Skin microbiome composition and diversity across *Anaxyrus boreas* developmental stages

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ABSTRACT

In the last decade our understanding of host-associated microbes has grown immensely, however there are still many questions to be answered. Few studies have examined the assembly of the microbiome throughout organismal development, and even fewer have attempted to observe this phenomenon through a natural study. We sampled three high elevation wetland habitats in Colorado, USA, where boreal toads (*Anaxyrus boreas)* had successfully reproduced and deposited egg masses. In total, we sampled 12 distinct life stages ranging from eggs to adults along with two types of environmental samples during May through September 2015. We used barcoded sequencing of the 16S rRNA marker gene to characterize the diversity and composition of bacterial taxa living on the skin of the boreal toads. We found that microbiome composition was highly dependent on amphibian life stage. The bacterial communities on egg stages and early tadpoles were very similar to the environmental bacterial community. The skin community on later-stage tadpoles began to diverge from the environmental community as development continued, then shifted again following metamorphosis. Bacterial diversity on the *A. boreas* decreased soon after the tadpoles hatched form the egg but then increased following metamorphosis. The relative proportion of the bacterial order Burkholderiales increased during the tadpole stages and decreased after metamorphosis. Stramenopiles were dominant during the egg stage but nearly absent on adults. Actinomycetales were absent or rare throughout development until becoming the most proportionally abundant order of bacteria on the subadult and adult stages. Our results suggest that the microbiome of amphibians is deterministic and extremely selective as tadpoles develop which indicates that the skin microbiome of larval amphibians may perform specific functions for the host.

BACKGROUND

'Host-associated microbiome' is a term used to describe the ecosystem of symbiotic bacteria on and inside an organism. In the last decade we have begun to understand how important these microbes are to an organism's overall health and development. For example, we have found that microbial symbionts seem to aid digestion (Zhu et al, 2011), play a role in the development of the immune system (Lee et al, 2010), and even have an influence over anxiety and depression (Foster et al, 2013). We are just beginning to understand this micro-world, but it is already clear that an individual's unique microbiome has a great influence on health.

Amphibians are the only vertebrate class that undergoes metamorphosis, a process that involves significant morphological and physiological changes from birth to adulthood. Typically, for aquatic anurans, the adults lay their eggs in water, and the eggs develop for fifteen days (depending on the species of amphibian). The free living larvae emerge from their eggs adapted for a fully aquatic lifestyle. The period of time spent as a larval anuran is species specific, but eventually the tail will be reabsorbed into the body, the skin will become **keratinized**, and the gills will disappear; at this point they are referred to as 'metamorphic' individuals, or 'metamorphs'. Following metamorphosis, the time to sexual maturity varies across species. K.A. Gosner created a detailed table (Table 2) representing the process of anuran metamorphosis with 46 distinct stages. This table is used to estimate the age of anurans based on their physical characteristics (Gosner, 1960).

Anuran skin is extremely different from the skin of mammals as it is very thin and contains many mucous glands. These glands help the skin stay moist. Anuran skin is considered a mucosal tissue much like the mouth, gut, and intestines of mammals. This makes it a prime habitat for many microbial species (Woodhams et al, 2014). The anuran skin plays many

different roles, aiding in respiration, temperature control, and providing protection from predators and pathogens (Greven et al, 1995). During the tadpole stage of development, keratinized **epithelium** is found only in the mouth parts. During the metamorph stage keratinized cells are also found on the feet. However, shortly after anuran metamorphosis the skin becomes fully keratinized (Budtz & Larsen, 1975). It is known that *Batrachochytrium dendrobatidis* **zoospores** infect these keratinized epithelial cells and utilize the host to develop into **zoosporangia** and reproduce. This results in the development of the infectious disease **chytridiomycosis**.

The chytrid fungus**,** *B. dendrobatidis* (*Bd*), invades and reproduces within amphibian skin, disrupts **osmoregulation**, and causes mortality (Voyels et al, 2009). This pathogen is responsible for the extinction of numerous amphibian species worldwide, a pandemic that has been regarded by many researchers as the most substantial loss of vertebrate biodiversity due to an infectious disease in all of recorded history (Kilpatrick et al. 2010). In fact, 39% of **extant** amphibian species are either endangered or extinct which makes them the most threatened group of vertebrates (Stuart et al, 2004).

Researchers attempting to prevent amphibian extinction have explored many intervention options, one of which is **probiotic** or **bioaugmentation** treatments (Bletz, 2010). A recent study showed that inoculating *Rana mucosa* frogs with *Janthinobacterium lividum* (a species of bacteria that produces antimicrobials) attenuated morbidity and mortality due to *Bd* infections (Harris et al, 2009). Another study found that *R. muscosa* populations with high proportions of anti-*Bd* skin bacteria saw better survival than *R. muscosa* populations with lower proportions of anti-*Bd* skin bacteria (Lam et al, 2010). This research supports the hypothesis that the

composition of the microbiome could be indicative of a species' ability to tolerate *Bd* infections (Bletz et al, 2013).

The boreal toad (*Anaxyrus boreas*) is found in the southern Rocky Mountains at high elevations (7,000-12,000 feet). Females typically lay 3,000-8,000 eggs during the breeding season from May to July. The larval amphibians take about two months to fully metamorphose into terrestrial adults (Hammerson, 1999). This species has experienced dramatic population declines in the last decade due to the chytrid fungus pandemic (Pilliod et al, 2010). They are now listed as an endangered species in New Mexico and Colorado.

INTRODUCTION

We know that amphibian species have distinct skin bacterial communities, even when they cohabit the same environment (McKenzie et al, 2012), and that larval amphibians have bacterial skin communities that are distinct from adults (Kueneman et al. 2014). Research has also shown that late stage larval amphibians and adult amphibians have skin bacterial communities that are distinct from the microbial communities of the environment (Walke et al, 2014). This then raises the question about the assembly of the skin bacterial community and when in development it differs from the surrounding environment. Previous studies have indicated that the tadpole stage of development is unique in its microbial composition. For example, the tadpole stage has been found to have the highest proportion of bacterial **OTUs** of known fungal inhibitors (Kueneman et al, 2013). However, many studies of the amphibian microbiome have not teased apart this early stage in development.

The overarching question of this study is: how do the skin bacterial communities of early boreal toads (*Anaxyrus boreas)* assemble under natural conditions? To answer this question, we

undertook an approach that involved observing natural populations of boreal toads as they were undergoing development from the egg stage through metamorphosis by sampling them at different points in their development. Specifically, we are addressed:

Q1: Do the bacterial communities of the earliest lifestages of boreal toads resemble the bacterial communities of their environment?

Q2: How does the *A. boreas* microbiome change throughout development, in terms of diversity and composition?

Q3: How do these patterns inform further research focused on the functioning of these microbial communities throughout amphibian development?

MATERIALS AND METHODS

Study system and field collections

During the 2015 summer field season (June-August), we sampled boreal toads (*Anaxyrus boreas*) from three freshwater habitats in Chaffee County, Colorado: Four Mile, South Cottonwood, and Denny's Creek (Table 1). These sites harbor some of the remaining robust populations of boreal toads (*Anaxyrus boreas*) in Colorado. Amphibians were collected at these sites approximately every two weeks throughout the summer. Individuals were sampled within an hour of collection. The life stage of each amphibian is provided in Table 1. Boreal toads from various developmental stages (eggs, tadpoles, metamorphs, subadults, and adults) were sampled. Permits and authorization were granted by the Colorado Parks and Wildlife department and the University of Colorado Institutional Animal Care and Use Committee (permit #: 1505.04). All *A. boreas* individuals were handled with new nitrile gloves and gloves were changed between each speciman. Before collecting a skin swab from the specimen, the individual was rinsed twice with 50mL of sterile water to rinse away any transient microbes and to ensure that only skin-associated microbes remained (McKenzie et al. 2012). Toads were then sampled using sterile

cotton inoculating swabs that were brushed over each toad's ventral surface and limbs. These swabs were placed back into their sterile vials, stored on ice in a cooler, and transferred to a -20°C freezer prior to DNA extraction.

DNA extraction, sequencing, and bioinformatics

The MoBio Power Soil Extraction kit and protocol were used for DNA extraction. The sample was prepared by adding the sample to the bead tube along with SDS solution which was then vortexed. The cells were then **lysed** by adding two inhibitor remover solutions and incubating at 4°C. The DNA was then bound by adding a highly concentrated salt solution, washed by adding an ethanol based solution and **eluted** via a sterile elution buffer. The PCR primers F515 and R806 were used to isolate the V4 region of the 16S rRNA for bacterial identification. **PCR** conditions were as follows: **denaturation** at 94°C for 3 min, 35 cycles at 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s, and final **extension** at 72°C for 10 min. The PCR was performed in triplicate (3 trials for each sample) and was combined after **amplification** (75 uL total). Quant-IT Picogreen dsDNA reagent was used to quantify **amplicons**. Equal concentrations of each amplicon were then combined into one sample per plate. MoBio UltraClean PCR clean-up DNA purification kit was used to clean these samples and then they were once again quantified using PicoGreen reagent and equal concentrations were again combined. A NanoDrop spectrophotometer determined the purity and DNA concentration of the sample before final DNA sequencing. The samples were **sequenced** using an Illumina MiSeq instrument at the BioFrontiers Institute Next-Generation Genomics Facility at the University of Colorado, Boulder.

The amplicons were sequenced and produced 150 **bp** reads. The program QIIME v1.9.0 was used first to filter for quality, then for sequence analysis. Bacterial OTU's were assigned

using the 13.5 version of the Greengenes reference and the UParse algorithm. An OTU is an 'operational taxonomic unit', a term that is commonly used when referring to different bacteria. OTUs are typically determined by 97% sequence similarity of the 16S ribosomal RNA marker gene. All OTUs which had fewer than 0.0005 percent of the reads, or those only present in one sample, were filtered out, which left 5,452,606 sequences remaining, which clustered into 1,807 unique OTUs with a 97% confidence interval using the RDP Classifier.

Analysis Methods

Does the microbiome of early amphibians resemble the environment?

One of our aims is to determine if microbiome composition in early larval stages of amphibians reflects the environment the larval stages develop within. In order to analyze **beta-diversity**, any samples with fewer than 7,800 sequences were removed. This resulted in 208 samples in the final data set (Table 1). We then used the **UniFrac algorithm** to determine similarities in microbiome composition between early life stages and the environment. These beta-diversity patterns were visualized via **PCoA plots** for the two sites for which we had samples from eggs and early tadpole life stages as well as the environment (4 Mile and Denny's Creek). The South Cottonwood site lacked egg and early tadpole samples, and was therefore excluded from this particular analysis (Figure 1). In order to determine the significance of the similarities in the microbiome composition between larval amphibian and environment we employed a single factor ANOSIM (an analysis of similarity) to compare the community similarity between environmental samples, eggs, and early tadpoles.

Does the diversity of the bacterial community change throughout development?

In order to determine how the diversity of the bacterial community changes throughout amphibian development, we analyzed the **alpha diversity** of all of the samples from all sites.

Alpha diversity was calculated at the bacterial OTU level using the Shannon metric for all developmental stages in QIIME. The calculated alpha diversity was represented with a box and whisker plot made in the program R (version 3.2.3) (Figure 2).

Does the composition of the microbiome change throughout development?

To examine what OTUs were present on different life stages we visualized the proportional relative abundance of all OTUs throughout developmental time. The OTUs are clustered by bacterial order and represented as differently colored stacked bar plots using the R statistical programming language (version 3.2.3, package: ggplot2) (Figure 3).

RESULTS

Sequencing resulted in 6,752,156 total sequences and 133,815 unique OTUs before filtering in QIIME. Bacterial OTUs were primarily found, but some **microeukayotic** OTUs were also observed despite the use of 16S sequencing. The *Anaxryus boreas* skin microbiome was primarily composed of the orders Burkholderiales, Actinomycetales, Flavobacteriales, and Stramenopiles (Figure 3). The bacterial orders most commonly found in the environment and egg samples consisted of the bacterial order Burkholderiales and the microeukaryotic order Stramenopiles. Burkholderiales was the dominant order found on tadpoles, other orders were rare. OTUs belonging to orders Flavobacteriales, Burkholderiales, and Sphingomonadales were found on the metamorphic life stage (Figure 3). Subadult and adult toads were found to have large communities of Actinomycetales and smaller communities of Burkholderiales. The microeukaryotic order Stramenopiles were found in all life stages, however the abundance decreased as developmental time went on (Figure 3).

Do the microbiomes of early amphibians resemble the environment?

To answer this question, we conducted an analysis of bacterial beta-diversity patterns from the environment, eggs, and early tadpoles at the sites 4 Mile and Denny's Creek. The South Cottonwood site was excluded due to an absence of samples from egg stages and early tadpoles. A PCoA plot (Figure 1) was constructed from this analysis in order to visualize data trends. Figure 1 shows that lake water, sediment, eggs, and early tadpoles are different from each other. The largest difference appears to be between early tadpoles and the egg and environmental samples, as they are separated along the PC1 axis (which accounts of 39% of the variability in the data). It appears that eggs and sediment are slightly different, as they are separated along the PC2 axis (21% of the variability). This trend is statistically significant (ANOSIM, **p-value**: 0.001) despite the two lake water outlier samples which were most likely due to sampling or sequencing issues.

Does the diversity of the bacterial community change during amphibian development?

This question required examination of the alpha diversity of life stages. To do this, a Shannon index (which measures bacterial evenness and richness) was used and a box and whisker plot was generated to visualize this data (Figure 2). The bacterial community has high diversity in the egg stage and the environmental samples. However, as developmental time goes on, the alpha diversity dramatically declines. For example from tadpole 23-25 to tadpole 36-39 the diversity is the lowest relative to other life stages. Alpha diversity begins to increase again starting at the metamorph, subadult, and adult life stages becoming intermediately diverse. The general trend of alpha diversity is illustrated in Figure 4.

How does the composition of the microbiome change throughout developmental time?

In order to examine the change in bacterial community composition over developmental time, a stacked bar plot representing the proportional abundance of various OTUs grouped by order was constructed (Figure 3). The figure shows changes in the proportion of Burkholderiales, Stramenopiles, and Actinomycetales over developmental time. Burkholderiales populations steadily increase during the early tadpole stage before reaching a peak at the stages tadpole (25-27) to tadpole (36-39). As the amphibian enters the metamorph stage of development the population sharply decrease and continue to steadily lower. The general trend of the Burkholderiales is summarized in Figure 5. Stramenopiles seem to steadily decrease in proportional abundance as developmental time goes on, with the highest proportion in the egg stage and the lowest in the adult stage. This trend is summarized in Figure 6. Actinomycetales increase as developmental time goes on, comprising a small amount of the proportional abundance in early lifestages (such as eggs and early tadpoles: 23-25) and non-existent between the stages of tadpole (25-27) to tadpole (36-39). The order Actinomycetales reappear in the metamorph stage, then becomes the predominant OTU in the subadult and adult phase of development. This trend is summarized in Figure 7.

DISCUSSION

Our knowledge about the amphibian microbiome has grown in the last decade. However, there are still gaps in our knowledge that must be addressed. One of these gaps is how the unique process of metamorphosis effects the composition and dynamics of the developmental microbiome. This study indicates that the natural amphibian microbiome is dynamic. The composition of the microbiome changes, with some OTUs appearing and disappearing throughout development, but in a predictable and consistent manner across *A. boreas* individuals

and across sites. Between the egg and adult life stages, there appear to be three distinct 'colonization events' (Figure 8). Though these findings require more research to confirm whether this pattern is consistent across amphibians broadly, they may have important implications for *Bd* mitigation strategies.

Do the earliest lifestages (e.g., eggs and early tadpoles) resemble the bacterial communities of their environment?

The data suggests (Figure 1) that the bacterial communities on eggs largely overlapped with their environment, however as the tadpole emerges from the egg and begins to move freely in the environment the bacterial communities begin to diverge. The toad egg stage and the environment had similar levels of bacterial diversity (Figure 2). The bacterial diversity found in sediment, lake water, and on the outer surface of toad eggs are all the highest relative to later life stages of the toads. Eggs and environmental samples also have similar microbiome compositions (Figure 3); lake water, sediment and eggs have comparable proportions of Stramenopiles, Burkholderiales, Rhizobiales, Shingomonadales, Actinomycetales, and Flavobacteriales.

How does the microbiome change throughout development, in terms of diversity and composition of the microbiome?

When examining the changes in diversity and composition of the skin microbiome found throughout amphibian life stages, it becomes clear that there are three distinct points of bacterial community change over the course of developmental time. These considerable changes in the bacterial communities seem to correlate with substantial points in development where the organism undergoes dramatic physiological and anatomical changes: when the tadpole emerges from the egg, when the tadpole transitions into a metamorph, and when the metamorph reabsorbs

its tail and shifts from the aquatic to the terrestrial environment. The first recolonization event seems to occur when the tadpole leaves the protective coating of the egg and begins to move through the environment (Figure 8). The microbiome on the outer surface of the amphibian egg is the most diverse of any life stage (Figure 2); it closely resembles its environment with the largest proportion of microbes being eukaryotic Stramenopiles (Figure 3). As the tadpole emerges it begins to diverge from the environmental bacterial community (Figure 1). The tadpole microbiome also becomes more restricted as bacteria on the tadpole appear to colonize in a selective fashion (Figure 2). The second recolonization event (Figure 8) occurs when the tadpole sheds its skin while transitioning into metamorph form, the microbes present on the tadpole skin are largely lost and the new metamorph becomes susceptible to a wide range of potential colonizing microbes (Figure 3).

How do these patterns inform further research focused on the functioning of these bacterial communities through amphibian development?

It is known that *Bd* infections most commonly occur during the metamorph stage of development (Kilpatrick et al, 2010). The timing of infection has been attributed to the emergence of keratinized skin cells, however this study suggests that a dramatic shift in the diversity and composition of the microbiome may also play a role in *Bd* susceptibility. For example, Burkholderiales proportionally make up the majority of the tadpole microbiome, but during the second recolonization event the proportion of Burkholderiales dramatically declines (Figure 3). Many Burkholderiales OTU's are anti-fungal and may have disease preventive roles (Woodhams et al, 2016). If these microbes provide protection against fungal pathogens like *Bd*, tadpoles would seem to be the most protected from *Bd* infection and metamorphs seem to be most vulnerable.

This study indicates that at some points in development the host has varying degrees of **colonizability** (Figure 9). Eggs have a high amount of **microbial richness** of symbiotic microbes (Figure 2), a fairly balanced **microbial evenness** (Figure 3), and have a relatively high colonizability. This pattern is evident at the 'order' taxonomic level, as seen in Figure 3. Tadpoles have a very low species richness (Figure 2), a low species evenness (Figure 3), and are the least colonizable life stage. Adults and subadults have moderate species richness and evenness (Figure 2, 3) and are relatively moderate in colonizability. It is unclear if there is a relationship between microbiome evenness/richness and colonizability.

In the past some experimental probiotic treatments have been successful, while others have failed for unknown reasons (Becker et al, 2011). Our data seems to indicate that there are distinct periods of microbial recolonization in amphibian development and that the success of adding beneficial microbes may depend largely on the timing of the introduction. Further research should include experiments testing the timing of probiotic introduction on *Bd* disease resistance. Another question that this research highlights is how the colonizability of hosts is determined. Though interspecific microbial interactions are most likely a large part of determining which microbes are selected for, the host may also be a determining factor. Host molecules (hormonal, immune, etc.) may inhibit or promote certain microbial species establishment and success on the host's skin.

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Committee Members: Dr. Pieter Johnson, Dr. Valerie McKenzie, Dr. Theresa Foley

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Anurans: An order of amphibians composed of frogs, toads, and tree frogs.

Keratinized: A process where epithelial cells are filled with keratin proteins n tissue transforms into a form containing keratin (a structural protein).

Epithelium: A type of animal tissue.

Zoospores: A motile asexual spore, used by fungi to propagate themselves

Zoosporangia: A protective case in which the zoospore develops.

Chytridiomycosis: An infectious disease caused by the chytrid fungus *Bd*

Osmoregulation: A balance of an organism's fluid and electrolytes concentration. Regulation occurs to insure the fluids to do not become too concentrated or too dilute.

Extant: In existence (as opposed to extinct).

Probiotic: Microbes intentionally added to an individual to increase the organism's health.

Bioaugmentation: The process of intentionally adding microbes to an organism's body in attempt to enhance its health.

OTUs: An 'operational taxonomic unit' determined by 97% sequence similarity of the 16S ribosomal RNA marker gene.

Lysed: Breaking open a cell.

Eluted: The separation of one material from another.

PCR: The polymerase chain reaction is a technology used to replicate a particular piece or strand of DNA.

Denaturation: The process when a protein is unfolded.

Extension: The process in PCR where the Taq polymerase adds nucleotides to the primer.

Amplification: A massive replication of a specific piece of DNA.

Amplicons: A piece of DNA that has been artificially replicated/amplified.

Sequenced: The process of determining the order of nucleotides in a specific region of DNA

bp: Short for 'base-pairs', how many nucleotides pairs are connected in double stranded DNA.

Beta-diversity: A comparison of microbial samples to one another, determines the distance/dissimilarity between every pair of samples collected.

UniFrac algorithm: A distance/dissimilarity measurement, a weighted UniFrace algorithm uses the abundance information of OTUs and phylogeny to calculate the measurement.

PCoA plots: A 'Principal Coordinates Analysis' visualizes the results of the beta-diversity distance matrix.

Alpha diversity: Used to measure the diversity in a sample, this study used a Shannon metric which measures the richness and evenness of a sample.

p-value: A measurement of statistical significance, a low p-value (like the one used in this study) indicates that the hypothesis 'microbiome composition is dependent on developmental stage' is true.

FIGURES AND TABLES

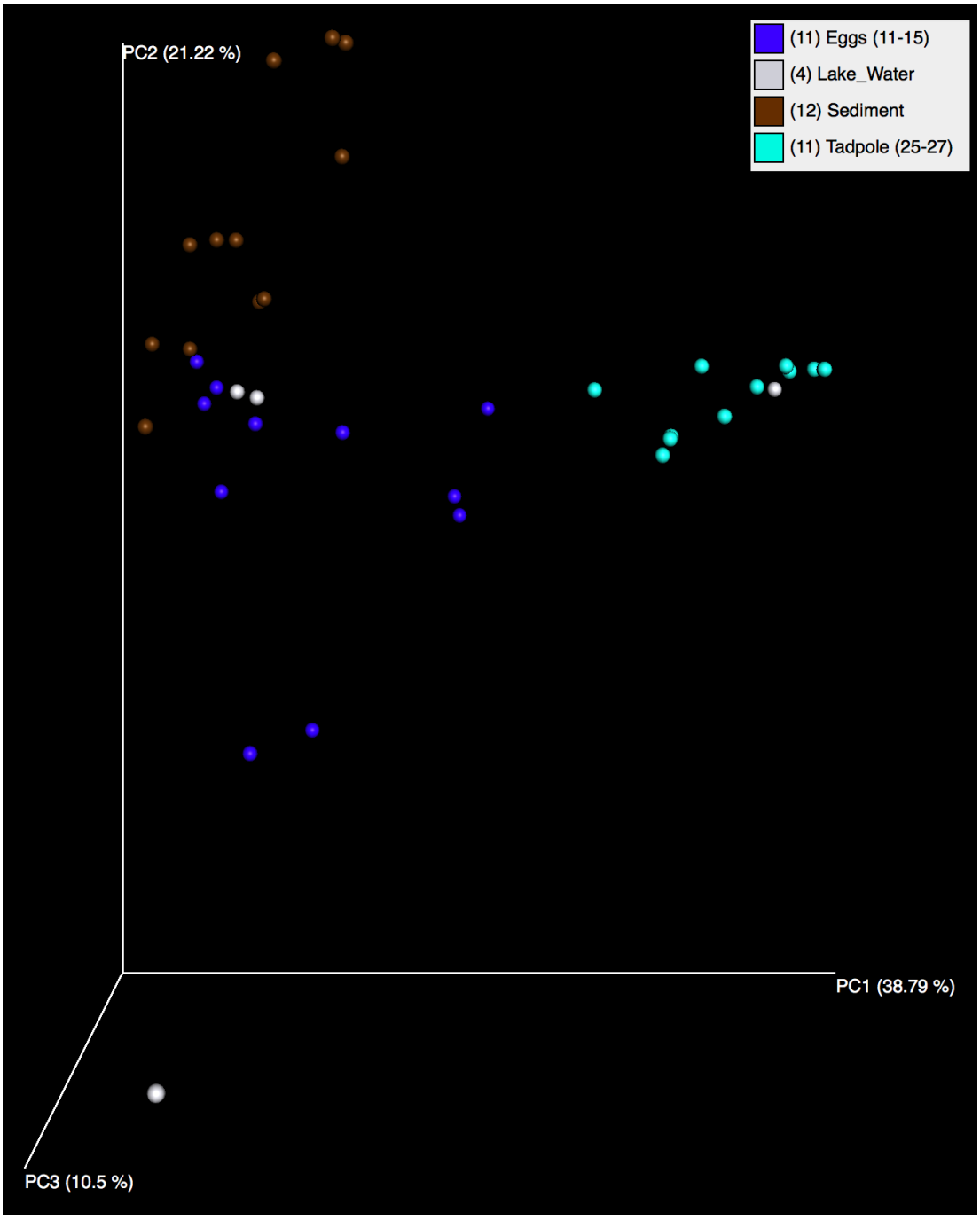

***Figure 1**: A PCoA plot visualizing the b-diversity distance matrix, the maximum amount of variation in the data set is found on the PC1 axis where environmental and egg samples are being pulled apart from early tadpole samples.*

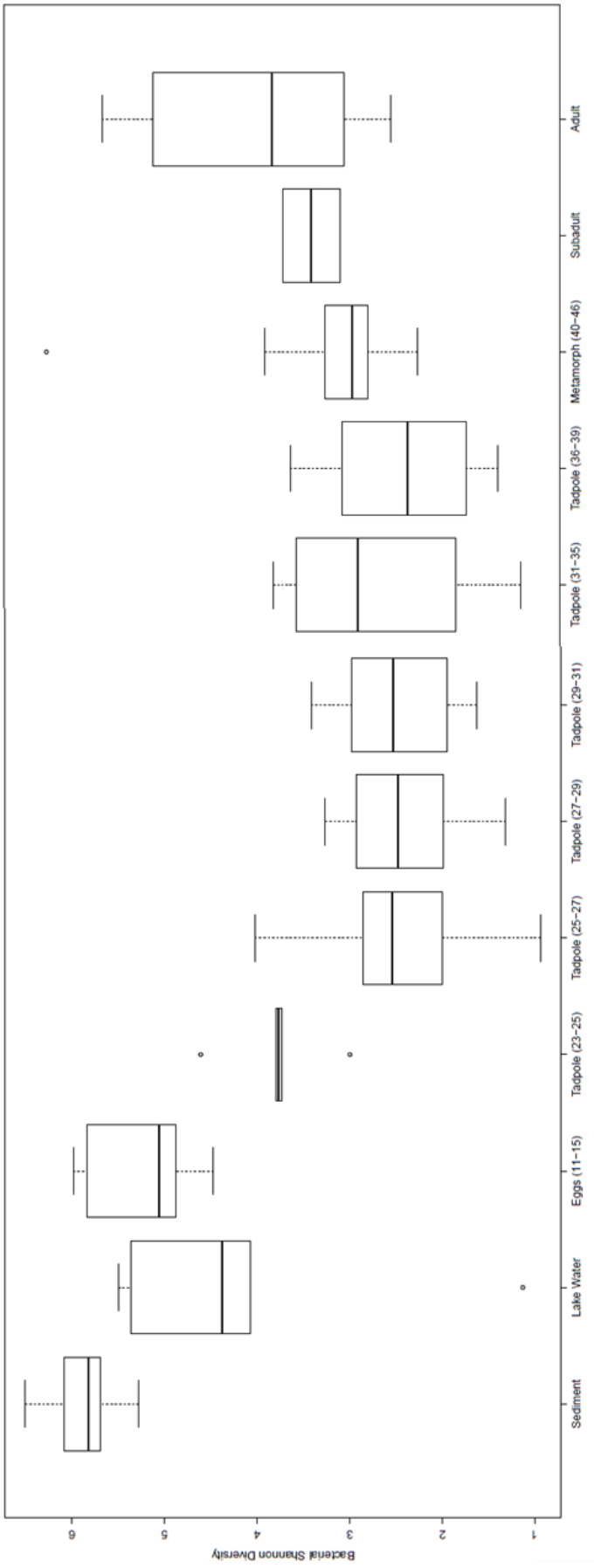

Figure 2*: A box and whisker plot of the bacterial Shannon diversity across life stages.*

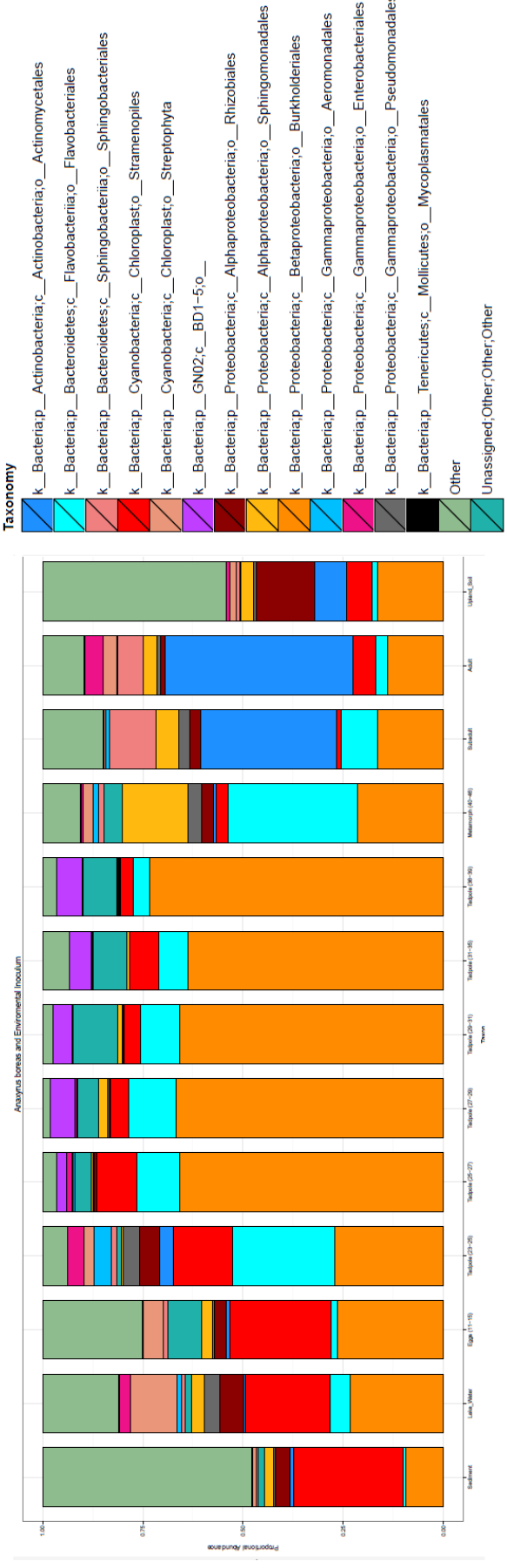

***Figure 3**: A stacked bar plot of proportional OTUs found on various Boreal toad lifestages.*

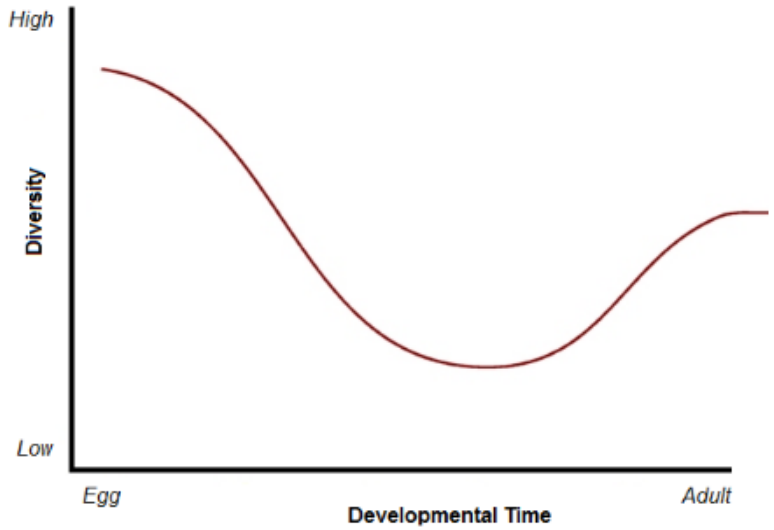

Figure 4 *An illustration of the general trend of microbial diversity based on Figure 2.*

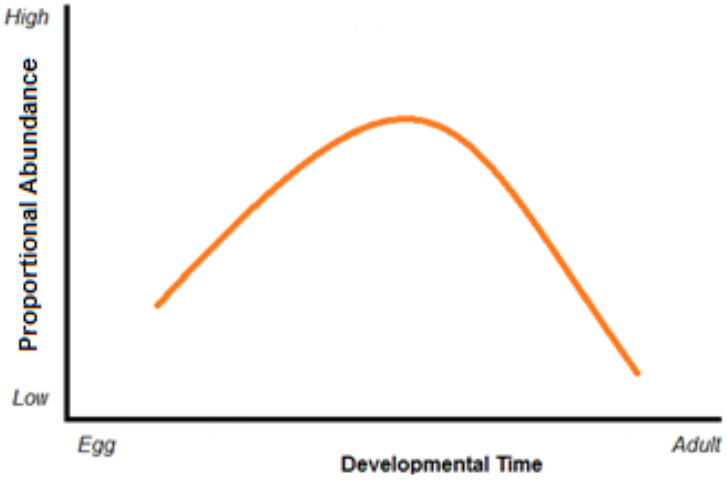

Figure 5: *A summary of the trend of Burkholderiales over developmental time.*

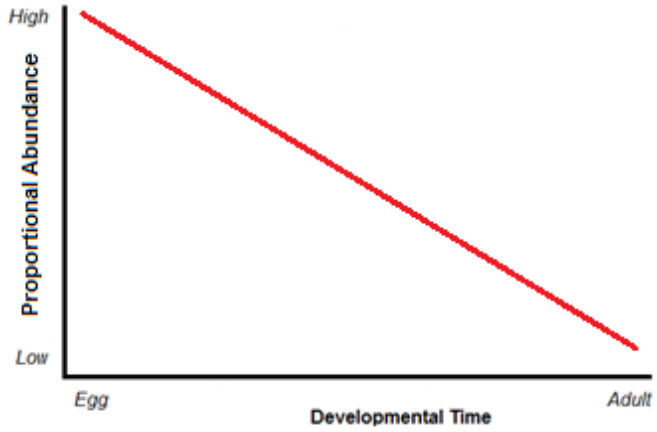

***Figure 6**: A summary of the trend of Stramenopiles over developmental time.*

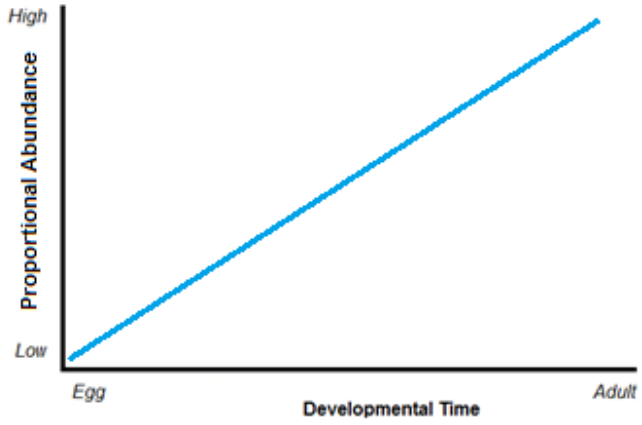

***Figure 7**: A summary of the trend of Actinomycetales over developmental time.*

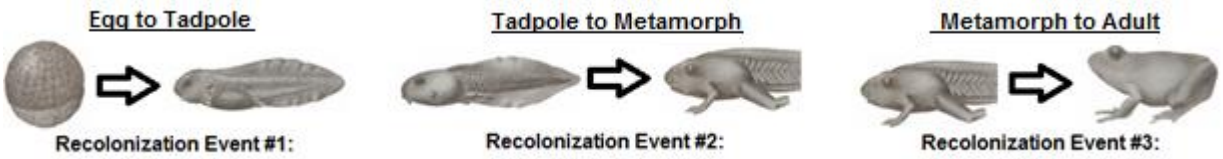

***Figure 8**: A representation of the three recolonization events in amphibian metamorphosis.*

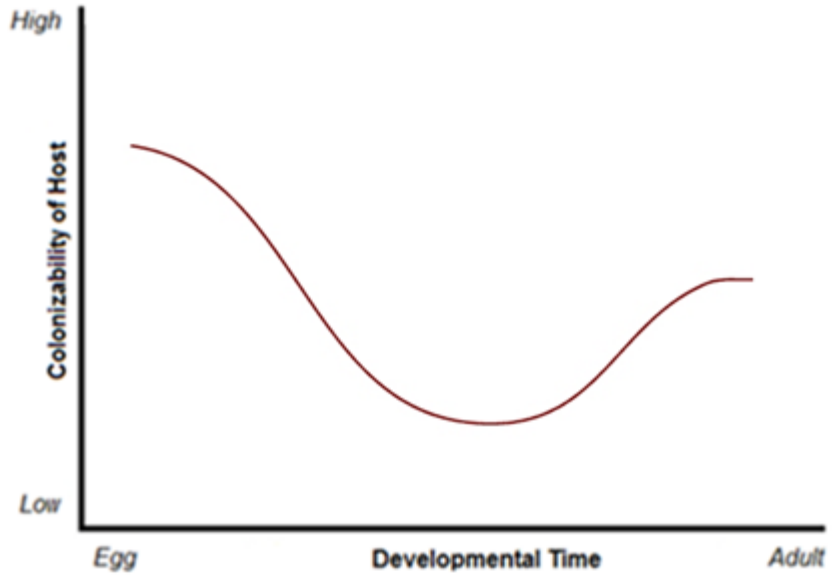

Figure 9*: A representation of the colonizability (a measure of microbial establishment) of hosts over developmental time*

Sample Type	***Site ID***		
	4 Mile	*South Cottonwood*	*Denny's Creek*
Upland Soil	0	3	0
Sediment	6	11	7
Lake Water	5	9	7
Eggs (11-15)	9	0	3
Tadpole (23-25)	0	9	0
Tadpole (25-27)	6	6	7
Tadpole (27-29)	3	16	25
Tadpole (29-31)	2	5	6
Tadpole (31-35)	3	6	0
Tadpole (36-39)	7	0	6
Metamorph (40-46)	2	32	0
Subadult	2	0	0
Adult	6	0	10

Table 1: *Anaxyrus boreas boreas sampling scheme from three sites in Chaffee County, CO.*

**Amphibian life stages were estimated using Gosner stages (Gosner, 1960, Table 2) and are shown in parentheses, only individuals included in the final analysis are represented (i.e., those samples for which quality sequences were obtained).*

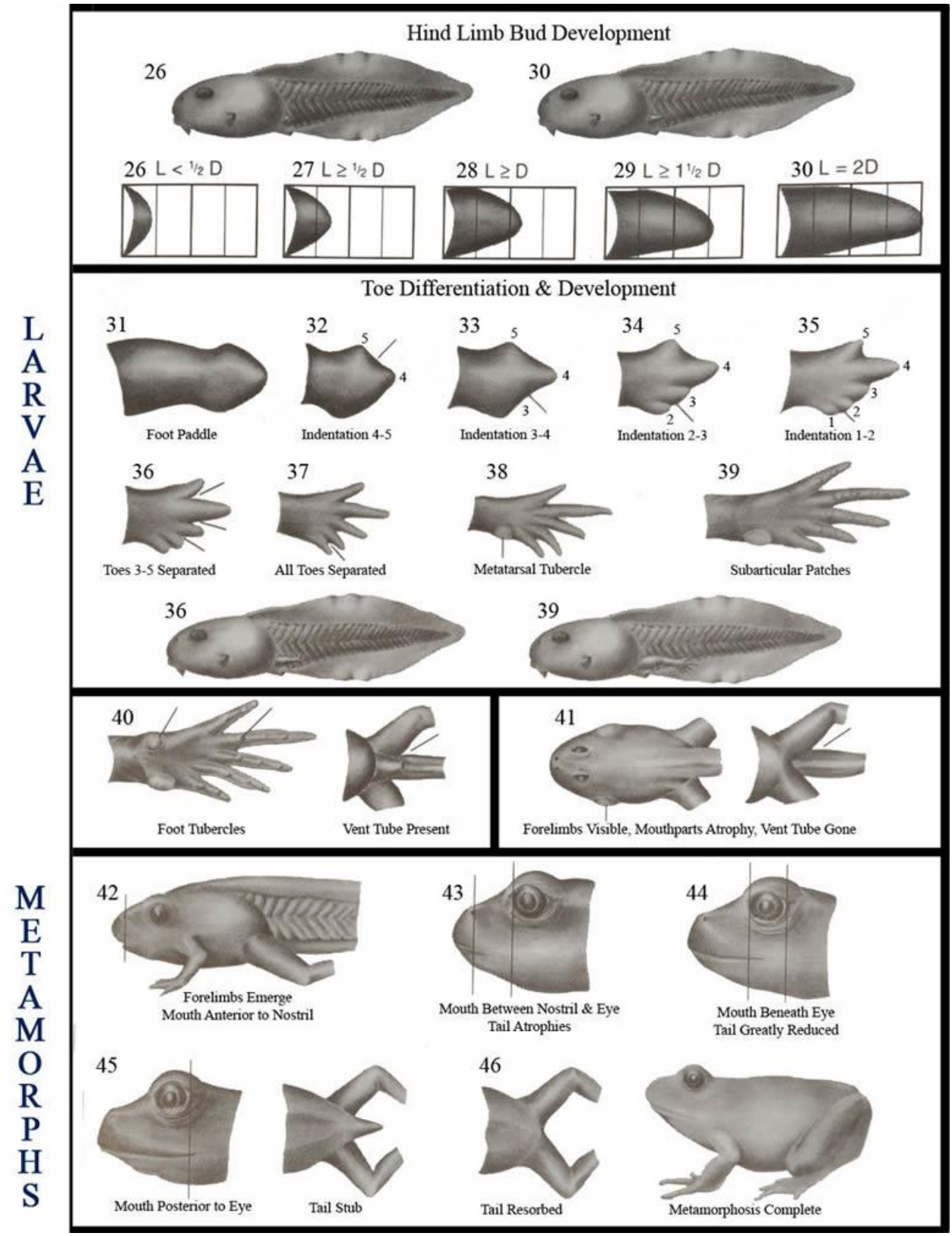

Table 2: *The Gosner staging table*

REFERENCES

1. Becker, M.H., Harris, R.N., Minbiole, K.P., Schwantes, C.R., Rollins-Smith, L.A., Reinert, L.K., Brucker, R.M., Domangue, R.J. and Gratwicke, B., 2011. Towards a better understanding of the use of probiotics for preventing chytridiomycosis in Panamanian golden frogs. Ecohealth, 8(4), pp.501-506.

2. Bletz MC, Loudon AH, Becker MH, Bell SC, Woodhams DC, et al. (2013) Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. Ecol Lett 16: 807– 820.

3. Brucker, R. M., Harris, R. N., Schwantes, C. R., Gallaher, T. N., Flaherty, D. C., Lam, B. A., & Minbiole, K. P. (2008). Amphibian chemical defense: antifungal metabolites of the microsymbiont Janthinobacterium lividum on the salamander Plethodon cinereus. Journal of chemical ecology, 34(11), 1422-1429.

4. Budtz, P. E., & Larsen, L. O. (1975). Structure of the toad epidermis during the moulting cycle. *Cell and tissue research*, *159*(4), 459-483.

5. Foster, J. A., & Neufeld, K. A. M. (2013). Gut–brain axis: how the microbiome influences anxiety and depression. Trends in neurosciences, 36(5), 305-312.

6. Gosner, K. L.. (1960). A Simplified Table for Staging Anuran Embryos and Larvae with Notes on Identification. *Herpetologica*, *16*(3), 183–190.

7. Greven, H., Zanger, K., & Schwinger, G. (1995). Mechanical properties of the skin of Xenopus laevis (Anura, Amphibia). *Journal of morphology*, *224*(1), 15-22.

8. Hammerson, G. A. (1999). Amphibians and reptiles in Colorado. University Press of Colorado; Colorado Division of Wildlife.

9. Harris, R. N., Brucker, R. M., Walke, J. B., Becker, M. H., Schwantes, C. R., Flaherty, D. C., ... & Minbiole, K. P. (2009). Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. The ISME journal, 3(7), 818-824.

10. Kilpatrick, A. M., Briggs, C. J., & Daszak, P. (2010). The ecology and impact of chytridiomycosis: an emerging disease of amphibians. Trends in Ecology & Evolution, 25(2), 109-118.

11. Lam, B. A., Walke, J. B., Vredenburg, V. T., & Harris, R. N. (2010). Proportion of individuals with anti-Batrachochytrium dendrobatidis skin bacteria is associated with population

12. Lee, Y. K., & Mazmanian, S. K. (2010). Has the microbiota played a critical role in the evolution of the adaptive immune system?. Science, 330(6012), 1768-1773.

13. McKenzie, V. J., Bowers, R. M., Fierer, N., Knight, R., & Lauber, C. L. (2012). Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. The ISME journal, 6(3), 588-596.

14. Pilliod, D. S., Muths, E., Scherer, R. D., Bartelt, P. E., Corn, P. S., Hossack, B. R., ... & Gaughan, C. (2010). Effects of amphibian chytrid fungus on individual survival probability in wild boreal toads. Conservation Biology, 24(5), 1259-1267.

15. Stuart, S. N., Chanson, J. S., Cox, N. A., Young, B. E., Rodrigues, A. S., Fischman, D. L., & Waller, R. W. (2004). Status and trends of amphibian declines and extinctions worldwide. Science, 306(5702), 1783-1786.

16. Voyles, J., Young, S., Berger, L., Campbell, C., Voyles, W.F., Dinudom, A., Cook, D., Webb, R., Alford, R.A., Skerratt, L.F. and Speare, R., 2009. Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. Science, 326(5952), pp.582-585.

17. Walke, J. B., Becker, M. H., Loftus, S. C., House, L. L., Cormier, G., Jensen, R. V., & Belden, L. K. (2014). Amphibian skin may select for rare environmental microbes. The ISME journal, 8(11), 2207-2217.

18. Woodhams, D. C., Bletz, M., Kueneman, J., & McKenzie, V. (2016). Managing Amphibian Disease with Skin Microbiota. *Trends in microbiology*,*24*(3), 161-164.

19. Woodhams, D. C., Brandt, H., Baumgartner, S., Kielgast, J., Küpfer, E., Tobler, U., ... & Knight, R. (2014). Interacting symbionts and immunity in the amphibian skin mucosome predict disease risk and probiotic effectiveness. PLoS One, 9(4), e96375.

20. Zhu, L., Wu, Q., Dai, J., Zhang, S., & Wei, F. (2011). Evidence of cellulose metabolism by the giant panda gut microbiome. Proceedings of the National Academy of Sciences, 108(43), 17714-17719.