Probiotic Solutions to *Bd*-Associated Declines in Boreal Toads
Lois Webster Fund Final Report, November, 2018

Until a few decades ago, the Boreal toad (*Anaxyrus boreas*) was an abundant amphibian in Colorado’s high elevation lakes and wetlands. A recently emerged fungal skin pathogen, *Batrachochytrium dendrobatidis* (*Bd*), has spread throughout the state and decimated Boreal toad populations. They have been extirpated from the majority of their former breeding sites and have significantly smaller populations wherever they have persisted. They are listed as endangered in Colorado, and Colorado Parks and Wildlife leads a number of efforts to ensure the persistence of these unique amphibians in the state.

An exciting potential direction for conservation efforts for amphibians affected by *Bd* is the use of probiotic bacterial treatments. In a number of amphibian systems (including Boreal toads), the skin microbial communities have been shown to play a role in defense against fungal pathogens like *Bd*. Some bacteria produce anti-fungal metabolites that inhibit the growth of *Bd*, and the higher proportion of these bacteria on the skin of amphibians, the more resistant the skin environment is to infection. The idea behind probiotic treatments is to expose the skin to high numbers of anti-fungal bacteria to increase the proportion of inhibitors in order to prevent an infection. To date, the vast majority of anti-*Bd* probiotic trials for amphibians have used a single bacterium, called *Janthinobacterium lividum* (*Jliv*), which produces a strongly anti-fungal purple pigment called violacein.

**Project Objectives**

- To locate remaining wild populations of Boreal toads, capture multiple individuals and swab their skin surface
- To grow the bacteria on found on their skin surface in culture
- To isolate as many unique strains from as many different toad life stages as possible
- To evaluate those isolates for their ability to inhibit *Bd* and be an effective probiotic candidate
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**Methods**

We surveyed seven wetlands that recently had Boreal toad populations on a total of 22 sampling trips. We encountered individuals at 5 of the sites, and collected swabs from 131 toads of various life stages (adults, subadults, metamorphs, tadpoles, and eggs). We spent approximately 82 hours conducting visual encounter surveys and processing samples in the field. Those swabs were rubbed out on R2A agar, a low nutrient media good at facilitating the growth of fastidious bacteria found in fresh water environments. Individual unique colonies were isolated from these field plates using standard microbiological methods in the lab, and were preserved for later analysis. *Bd* challenge assays are currently underway, and consist of growing *Bd* and the isolate together on tryptone agar and looking for a zone of inhibition of *Bd*.

**Results**

The majority of the swabs from the field yielded multiple culturable bacterial isolates, and most of those were successfully isolated and cryopreserved. In total, we isolated approximately 250 bacterial strains for evaluation as a potential probiotic. Some of those are likely the same species isolated from multiple toads (which are actually the isolates we are most interested in), but we won’t know that until we have their DNA sequenced. We have just begun the process of evaluating the isolates as potential probiotics, but we have already confirmed multiple *Bd* inhibitors in the collection. Of special note was the isolation of multiple new *Janthinobacterium* isolates, which serve as the probiotic organism in our lab’s current trials.

**Discussion**

The field survey portion of this project was quite successful. On any given visit, the chances of encountering toads are somewhat random, subject to the whims of weather and the exact timing of breeding. Given that, we were quite successful in finding toads to sample. The culturing and isolation of microbes was very successful, though we don’t know exactly how many of those are inhibitory yet. As we complete culture work over the next few months, I am confident we will find multiple potential new probiotics.
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**Lois Webster Fund**

The funds we received from the Audubon Society of Greater Denver were instrumental in completing this work. The 1,597 dollars allowed us to purchase field sampling equipment such as swabs and gloves, culturing supplies such as agar and petri dishes, as well as pay an undergraduate field assistant for over 30 hours of work. The sampling trips involved in this project were concurrently helpful to Colorado Parks and Wildlife, as they allowed us to get a picture of the status of Boreal toad populations at a few sites that could not be sampled by field technicians multiple times in the seasons.

**Fund Usage**

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<tr>
<th>Item</th>
<th>Quantity</th>
<th>Cost</th>
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<tr>
<td>Pay for undergraduate field assistant</td>
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<td>Petri Dishes (500)</td>
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<td>R2A Agar</td>
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<td>Sterile Swabs (200)</td>
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<td><strong>TOTAL</strong></td>
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Thank you so much for your support of this project and our endangered Boreal toad populations.

Tim Korpita
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