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The Use of Probiotic Applications in Early Life Stages to Mitigate *Batrachochytrium dendrobatidis* Infections in *Rana luteiventris* (Columbia Spotted Frogs)

Autumn N. Holley
Eastern Washington University

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Autumn Holley and Dr. Jenifer Walke, Eastern Washington University

Introduction

- Amphibian species are facing significant declines, with a third of species threatened with extinction (IUCN).
- Many declines are due to Bd (Skerratt et al. 2007), which has been spread globally by humans (Daszak et al. 2001).
- Symbiotic antifungal bacteria can provide Bd resistance (Lauer et al. 2007), but probiotic treatments with these bacteria have had inconsistent success (Becker et al. 2011), largely due to the probiotic species' failure to persist on amphibian skin.
- Amphibians have fewer known methods of microbiome regulation prior to metamorphosis (Rebollar et al. 2016), yet probiotic studies have largely been conducted on adults or juveniles.
- This means bacteria may colonize amphibian skin more easily in early life stages and have priority effects, which are the impact the first microbial colonizers have on which microbial species can subsequently grow (Sprockett et al. 2018).

Objectives

1. Determine if there are priority effects of a probiotic application and its impacts on Columbia spotted frog (*Rana luteiventris*) skin microbiome assembly.
2. Determine whether timing of probiotic additions impacts effective probiotic colonization, persistence, and protection against Bd.
3. Determine the influence of temperature on microbiome assemblies and probiotic effectiveness.

Hypotheses

1. Introducing the probiotic to eggs or tadpoles immediately after hatching will result in increased colonization rates, longer persistence, and thus greater protection against Bd infection than introducing the probiotic to recently metamorphosed frogs.
2. Increased temperature will limit infection intensity and contribute to probiotic success (Turner et al. 2021), and temperature treatment will play a role in microbiome composition (Muletz-Wolz et al. 2017).

Columbia spotted frog tadpole (Below) (Patla & Keinath 2005)

Columbia spotted frog egg masses (Left) (Patla & Keinath 2005)

Amphibian Probiotic Literature

Study	Host Species	Probiotic Species	Timing
Küng et al. 2014	*Colostethus panamansis*	*Lysinibacillus fusiformis*	Likely adults
Harris et al. 2009	*Rana mucosa*	*Janthinobacterium lividum*	Juveniles
Woodhams et al. 2019	*Rana sierrae*	*Pseudomonas fluorescens, Pedobacter cryoconitis, Chryseobacterium sp.*, and *Iodobacter sp.*	Juveniles
Becker et al. 2021	*Atelopus zeteki*	*Diaphorobacter* 63F genetically modified to produce violacein	Subadults/adults
Muletz et al. 2012	*Plethodon cinereus*	*J. lividum*	Adults
Becker et al. 2009	*P. cinereus*	*J. lividum*	Adults
Harris et al. 2009	*P. cinereus*	*Pseudomonas reactans*	Likely adults
Kueneman et al. 2016	*Anaxyrus boreas*	*Chyrseobacterium sp., Pseudomonas sp.* then *J.lividum*	Juveniles
Kearns et al. 2017	*Alytes obstetricans*	*Flavobacterium johnsoniae, J. lividum,* or *Penicillium expansum* (fungi)	Juveniles
Becker et al. 2011	*Atelopus zeteki*	*J. lividum*	Juveniles
Davis et al. 2017	*Alytes obstetricans*	*Pseudomonas fluorescens* and *Flavobacterium johnsoniae*	Tadpoles
Rebollar et al. 2016	*Lithobates clamitans*	*J. lividum*	Tadpoles
Woodhams et al. 2014	*A. obstetricans, Bombina variegata, Rana temporaria, Pelophylax esculentus*	*F. johnsoniae, J. lividum, P. expansum,* or *Serratia plymuthica*	Juveniles
Walke et al. 2015	*Lithobates catesbieanus*	*J. lividum*	Juveniles

Table 1. Review of amphibian probiotic literature by host species, probiotic, and life stage

Methods

- Columbia spotted frog eggs collected from Turnbull National Wildlife Refuge (TNWR) will be randomly separated into treatment groups (Table 2).
- The probiotic was chosen (following selection methods of Bletz et al. 2013) because it was found on frogs in TNWR (Campos 2020) and inhibited Bd *in vitro* in preliminary research (Dodd and Walke, unpublished data).
- Temperature treatments will mimic current temperatures for this region and projected future temperatures for 2070-2090 (Tasker et al. in review).
- Eggs will be raised in the lab and probiotics will be added via a probiotic bath (Walke et al. 2015).

Challenge assay for anti-Bd bacteria. Control (top) and *Pseudomonas sp.* (bottom) on a Bd lawn (Walke et al. 2017)

Columbia spotted frog (Washington Department of Fish and Wildlife)

Methods

- Frogs will be swabbed (Becker et al. 2011) one month after probiotic addition to the metamorph treatment group and infected with Bd (Harris et al. 2009).
- The 16S rRNA gene from the swabs will be extracted, amplified, and sequenced to analyze the microbiome and determine the presence and relative abundance of the probiotic bacterium, and Bd infection will be quantified using qPCR (Walke et al. 2015). DNA will be amplified via PCR and sequenced at the Dana Farber Cancer Institute at Harvard University.
- Frogs will be monitored for sublethal effects of Bd infection, including changes in growth rate (Walke et al. 2015) or reduced weight (Pearl et al. 2009).

Timing of Probiotic	Control/ No Probiotic	Eggs	Hatch/Emerge from Egg Jelly	Tadpoles 1 Week Post-Hatching	Metamorphs
Current Temperatures	N=9	N=9	N=9	N=9	N=9
Projected Climate Change Temperatures	N=9	N=9	N=9	N=9	N=9

Table 2. Treatment groups for probiotic experiment

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