1). A panel of DNA oligonucleotide primer pairs were designed against the genes of interest and evaluated for their efficacy in QPCR using a rigorous three-tier quality control procedure. Gene-specific QPCR primers that passed all quality control measures were used to evaluate mRNA abundance in liver from animals collected at reference sites and at test sites with elevated atrazine levels listed in Table 2. Transcript levels were then normalized to the invariant *rpL8* mRNA transcript and graphed as fold difference relative to the reference sites for each species. Since the data were non parametric, the Mann-Whitney U test was used to determine significance between sites where $p<0.05$ was deemed significant.

Table 1. Comparison of select expressed gene sequences isolated from Wyoming Toad and chorus frog liver and used in QPCR assay development.

Gene Target			*Xenopus laevis*		*Rana catesbeiana*	
			BLASTn	BLASTx	BLASTn	BLASTx
	rpL8	*B. baxteri*	89	99	90	99
		P. triseriata	89	99	91	99
	TR	*B. baxteri*	87	99	90	99
		P. triseriata	88	99	90	99
	ER	*B. baxteri*	83	98	NA	NA
		P. triseriata	84	97	NA	NA
	CYP19	*B. baxteri*	77	94	81	94
		P. triseriata	77	96	81	93
	VTG	*B. baxteri*	-	-	-	-
		P. triseriata	67	78	NA	NA

BLASTn analysis depicts percent nucleotide identity following pairwise alignment while BLASTx shows percent putative amino acid sequence similarity of pairwise alignement. NA=only non overlapping sequences available.

Table 2. Sampling locations for Wyoming Toads and chorus frogs

Species	Reference	Test Site
Wyoming toad	Red Buttes n = 10	Mortesen NWR n = 10
Chorus frog	Vedauwoo n = 7	Morteson NWR n = 10

Results:

Wyoming toad showed significantly reduced levels of *TR*, *ER*, and *CYP19* mRNA in the liver of animals from the Morteson NWR (p=0.0343, 0.0009, 0.0343, respectively; Figure 1) compared to the reference site. In contrast, chorus frog liver displayed no difference in these three mRNA between the two sites examined. *VTG* mRNA abundance was similarly unaffected by location of sampled chorus frogs.

Discussion:

Alteration of *TR* mRNA abundance is commonly associated with a perturbation of thyroid hormone-dependent metamorphosis and development [6-11]. Typically, as levels of *TR* mRNA increase, the rate of metamorphosis is also increased. We observed a decrease in *TR* mRNA levels that were consistent with the difference in time from hatching until metamorphosis supplied by K. Dickerson (Reference site: 30 ± 0 d; Test site: 67 ± 2.7 d).

Reduction in *ER* and *CYP19* mRNA at the atrazine contaminated site suggests an alteration in estrogen signalling. This reduction could be the result of activation of a negative feedback loop by an estrogenic substance to regulate the receptor important in estrogen signalling and the enzyme important in estrogen synthesis by converting testosterone to estrogen. Alternatively, it could represent a reduced capacity to respond to estrogens properly. However, these possibilities remain to be tested.

The inability to obtain *VTG* mRNA from Wyoming toad provides some indirect support for the possibility that these amphibians have a reduced capacity for estrogen signalling at the contaminated site. *VTG* is regarded as a "classic" estrogen-response gene in the liver [12]. We were unable to find any evidence for its expression in the metamorphs or a mature female obtained later during the study. This observation may be indicative of reproductive problems encountered by females as observed in killifish [13]. Subsequent attempts to clone *VTG* should include liver from a gravid female Wyoming toad.

We were unable to determine whether there were sex-specific differences in the transcript responses. Metamorphs have undergone sexual differentiation, but are not sexually mature. Once histological confirmation of sex for these animals is obtained, we will be able to evaluate transcript level with gonadal sex.

Project Summary:

We successfully developed and employed QPCR-based tools for the evaluation of the *TR* gene expression, which is an important component of the thyroid hormone-associated establishment of the metamorphic developmental process in anuran species, as well as tools that may identify a change in estrogen-regulated mRNA expression (*ER*, *CYP19*, and *VTG*). Preliminary analysis of small sample set of Wyoming toad and chorus frog metamorphs demonstrated significant reduction

in mRNA levels of Wyoming toad from the Morteson National Wildlife Refuge. The chorus frog showed no differences between sites suggesting that the Wyoming toad may be more sensitive to possible contamination differences between reference and test sites. Further work will be necessary to confirm this observation, determine causality, and to determine whether there are sex-related differences in sensitivity.

References Cited:

1. Reeder, A.L., *et al*., Forms and prevalence of intersexuality and effects of environmental contaminants on sexuality in cricket frogs (*Acris crepitans*). Environ Health Perspect, 1998. **106**(5): p. 261-6.
2. Hayes, T.B., *et al*., Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proc Natl Acad Sci U S A, 2002. **99**(8): p. 5476-80.
3. Hayes, T., *et al*., Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Rana pipiens*): laboratory and field evidence. Environ Health Perspect, 2003. **111**(4): p. 568-75.
4. Tavera-Mendoza, L., *et al*., Response of the amphibian tadpole *Xenopus laevis* to atrazine during sexual differentiation of the ovary. Environ Toxicol Chem, 2002. **21**(6): p. 1264-7.
5. Tavera-Mendoza, L., *et al*., Response of the amphibian tadpole (*Xenopus laevis*) to atrazine during sexual differentiation of the testis. Environ Toxicol Chem, 2002. **21**(3): p. 527-31.
6. Langlois, V.S., *et al*., Low levels of the herbicide atrazine alter sex ratios and reduce metamorphic success in *Rana pipiens* tadpoles raised in outdoor mesocosms. Environ Health Perspect, 2010. **118**(4): p. 552-7.
7. Shi, Y.-B., Amphibian Metamorphosis: From morphology to molecular biology. 2000, New York: Wiley-Liss.
8. Helbing, C.C., K. Ovaska, and L. Ji, Evaluation of the effect of acetochlor on thyroid hormone receptor gene expression in the brain and behavior of *Rana catesbeiana* tadpoles. Aquatic Toxicology, 2006. **80**: p. 42-51.

9. Zhang, F., *et al*., Evaluation of gene expression endpoints in the context of a *Xenopus laevis* metamorphosis-based bioassay to detect thyroid hormone disruptors. Aquatic Toxicology, 2006. **76**: p. 24-36.
10. Veldhoen, N., *et al*., The bactericidal agent Triclosan modulates thyroid hormone-associated gene expression and disrupts postembryonic anuran development. Aquatic Toxicology, 2006. **80**: p. 217-227.
11. Opitz, R., *et al*., Analysis of thyroid hormone receptor beta A mRNA expression in *Xenopus laevis* tadpoles as a means to detect agonism and antagonism of thyroid hormone action. Toxicology and Applied Pharmacology, 2006. **212**(1): p. 1-13.
12. Marin, M.G. and V. Matozzo, Vitellogenin induction as a biomarker of exposure to estrogenic compounds in aquatic environments. Mar Pollut Bull, 2004. **48**(9-10): p. 835-9.
13. Bugel, S.M., L.A. White, and K.R. Cooper, Decreased vitellogenin inducibility and 17beta-estradiol levels correlated with reduced egg production in killifish (*Fundulus heteroclitus*) from Newark Bay, NJ. Aquat Toxicol, 2011. **105**(1-2): p. 1-12.

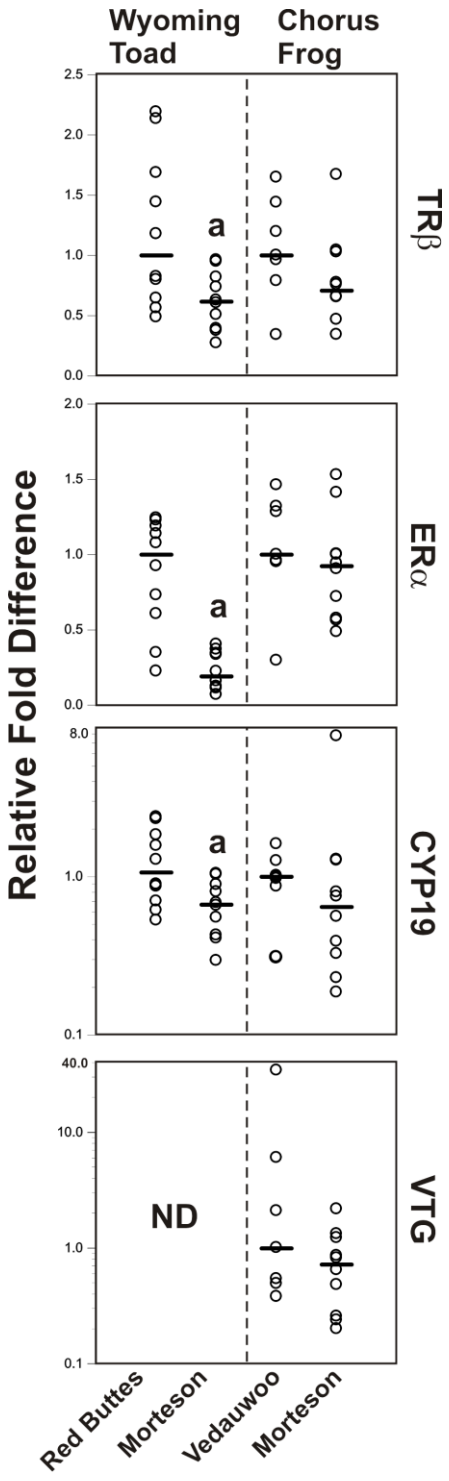
Wyoming Toad
Chorus Frog
Relative Fold Difference
TRβ
ERα
CYP19
VTG
a
a
a
ND
2.5
2.0
1.5
1.0
0.5
0.0
8.0
40.0
10.0
0.1
Red Buttes
Morteson
Vedauwoo
Morteson

Figure 1. Quantitation of specific mRNA abundance in liver from Wyoming toad (*P. baxteri*) and chorus frog *(P. triseriata*) using QPCR. Relative fold difference values of each individual are depicted by open circles. The median mRNA abundance is depicted by a horizontal bar. Significant difference in mRNA levels between reference and test sites is shown by an "a" ($p<0.05$ Mann Whitney U test).

Appendix 3. Report to the U.S. Fish and Wildlife Service: "Identification of sex and fungal infection screening of Wyoming anurans Part 1: Sex identification. as prepared by Dr. Mark A. Jordan, Indiana University-Purdue University, Fort Wayne, Indiana.

See attached pdf file: App 3 Jordan_final_sexid.pdf

Appendix 4. Report to the U.S. Fish and Wildlife Service: "Identification of sex and fungal infection screening of Wyoming anurans Part 2: Chytridiomycosis as prepared by Dr. Mark A. Jordan, Indiana University-Purdue University, Fort Wayne, Indiana.

See attached pdf file: App 4 Jordan_final_chytrid.pdf

Appendix 5. Concentrations of triazine, atrazine, atrazine metabolites (mg/POCIS) in extracts from Polar Organic Chemical Integrative Samplers along the Laramie River, Mortenson Lake National Wildlife Refuge, and Vedauwoo, Wyoming, 2008.

Sample	Location	Atrazine	desethyl Atrazine	desisopropyl Atrazine	desethyldesisopropyl Atrazine	Cyanazine	Metalachlor
ATPOJE01	Laramie River at WY/CO state line	<0.1	<0.1	<0.1	<0.1	<0.01	<0.01
ATPOM01	Laramie River at Monolith site, WY	<0.1	<0.1	<0.1	<0.1	<0.01	<0.01
ATPOMT01	Mortenson Lake at Mortenson NWR, WY	<0.1	<0.1	<0.1	<0.1	<0.01	<0.01
ATPOVV01	Vedauwoo, WY	<0.1	<0.1	<0.1	<0.1	<0.01	<0.01

Appendix 5. cont.

Sample	Metribuzin	Norflurazone	Propazine	Simazine
ATPOJE01	<0.01	<0.2	<0.1	<0.1
ATPOM01	<0.01	<0.2	<0.1	<0.1
ATPOMT01	<0.01	<0.2	<0.1	<0.1
ATPOVV01	<0.01	<0.2	<0.1	<0.1

Appendix 6. Concentrations of triazines and atrazine metabolites (mg/POCIS) in extracts from Polar Organic Chemical Integrative Samplers along the Laramie River, Mortenson Lake National Wildlife Refuge, and Vedauwoo, Wyoming, 2009.

Sample	Location	Date Collected	Atrazine	desethyl Atrazine	desethyldesisoproyl Atrazine	desisopropyl Atrazine	Cyanazine
POATWL01	Laramie River at WY/CO state line	6/29/2009	<0.01	<0.01	<0.01	<0.01	<0.01
POATML01	Laramie River at Monolith Site, WY	6/29/2009	<0.01	<0.01	<0.01	<0.01	<0.01
POATMT01	Mortenson Lake at Mortenson NWR, WY	6/29/2009	<0.01	<0.01	<0.01	<0.01	<0.01
POATVW01	Vedauwoo, WY	7/1/2009	<0.01	<0.01	<0.01	<0.01	<0.01

Appendix 6. cont.

Sample	Metolachlor	Metribuzin	Norflurazon	Propazine	Simazine
POATWL01	<0.01	<0.01	<0.01	<0.01	<0.01
POATML01	<0.01	<0.01	<0.01	<0.01	<0.01
POATMT01	<0.01	<0.01	<0.01	<0.01	<0.01
POATVW01	<0.01	<0.01	<0.01	<0.01	<0.01

Appendix 7. Concentrations of inorganics (mg/L wet weight) in water samples collected from Mortenson Lake National Wildlife Refuge and Vedauwoo, Wyoming, 2009.

Location	Sample	Al	As	B	Ba	Be	Cd	Cu	Fe	Hg
Mortenson Lake NWR, WY	MTWAME01	50	0.5	40	42	0.5	0.5	2	100	0.5
Vedauwoo, WY	VWWAME01	390	0.5	40	7.6	0.5	0.5	2	1,800	0.5

Appendix 7. cont.

Location	Sample	Mg	Mn	Mo	Ni	Pb	Se	Sr	V	Zn
Mortenson Lake NWR, WY	MTWAME01	17,500	24	20	5	5	0.2	646	1	6
Vedauwoo, WY	VWWAME01	2,370	120	20	5	5	0.2	57.6	2	10

Appendix 8. Concentration of triazine herbicides (ug/g wet weight) in algal samples from Mortenson Lake National Wildlife Refuge, and Vedauwoo, Wyoming, 2009.

Sample	Location	Sample Weight (g)	Atrazine	desethyl Atrazine	desethyldesiso-propyl Atrazine	desisopropyl Atrazine	Cyanazine	Propazine	Simazine
MTALAT01	Mortenson Lake at Mortenson NWR, WY	173	<0.001	<0.005	<0.02	<0.01	<0.005	<0.001	<0.005
MTALAT02	Mortenson Lake at Mortenson NWR, WY	236	<0.001	<0.005	<0.02	<0.01	<0.005	<0.001	<0.005
VWALAT01	Vedauwoo, WY	260	<0.001	<0.005	<0.02	<0.01	<0.005	<0.001	<0.005
VWALAT02	Vedauwoo, WY	200	<0.001	<0.005	<0.02	<0.01	<0.005	<0.001	<0.005

Appendix 9. Concentrations of nutritional components in commercial algae fed to Wyoming toad tadpoles at Red Buttes Laboratory, and algal samples from Mortenson Lake at National Wildlife Refuge, and Vedauwoo, Wyoming, 2009.

Location	**Moisture** (%)	**Crude Fat** (%)	**Ash** (%)	**Protein** (%)	**Carbohydrate** (%)	**Calories** (kcal)	**Alanine** (mg/100g)	**Arginine** (mg/100g)
Red Buttes Laboratory	NV[1]	0.58	8.66	65.32	NV	NV	4240	4400
Mortenson Lake at Mortenson NWR, WY	4.88	0.2	36.75	13.93	44.24	234	493	417
Vedauwoo, WY	10.60	0.33	15.15	7.95	NV	NV	738	413

NV[1] = No Value

Appendix 9. cont.

Location	**Aspartic Acid** (mg/100g)	**Cystine** (mg/100g)	**Glutamic Acid** (mg/100g)	**Glycine** (mg/100g)	**Histidine** (mg/100g)	**Isoleucine** (mg/100g)	**Leucine** (mg/100g)	**Lysine** (mg/100g)
Red Buttes Laboratory	5750	497	8240	2370	808	3470	5620	3050
Mortenson Lake at Mortenson NWR, WY	729	77	808	443	85	332	560	330
Vedauwoo, WY	728	66.5	801	396	119	381	680	443

Appendix 9. cont.

Location	**Methionine** (mg/100g)	**Phenylalanine** (mg/100g)	**Proline** (mg/100g)	**Serine** (mg/100g)	**Threonine** (mg/100g)	**Tryptophane** (mg/100g)	**Tyrosine** (mg/100g)	**Valine** (mg/100g)
Red Buttes Laboratory	1400	2790	2370	2590	2900	1300	2800	3480
Mortenson Lake at Mortenson NWR, WY	137	359	379	336	352	156	281	419
Vedauwoo, WY	127	429	391	332	325	217	290	433

Appendix 10. Date Wyoming toad tadpoles hatched, date of metamorphosis, and weight and snout-vent length (SVL) of metamorphed individuals from Mortenson Lake NWR and Red Buttes Laboratory, Wyoming, 2009.

Mortenson Lake Tadpole ID	Date Tadpole Hatched	Metamorphosis Date	Weight (g)	SVL (cm)
MTWTAT01	5/19/2009	7/22/2009	0.17	1.3
MTWTAT02	5/19/2009	7/22/2009	0.21	1.4
MTWTAT03	5/19/2009	7/22/2009	0.18	1.4
MTWTAT04	5/19/2009	7/22/2009	0.21	1.3
MTWTAT05	5/19/2009	7/22/2009	0.23	1.4
MTWTWB01	5/19/2009	7/22/2009	0.21	1.3
MTWTWB02	5/19/2009	7/22/2009	0.21	1.2.
MTWTWB06	5/19/2009	7/23/2009	0.24	1.3
MTWTWB07	5/19/2009	7/23/2009	0.18	1.3
MTWTWB10	5/19/2009	7/24/2009	0.19	1.2
MTWTWB11	5/19/2009	7/24/2009	0.13	1.3
MTWTWB22	5/19/2009	7/27/2009	0.08	0.9
MTWTWB23	5/19/2009	7/27/2009	0.17	1.1
MTWTWB27	5/19/2009	7/29/2009	0.11	0.9
MTWTWB28	5/19/2009	7/29/2009	0.11	1

Appendix 10. cont.

Red Buttes Tadpole ID	Date Tadpole Hatched	Metamorphosis Date	Weight (g)	SVL (cm)
RBWTAT01	5/19/2009	6/18/2009	0.101	1.2
RBWTAT02	5/19/2009	6/18/2009	0.207	1.2
RBWTAT03	5/19/2009	6/18/2009	0.129	1.1
RBWTAT04	5/19/2009	6/18/2009	0.172	1.2
RBWTAT05	5/19/2009	6/18/2009	0.135	1
RBWTWB01	5/19/2009	6/18/2009	0.24	1.2
RBWTWB02	5/19/2009	6/18/2009	0.148	1.2
RBWTWB03	5/19/2009	6/18/2009	0.133	1.1
RBWTWB04	5/19/2009	6/18/2009	0.152	1.2
RBWTWB05	5/19/2009	6/18/2009	0.2	1.2
RBWTWB06	5/19/2009	6/18/2009	0.161	1.3
RBWTWB07	5/19/2009	6/18/2009	0.21	1.3
RBWTWB08	5/19/2009	6/18/2009	0.167	1
RBWTWB09	5/19/2009	6/18/2009	0.215	1.2
RBWTWB10	5/19/2009	6/18/2009	0.264	1.2

Appendix 11. Date chorus frog tadpoles collected, date of metamorphosis, and weight and snout-vent length (SVL) of metamorphed individuals from Mortenson Lake NWR and Vedauwoo, Wyoming, 2009.

Mortenson Lake Tadpole ID	Date Tadpole Collected	Metamorphosis Date	Weight (g)	SVL (cm)
MTCFAT01	5/26/2009	7/24/2009	0.15	1
MTCFAT02	5/26/2009	7/24/2009	0.1	1
MTCFAT03	5/26/2009	7/24/2009	0.4	1.1
MTCFAT04	5/26/2009	7/24/2009	0.16	1
MTCFAT05	5/26/2009	7/24/2009	0.1	1.1
MTCFWB01	5/26/2009	7/27/2009	0.15	1.1
MTCFWB02	5/26/2009	8/3/2009	0.11	1.1
MTCFWB03	5/26/2009	8/7/2009	0.17	1
MTCFWB04	5/26/2009	8/7/2009	0.12	1.2
MTCFWB05	5/26/2009	8/10/2009	0.12	1
MTCFWB06	5/26/2009	8/19/2009	0.11	1.1
MTCFWB07	5/26/2009	9/7/2009	0.15	1
MTCFWB08	5/26/2009	9/9/2009	0.16	1.1
MTCFWB09	5/26/2009	9/12/2009	0.17	1.2
MTCFWB10	5/26/2009	9/15/2009	0.13	1

Appendix 11. cont.

Vedauwoo Tadpole ID	Date Tadpole Collected	Metamorphosis Date	Weight (g)	SVL (cm)
VWCFAT01	5/27/2009	7/8/2009	0.149	1
VWCFAT02	5/27/2009	7/8/2009	0.141	1
VWCFAT03	5/27/2009	8/10/2009	0.13	1.2
VWCFAT04	5/27/2009	8/31/2009	0.17	1
VWCFAT05	5/27/2009	8/31/2009	0.19	1
VWCFWB01	5/27/2009	8/27/2009	0.142	1
VWCFWB02	5/27/2009	8/27/2009	0.129	1.2
VWCFWB03	5/27/2009	8/27/2009	0.188	1.2
VWCFWB04	5/27/2009	9/3/2009	0.15	1.3
VWCFWB05	5/27/2009	9/4/2009	0.24	1.1
VWCFWB06	5/27/2009	9/2/2009	0.16	1.2
VWCFWB07	5/27/2009	9/7/2009	0.19	1.2

Appendix 12. Chorus frog toad metamorph sample identification number, corresponding liver sample identification number, and sex determination from Mortenson Lake NWR and Vedauwoo, Wyoming, 2009.

Whole Body Sample #	Location	Date Collected	Corresponding Liver Sample #	Sex Determination
MTCFWB01	Mortenson	7/27/2009	MTCFLV01	Female
MTCFWB02	Mortenson	8/03/2009	MTCFLV02	Female
MTCFWB03	Mortenson	8/07/2009	MTCFLV03	Female
MTCFWB04	Mortenson	8/07/2009	MTCFLV04	Female
MTCFWB05	Mortenson	8/10/2009	MTCFLV05	Male
MTCFWB06	Mortenson	8/19/2009	MTCFLV06	Male
MTCFWB07	Mortenson	9/07/2009	MTCFLV07	Male
MTCFWB08	Mortenson	9/09/2009	MTCFLV08	Male
MTCFWB09	Mortenson	9/12/2009	MTCFLV09	Female
MTCFWB10	Mortenson	9/15/2009	MTCFLV10	Male
MTCFWB01	Vedauwoo	8/27/2009	MTCFLV01	Female
MTCFWB02	Vedauwoo	8/27/2009	MTCFLV02	Female
MTCFWB03	Vedauwoo	8/27/2009	MTCFLV03	Female
MTCFWB04	Vedauwoo	9/03/2009	MTCFLV04	Female
MTCFWB05	Vedauwoo	9/04/2009	MTCFLV05	Female
MTCFWB06	Vedauwoo	9/02/2009	MTCFLV06	Male
MTCFWB07	Vedauwoo	9/07/2009	MTCFLV07	Female

Appendix 13. Wyoming toad metamorph sample identification number and corresponding liver sample identification number from Mortenson Lake NWR and Red Buttes Laboratory, Wyoming, 2009.

Whole Body Sample #	Location	Date Collected	Corresponding Liver Sample #	Sex Determination
MTWTWB01	Mortenson	7/22/2009	MTWTLV01	Possible female
MTWTWB02	Mortenson	7/22/2009	MTWTLV02	Possible female
MTWTWB06	Mortenson	7/23/2009	MTWTLV06	Possible male
MTWTWB07	Mortenson	7/23/2009	MTWTLV07	Undifferentiated
MTWTWB10	Mortenson	7/24/2009	MTWTLV10	Possible male
MTWTWB11	Mortenson	7/24/2009	MTWTLV11	Possible female
MTWTWB22	Mortenson	7/27/2009	MTWTLV22	Possible female
MTWTWB23	Mortenson	7/27/2009	MTWTLV23	Undifferentiated
MTWTWB27	Mortenson	7/29/2009	MTWTLV27	Possible female
MTWTWB28	Mortenson	7/29/2009	MTWTLV28	Undifferentiated
RBWTWB01	Red Buttes	6/18/2009	MTWTLV01	Male
RBWTWB02	Red Buttes	6/18/2009	MTWTLV02	Possible male
RBWTWB03	Red Buttes	6/18/2009	MTWTLV03	Possible female
RBWTWB04	Red Buttes	6/18/2009	MTWTLV04	Possible male
RBWTWB05	Red Buttes	6/18/2009	MTWTLV05	Possible female
RBWTWB06	Red Buttes	6/18/2009	MTWTLV06	Possible female
RBWTWB07	Red Buttes	6/18/2009	MTWTLV07	Undifferentiated
RBWTWB08	Red Buttes	6/18/2009	MTWTLV08	Possible female
RBWTWB09	Red Buttes	6/18/2009	MTWTLV09	Possible male
RBWTWB10	Red Buttes	6/18/2009	MTWTLV10	Undifferentiated

Appendix 14. Concentrations of triazine herbicides in (ug/g wet weight) in chorus frog and Wyoming toad metamorphs from Mortenson Lake NWR, Wyoming, 2009.

Sample	Sample Weight (g)	Date Collected	Lipid (%)	Atrazine	desethyl Atrazine	desisopropyl Atrazine	Cyanazine	Propazine	Simazine
MTCFAT01	0.15	7/24/2009	0.102	<0.01	<0.04	<0.03	<0.01	<0.01	<0.01
MTCFAT02	0.1	7/24/2009	0.091	<0.01	<0.04	<0.03	<0.01	<0.01	<0.01
MTCFAT03	0.1	7/24/2009	0.466	<0.01	<0.04	<0.03	<0.01	<0.01	<0.01
MTCFAT04	0.16	7/24/2009	1.08	<0.01	<0.04	<0.03	<0.01	<0.01	<0.01
MTCFAT05	0.09	7/24/2009	0.408	<0.01	<0.04	<0.03	<0.01	<0.01	<0.01

Sample	Sample Weight (g)	Date Collected	Lipid (%)	Atrazine	desethyl Atrazine	desisopropyl Atrazine	Cyanazine	Propazine	Simazine
MTWTAT01	0.17	7/22/2009	0.866	<0.004	<0.016	<0.012	<0.004	<0.004	<0.004
MTWTAT02	0.21	7/22/2009	1	<0.004	<0.016	<0.012	<0.004	<0.004	<0.004
MTWTAT03	0.18	7/22/2009	1.06	<0.004	<0.016	<0.012	<0.004	<0.004	<0.004
MTWTAT04	0.21	7/22/2009	1.16	<0.004	<0.016	<0.012	<0.004	<0.004	<0.004
MTWTAT05	0.23	7/22/2009	0.925	<0.004	<0.016	<0.012	<0.004	<0.004	<0.004

Appendix 15. Concentrations of triazine herbicides in (ug/g wet weight) in chorus frog metamorphs from the reference site at Vedauwoo, Wyoming, 2009.

Sample	Sample Weight (g)	Date Collected	Lipid (%)	Atrazine	desethyl Atrazine	desisopropyl Atrazine	Cyanazine	Propazine	Simazine
VWCFAT01	0.15	7/8/2009	1.15	<0.007	<0.012	<0.021	<0.007	<0.007	<0.007
VWCFAT02	0.14	7/8/2009	1.17	<0.007	<0.012	<0.021	<0.007	<0.007	<0.007
VWCFAT03	0.13	8/10/2009	2.22	<0.007	<0.012	<0.021	<0.007	<0.007	<0.007
VWCFAT04	0.17	8/31/2009	0.505	<0.007	<0.012	<0.021	<0.007	<0.007	<0.007
VWCFAT05	0.19	8/31/2009	1.3	<0.007	<0.012	<0.021	<0.007	<0.007	<0.007

Appendix 16. Concentrations of triazine herbicides in (ug/g wet weight) in Wyoming toad metamorphs from Red Buttes Laboratory, Wyoming, 2009.

Sample	Sample Weight (g)	Date Collected	Lipid (%)	Atrazine	desethyl Atrazine	desisopropyl Atrazine	Cyanazine	Propazine	Simazine
RBWTAT01	0.1	6/18/2009	1.55	<0.008	<0.025	<0.025	<0.008	<0.008	<0.008
RBWTAT02	0.21	6/18/2009	1.33	<0.008	<0.025	<0.025	<0.008	<0.008	<0.008
RBWTAT03	0.12	6/18/2009	2.32	<0.008	<0.025	<0.025	<0.008	<0.008	<0.008
RBWTAT04	0.17	6/18/2009	1.65	<0.008	<0.025	<0.025	<0.008	<0.008	<0.008
RBWTAT05	0.13	6/18/2009	1.11	<0.008	<0.025	<0.025	<0.008	<0.008	<0.008