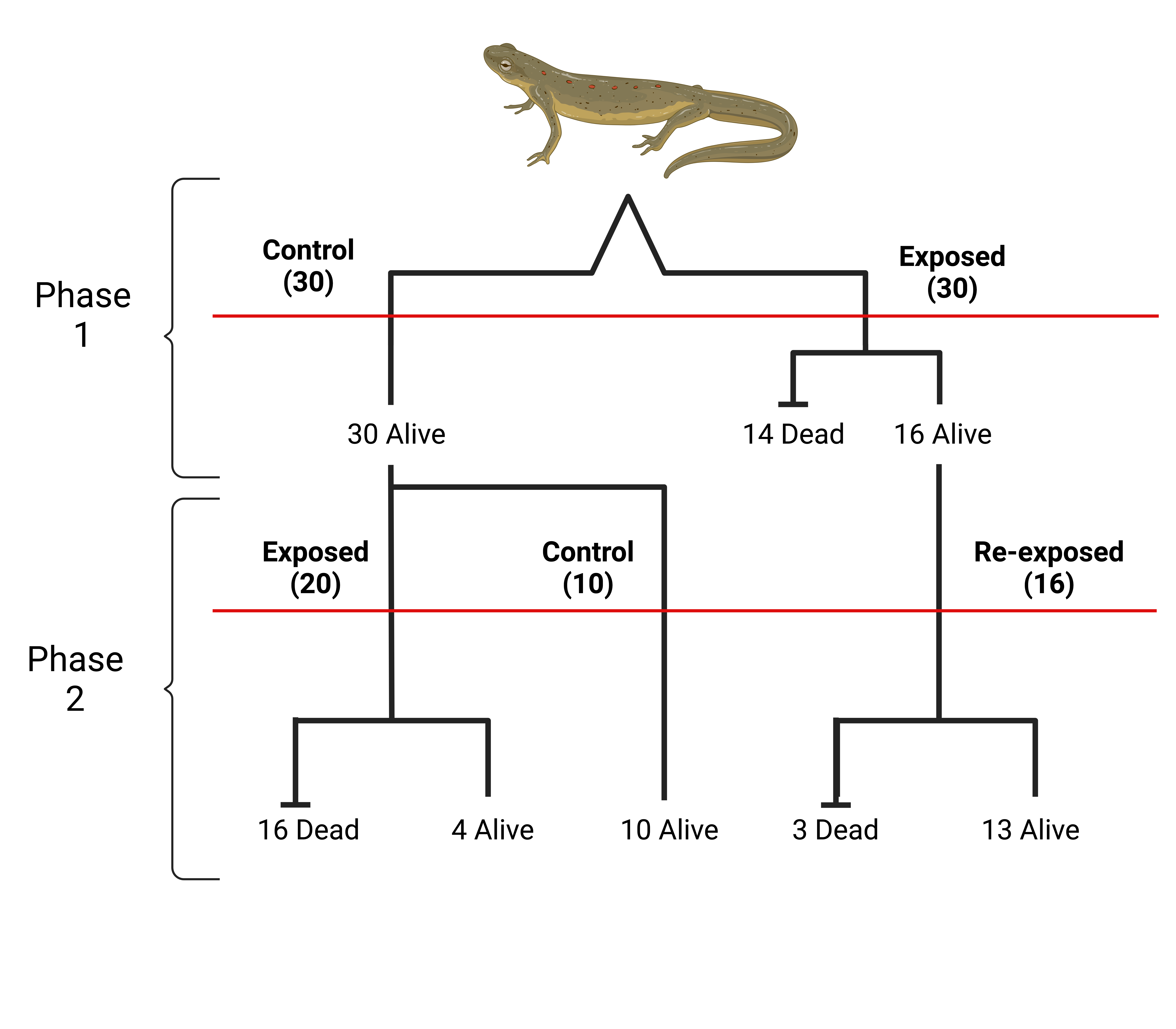
**Adult Exposure Experiment**

**11/8/2022**

**Main Question:** Does an individual exposed to *Bsal* for the first time respond differently than an individual that has survived a prior exposure?

**Microbiome Data Question:** Do we see differences in exposure history and disease outcome reflected in the microbiome?



**Figure 1.** Visual representation of the experimental Design

**Files included**

* Metadata file - All metadata available for the samples. See Key tab for explanations
* ASV table – Rarefied ASV table. Counts are number of 16S gene copies estimated with the plasmid
* Taxonomy file – Taxonomic assignments and confidence for all ASVs. Assignments made with GreenGenes v13-8 99% classifier (QIIME v2021.8)
* Repset Sequences file
* Subsetting R file

**Methodology**

*Live Animal Collection and Bd-Clearing*

We collected 60 adult *N. viridescens* from an aquatic habitat in Tennessee (TWRA Collection Permit# 2976 / IACUC Protocol# M1800078-01). Upon arrival to the laboratory, we performed skin swabs and collected body mass, snout-vent length, and tail length measurements. The skin swabs were tested using qPCR to determine *Bd* infection status at time of capture. To assist the newts in clearing potential *Bd* infections, we administered a heat-treatment of 30 °C for 10 days, adjusting the temperature by 2.5 °C per day. After the heat-treatment, we swabbed the newts for *Bd* again, and ensured that all the previously infected animals tested negative for *Bd* by qPCR.

*Animal Husbandry*

We acclimated the newts to a cycle of 10-hour day and 14-hour night and a constant temperature of 17.5 °C. This temperature was chosen because it is within the tolerance range of the host and pathogen, and lower temperatures led to nearly total incidence of mortality in previous experiments with *N. viridescens* (Carter et al. 2021). To ensure uniform conditions, we housed the newts in separate 750 mL aquaria inside of a programmable environmental chamber. We changed the aquarium water and fed the newts freeze-dried Tubifex worms at 3% of their body mass once per week.

*Bsal Exposures*

We exposed 30 of the newts to a dose of 2.5x103 *Bsal* zoospores in 10 mL of artificial pond water for 24 hours, and simultaneously gave the other 30 newts a sham exposure treatment of 10mL of artificial pond water. After the exposures were complete, we returned the newts to their 750mL aquaria and monitored their survival with daily health checks throughout the experiment. We performed weekly skin swabs and body mass measurements for each newt starting on day 4 after exposure, and continuing until day 45.

After 45 days, we gave all the newts a heat treatment of 30 °C for 10 days, using a 2.5 °C daily adjustment, to assist the newts to clear remaining *Bsal* infections from the first round of the experiment. At the end of the heat treatment, we swabbed the newts and ensured that they all tested negative for *Bsal* infection by qPCR, and the newts were allowed to re-acclimate to the constant 17.5 °C conditions for seven days.

Once re-acclimated, we exposed all the survivors of the previously exposed group, as well as 20 of the previously unexposed newts to a dose of 2.5x103 *Bsal* zoospores in 10mL of artificial pond water for 24 hours. The remaining 10 unexposed newts got a sham exposure treatment of 10mL of artificial pond water. At the end of this second round of exposures, we again returned the newts to their 750mL aquaria and monitored their survival with daily health checks. We performed weekly skin swabs and body mass measurements for each newt starting on day 4 after exposure, continuing until day 45.

After day 45, skin secretions were collected from the surviving newts and the newts were euthanized using immersion in a bath of 2% Tricaine Methanesulfonate. We then collected tissue samples for histological confirmation of infection and serum for *Bsal* antibody testing.

*Sample Collection and Preparation*

The pre-trial skin swabs and all swabs collected following *Bsal* exposure consisted of 10 passes along the newt’s abdomen followed by 10 swipes on the bottom of each foot. After swabbing, we immediately placed the swab into a sterile 1.5mL tube in a dry ice bath, and then moved it to long term storage in a -20 °C freezer until the extraction. To ensure we could use the extracted DNA from skin swabs for both *Bsal* DNA assays and microbiome assays, we performed all extractions using a DNeasy® Blood and Tissue kit (Qiagen, Valencia, CA) protocol with the manufacturer’s pre-treatment protocol for gram-positive bacteria, which is optimized for the dual purpose of *Bsal* DNA detection and quantification by qPCR and amphibian skin microbiome analysis. *Bsal* DNA was quantified using a qPCR assay with a serially diluted standard made from extracted DNA from a known number of *Bsal* zoospores (Blooi et al. 2013).

*Library Preparation and Sequencing*

The V4 region of the 16S rRNA gene was targeted for next generation sequencing. All samples were individually barcoded with a single index on the forward primer and amplified in duplicate following the Earth Microbiome Protocol (515F-806R, EMP 2018). A known amount of a custom mock 16S V4/ITS plasmid was added to PCR reactions to allow for the calculation of estimated true reads in a given sample (Pisces Molecular, Boulder, Colorado, USA). Briefly, PCR reactions consisted of 2 μL sample DNA, 0.3 μL BSA (for reaction efficiency), 12.5 μL Azura HS Taq 2x Red MasterMix, 0.5 μL of each primer (10 uM), 0.1 μL of plasmid spike-in (105 copies mL-1, Pisces Molecular), and 9.25 μL of DNA-free water. PCR conditions were as follows: 1 minute at 94 °C, 35 cycles of 94 °C for 20 seconds, 55 °C for 20 seconds, and 72 °C for 40 seconds, then 72 °C for 10 minutes. Extraction negatives pooled into a single sample and PCR negatives for each plate were also included in sequencing. Samples were normalized using the Mag-Bind EquiPure Library Normalization Kit (Omega Bio-Tek, Norcross, Georgia USA). All samples were run on an Illumina MiSeq at the University of Massachusetts, Boston, with a 2 x 150 v2 chemistry kit, with 300 cycles in the forward direction and a 15% PhiX spike-in.

*Sequence Read Data Processing*

Sequences were first processed using QIIME2 (Bolyen et al. 2019; version 2021.8). Raw samples were demultiplexed, filtered to a minimum quality score of 20, and trimmed to 150 base pairs. sOTUs or ASVs (sub-operational-taxonomic-units, or amplicon sequence variants) were clustered with Deblur (Amir et al. 2017). Contaminants were identified in R with the package “decontam” (Davis et al. 2018; version 1.8.0), using the “prevalence” method and a threshold of 0.5, with pooled extraction and PCR controls used to identify contamination.

Samples were then corrected to ‘true’ number of reads based on the presence of plasmid in the sample. This was calculated by dividing the known added amount of plasmid copies spiked in by the total plasmid reads and multiplying by the total reads in the sample plus the plasmid reads (Eq. 1). To calculate the total 16S copies for an individual ASV in a sample, the number of reads for that ASV was divided by the total number of reads for that sample and multiplied by the total 16S copies for a sample (Eq. 2). All plasmid corrected ASV counts were rounded to whole numbers for downstream analysis.

**Equation 1**

**Equation 2**

Previously identified contaminants were removed after the correction to ensure that the calculations were accurate. This table was reimported into QIIME2 in order to remove chloroplasts and mitochondria sequences. Phylogeny was constructed using the align-to-tree-mafft-fasttree pipeline (Price et al. 2010; q2-phylogeny), and taxonomy assigned with the q2-feature-classifier using classify-sklearn (Bokulich et al. 2018) and Greengenes 13\_8 99% reference sequences (McDonald et al. 2012). Due to variation in sequencing depth, samples were rarefied at a depth of 10,000 to prevent bias in downstream analyses.

Alpha (Faith’s Phylogenetic Diversity, Richness, Shannon Entropy, Evenness) and Beta (Weighted and Unweighted Unifrac) diversity metrics were calculated in QIIME2 using the core-metrics-phylogenetic pipeline in the q2-diversity plugin following rarefaction to 10,000 16S copies per sample to account for variation in sequencing depth (Bolyen et al. 2019; q2-diversity). The proportion of ASVs that had predicted antifungal function were calculated based on 99% sequence identity to members of the Antifungal Isolates Database (Woodhams et al. 2015; updated database and code M. Bletz personal comm.) that were found to be strictly inhibitory only, with no facilitating or neutral isolates.