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The genetics of metamorphosis: expression profile across three life stages in *Lithobates catesbeianus* shows the enrichment of 29 gene sets using rotational gene set testing

By
Alicia M. Latta

A Thesis
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Committee Approval

To the Graduate Faculty:

The members of the committee appointed to examine the thesis of **Alicia M. Latta** find it satisfactory and recommend that it be accepted.

_____

Curtis W. Anderson, Ph.D.
Major Advisor

_____

Michael Thomas, Ph.D.
Committee Member

_____

Michele Brumley, Ph.D.
Graduate Faculty Representative

Idaho State
UNIVERSITY

Office for Research Integrity
921 South 8th Avenue, Stop 8046 • Pocatello, Idaho 83209-8046

March 8, 2016

Curtis Anderson, Ph.D.
Mail Stop 8007
Biological Sciences
Pocatello, Id 83209

RE: Your application dated 2/22/2016 regarding study number 741: Neurotransmitters in the developing brainstem of the bullfrog, Rana catesbiana

Dear Dr. Anderson:

Your request for approval of the new protocol listed above was reviewed at the 3/8/2016, meeting of the Idaho State University IACUC. This is to confirm that your protocol was approved. Your protocol number is 741.

You are free to proceed with your study as described in your protocol effective immediately.
The study is subject to annual review on or before 3/8/2017, unless closed before that date.

Please note that any changes to the protocol as approved must be immediately reported and approved. Contact me (208-282-2179; fax 208-282-4723; email: anmlcare@isu.edu) if you have any questions or require further information.

Sincerely,

Tom Bailey
IACUC Coordinator

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Exploring changes in the rate of expression of 54 gene sets during the metamorphic process in *Lithobates catesbeianus*

Thesis Abstract
Idaho State University (2016)

Metamorphosis is the process of transforming from larval to adult form. This extensive change can be explored using a model organism, the American bullfrog (*Lithobates catesbeianus*). To date, very little research has looked at the transcriptional changes occurring in amphibians as they move through the metamorphic process. The goal of our research is to investigate the changes in the rate of gene set expression during the three stages of life in the bullfrog, providing a basis to understanding the genetic alterations occurring during the metamorphic process. Using ROAST, rotational gene set analysis, we studied changes in gene set expression of 54 gene sets we hypothesize to be associated with the metamorphic process. When we compared expression rates between stage one and stage three, we identified four gene sets were significantly down-regulated and 25 gene sets were significantly up-regulated. Comparison between stage two and stage three animals yielded 24 significantly up-regulated gene sets and four significantly down-regulated gene sets. Some of these enriched gene sets include genes for nervous system development (e.g. *axonogenesis*, *neurite development* and *neuron differentiation*), tissue and organ development (e.g. *organ development* and *system development*), *growth factor activity*, *regulation of growth*, and cell and organism responsiveness to stimuli (e.g. *response to abiotic stimulus*, and *response to stress*). Down-regulated gene sets include *response to UV*, *glutamate receptor activity* and *glutamate signaling pathways*, and

feeding behavior. This study allows us to provide an explanation of the morphologic changes occurring during metamorphosis from a genetic basis. The major changes associated with the metamorphic process correspond with an up-regulation of gene expression later in life.

CHAPTER 1

INTRODUCTION

Alterations to the expression of several genes must occur to result in the phenotypes observed during the development of an organism. To date, very little research has looked at the extent to which gene expression is altered in amphibians during the metamorphic process. Our goal was to determine how the expression of the transcriptome changes during the development of the American bullfrog, *Lithobates catesbeianus*. This will provide a more thorough understanding of the genetic alterations that occur to anurans during the metamorphic process and, possibly other amphibian species. Metamorphosis is the process of transforming from an immature form to an adult form {Duellman 1986}. The bullfrog can spend up to two years in the larval (tadpole) stage. Once triggered to do so, tadpoles begin a metamorphic process that prepares them for terrestrial life {Gosner 1960}. Believed to be cued through environmental triggers, the tadpole's brain will signal for an increase in thyroid hormone production. This elevation of thyroid hormones and the decrease in prolactin lead to the dramatic changes observed throughout metamorphosis {Duellman 1986}. Metamorphosis is characterized by modification to all major organs. As the tadpole approaches metamorphic climax the appearance of forelimbs and hind limbs occur while the tail, the primary means of locomotion, begins to recede {Duellman 1986}. Other changes include alteration to sensory organs, the elimination of gills in exchange for lungs, shortening of the intestinal tract and alterations in hemoglobin structure.

Studies have been conducted to investigate physiological and morphological changes during the stages of metamorphosis as well as the expression of individual genes {Ishizuya-Oka 2010: Ishizuya-Oka 2011: Yamane 2013} however limited research has gone into examining the changes in the rate of expression of entire gene sets. For example the expression of *myf5* (myogenic factor 5) is found to be up-regulated in the adult frog as compared with the tadpole {Yamane 2013}. This gene is crucial in regulating muscle differentiation {Yamane 2013}. Studying many genes related to a single function is very beneficial. The expression of individual genes has been linked to numerous diseased phenotypes. A couple examples include HBB (hemoglobin subunit beta) gene and sickle cell disease or HEXA (hexosaminidase subunit alpha) gene and Tay-Sachs disease in humans {Mestrovic 2015: Ngo 2015: Triggs-Raine 1991}. While these Mendelian or single gene disorders do occur, the vast majority of phenotypes are created through the combined expression of many genes and the interactions of their products {Bateman 2014}. Gaining a better understanding of the genetic basis of metamorphosis can be highly beneficial to many areas of study. This study can strengthen the knowledge of genetic alterations possibly causing the observed morphology and behavioral patterns at different life stages of the amphibian from a genetic standpoint. Last, the study can provide a foundation to understanding metamorphosis in other amphibians.

In the following chapter, "The genetics of metamorphosis: expression profile across three life stages in Lithobates catesbeianus shows the enrichment of 29 gene sets using rotational gene set testing" we used rotational gene set analysis, ROAST, to study changes in the expression of 54 gene sets we hypothesize to be associated with changes

observed during amphibian metamorphosis. Using this type of analysis we are able to determine the extent to which gene sets are up-regulated or down-regulated before metamorphosis begins, during the metamorphic process, or once the animal has reached full adulthood. We expect genes related to development and growth to be up-regulated in tadpoles during metamorphosis and adult frogs, with lower expression rates in tadpoles that have yet to enter metamorphosis. Because major changes occur in almost all aspects of the frog as they develop, it would be expected that changes to the nervous system must occur in order to accommodate this. We expect most gene sets related to nervous system development will be up-regulated in later stages of life. The transition from an aquatic to terrestrial environment presents opportunity to be exposed to additional stimulus, such as drastic changes to temperature and light. We predict that genes associated with cell and organismal activity change in response to various stimuli will be up-regulated in the terrestrial-inhabiting adult frog. Other gene sets include those related to glutamate activity, and feeding behavior. A complete list of hypothesized changes can be found in Table One.

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CHAPTER 2

The genetics of metamorphosis: expression profile across three life stages in *Lithobates catesbeianus* shows the enrichment of 29 gene sets using rotational gene set testing

ABSTRACT

Metamorphosis is the process of changing from larval to adult form. This process has been observed in invertebrates, (e.g. nematodes and insects) and non-mammalian vertebrates (e.g. amphibians like the American bullfrog, *Lithoates catesbeianus*). Using ROAST, rotational gene set analysis, we tested 54 gene sets across three different life stages in the bullfrog. Of these we predicted 50 gene sets would be more highly expressed in adult frogs and 4 gene sets would to be down-regulated in the adult stage. No gene sets were discovered to be enriched in comparing tadpoles who have yet to begin metamorphosis (stage one) and tadpoles currently in the process of metamorphosis (stage two). We observed 29 gene sets enriched in adult frogs (stage three) when compared with pre-metamorphic tadpoles (stage one) and again with tadpoles undergoing metamorphosis. Of these 29, only four gene sets where down-regulated in stage three when compared with stage one animal. One additional gene set was discovered to be down-regulated in stage three when compared with stage two animals. Because the majority of observable phenotypes are created by the combined expression of a multiple genes and the interactions of their products, studying the changes in expression of several

genes within a set is crucial to understanding metamorphosis. This study provides an explanation of the morphologic changes occurring during metamorphosis caused by the interaction of several genes necessary for a specific function. This information can be used to understand the metamorphic process in other species of amphibians (i.e. salamanders, newts, and caecilians). Last, this study produces a foundation for further research in gene set analysis during metamorphosis. In particular it would be highly beneficial to look for gene expression rates at each stage in the developmental process, instead of the three broad life stages.

INTRODUCTION

Metamorphosis is the process of transforming from an immature form to an adult form in two or more distinct stages {Duellman 1986}. This transition can be observed in several taxa, including insects and amphibians. Each life stage of a species undergoing metamorphosis is characterized by a unique trait or function of the individuals {Gilbert 1981}. For example the larval caterpillar is able to inch its way along but not able to travel great distances {Jabr 2012}. However during metamorphosis this organism transforms into an adult butterfly capable of flight {Jabr 2012}.

Amphibian metamorphosis is characterized by transition from an aquatic lifestyle to a terrestrial one {Duellman 1986}. This transition can be noted by individuals moving through three periods: premetamorphosis; prometamorphosis; and metamorphic climax {Gosner 1960}. Those periods are further broken down into stages based on ratios between limb and tail length and other distinct features {Gosner 1960}. The premetamorphic stage is recognized by the growth of hind-limb buds {Gosner 1960}.

Those limbs continue to develop and the tail begins to recede during the prometamorphic stage {Gosner 1960}. Finally, the emergence of forelimbs and complete reabsorption of the tail can be noted in metamorphic climax. In addition to the limbs, changes in the lungs, intestinal tract, eyes, and several other organs occur {Shi 2000: Tata 1993}. These physical changes are linked to physiological and behavioral alterations {Duellman 1986: Ishizuya-Oka 2010}.

Metamorphosis is controlled by the thyroid gland {Gundernatsch 1912}. Environmental stimulation cues the central nervous system to begin the release of precursor hormones until the eventual creation of thyroid hormone (TH) {Denver 2009: Gilbert 2000: Gundernatsch 1912: Paris 2010}. TH can enter cells through several types of membrane transporter proteins {Bowen 2010}. Upon entering the cell, hormones migrate to the nucleus where they bind with nuclear receptors {Bowen 2010}. The now complete hormone-receptor complex binds to thyroid hormone response elements along the DNA strand {Bowen 2010}. This sequence of events allows for either the stimulation or inhibition of transcription of specific genes, dependent on the tissue {Bowen 2010}. Tissues throughout the body that undergo major changes later in metamorphosis (i.e. liver, brain, limb buds, tail) have higher concentrations of thyroid hormone receptor mRNA {Tata 2011}. Increased concentration of TH triggers apoptosis, leading to complete regression of the tail and gill structures through the activation of lytic enzymes such as collagenases, nucleases, phosphatases, and matrix metalloproteinases {Robinson 199: Tata 2011}. Thyroid hormones are associated with a decrease in protein synthesis in the striated muscles of the tail and induction of myosin heavy chain in muscles distributed in limbs {Little 1973: Tata 2011}. Lastly TH has been linked to the

restructuring of the liver, the induction of albumin and urea cycle enzymes, and development within the head, epidermis, and intestines Ishizuya-Oka 2011: Miller 2013: Suzuki 2009: Tata 2011}.

Although research has been conducted to investigate the morphologic changes occurring throughout metamorphosis within amphibians, little has examined changes in gene expression between these metamorphic stages. Understanding the change in expression of gene sets throughout metamorphosis serves to potentially act as an explanation of notable morphologic changes. The changes in genetic expression behind metamorphosis will aid in explaining alterations in morphology and behavioral patterns observed at different life stages of the amphibian. Most importantly, understanding the process of metamorphosis in bullfrogs can provide a foundation to understanding metamorphosis in other amphibian species. Last, creating a transcriptome database for bullfrogs under normal conditions can be used in understanding the role of environmental pollutants on genetic alterations during the metamorphic process.

To date few studies have explored whole transcriptome analysis among all stages of frog development. Using dendrogram analysis, Baldessari's laboratory was able to successfully identify the close relatedness in *Xenopus laevis* between in stage 10, stage 13, and stage 15 embryos and their distinction to feeding tadpoles {Baldessarie 2005}. Those stages refer to staging techniques developed by Nieuwkoop and Faber specifically for the genus *Xenopus* {Segerdell 2013}. Stage 10 is considered a blastocyst and stage 13 and 15 are gastrula {Segerdell 2013}. Langlois's laboratory was able to further support these finding using microarray data {Langlois 2013}. Their work highlighted an increase in gene networks essential for cell division, cell proliferation, and DNA replication in

Nieuwkoop and Farber stage 2 (2-cell organism) with no effect in later stage animals {Langlois 2013}. Gene networks for organ formation and morphogenesis, such as neurotransmitter transport and brain development genes, were found to be more highly expressed in later stages (NF 34) {Langlois 2013}. Note however that these studies only looked at embryological development and not changes that occur upon entering the metamorphic cycle. Other studies have looked at changes in transcript expression in frogs exposed to amphibian pathogens. Up-regulation in adaptive immune response after exposure to pathogens was noted {Price 2015}. Major work on transcriptional differences between tadpoles and adult frogs is necessary to fully understand the developmental process.

This study hypothesizes the rates of gene expression in the brainstem of the American bullfrog, *Lithobates catesbeianus*, will be enriched throughout what we define as three different stages of life. Our methods do not follow staging characterized by Taylor and Kollros for the genus *Lithobates,* formally *Rana*. Instead of defining each stage by morphologic characteristics, we grouped several of the traditional stages together. Stage one animals are tadpoles showing no indication of beginning metamorphosis. Stage two animals have begun to develop hind-limbs, signifying entrance into metamorphosis. Lastly stage three animals are adult frogs that have completed metamorphosis. They have fully developed limbs and the tail is completely receded. We used this means of staging in order to form a better understanding of the gene expressions involved in large periods of life, rather than each individual stage. Additionally, upon entering into the metamorphic process, the animal transforms extremely quickly from the

larval to the adult stage. Each individual stage during the metamorphic process would be extremely hard to accurately gage and extract from in a quick enough time.

Through gene set analysis, we selected and compared expression rates of 54 sets of genes known to be involved with developmental functions in vertebrates. A gene set is a group of genes that share a specific function. We looked specifically at GO or gene ontology sets from the Molecular Signature Database. These sets are involved with three different areas; molecular function, cellular function, or biological process. All gene sets contained a minimum of 20 genes and were selected because of their relatedness to metamorphosis. In particular all gene sets selected contain genes known to be involved with the developmental process or contain genes associated with changes known to occur during the metamorphosis and development. A complete list of the 54 gene sets selected and their specific function are described in Table Two.

We hypothesize that adult stage will have a statistically significant increase in the rate of gene expression of 17 gene sets associated with organ and tissue development as the majority of major organ remodeling and refunctioning occurs toward the end of metamorphosis. We expect enrichment of 10 gene sets related to the nervous system, including *brain development* and *neuron development*. For example *neuronal cell death* would be expected to be up-regulated during stage two and stage three animals in comparison to tadpole who have yet to enter metamorphosis. It has been suggested by White and Nicholl that genes associated with cell death, including neuron apoptosis, are always active, however suppressed during cell growth cycle. In frogs this suppression is caused by prolactin {White 1978}. The rapid input of thyroid hormones during initiation of metamorphosis is able to overcome the suppression of neuronal apoptosis by prolactin

in order to cause appropriate cell death in the amphibian brain {Tomei 1991}. We predict that ten gene sets containing genes associated with growth and the regulation of that process will experience enrichment during the metamorphic process. Because behavioral changes, such as the switch to actively hunting prey, are noted in adult frog versus tadpole we predict enrichment of the expression of ten gene sets containing genes that show response to various stimuli. Movement from an aquatic to terrestrial environment forces the degradation of gills in exchange for lungs, leading us to predict an increase expression of genes within the set *respiratory gaseous exchange* in adult frogs compared with tadpoles. Last, changes in behavioral patterns and gastrointestinal organs caused by metamorphosis leads us to predict enrichment in gene sets *digestion* and *feeding* will occur. Predicated changes for all 54 gene sets can be found in Table One.

MATERIALS AND METHODS

Tissue Extraction

We purchased tadpole and adult bullfrogs from a commercial supplier (Rana Ranch, Twin Falls, Idaho, USA). Animals were maintained in the laboratory within 57-L aquariums until time of tissue extraction. Tadpoles were provided aerated, dechlorinated tap water and adult frogs were given a dry area, and de-chlorinated tap water. All tap water was de-chlorinated with AquaSafe water conditioner (Tetra, Blacksburg, Virginia, USA) and allowed to sit for 24 -hrs before animals were transferred into clean water. This allowed the water to reach room temperature (20-22° C). Tadpoles were fed TetraVeggie algae wafers (Tetra, Blacksburg, Virginia, USA) once a week and adult frogs were fed small to medium size crickets (*Gryllidae*) twice a week. All maintenance and

experimental protocols were approved by the Idaho State University Animal Care and Use Committee (protocol number 711), and followed the National Research Council Committee recommendations {National Research Council Committee 2011}.

We collected samples of brainstem tissue from frogs in one of the life stages previously described (n=3 animals/group). The brainstem was selected because of its role in many functions necessary for life. The brainstem also acts a communicator between the brain and the rest of the body. Upon time of tissue extraction, tadpoles were euthanized through immersion in 0.05% dilute tricaine methanesulfonate, MS-222, (Western Chemical Inc., Ferndale, WA) for a minimum of 10 min and adult frogs were euthanized through immersion in MS-222 for a minimum of 45 min {Torreilles 2009}. To ensure animals were fully euthanized, breathing movement was monitored until it had ceased completely {Torreilles 2009}. In addition the withdrawal reflex in the legs and eye blink reflex were examined until no longer present {Torreilles 2009}.

All tissue extraction was completed in a Baker EdgeGaurd Laminar Flow Clean bench that was sterilized with both ultraviolet light and 75% ethanol. This helped to reduce the potential of sample degradation. All excision tools were sterilized with the Geminator 500 dry sterilizer (Careforde Safety and Scientific, Chicago, Illinois). To extract tissue, we made an incision along the top of the head, exposing the brain and brainstem. The brainstem, from the 3rd ventricle down, was excised and placed in ribonuclease (RNase) free 1.5-mL microcentrifuge tubes (ThermoFisher Scientific, Waltham, Massachusetts) containing 100-µL RNALater (Ambion, Carlsbad, CA). Tissues were left submerged in solution for 24 -hrs at 4 degrees Celsius (°C) before the RNALater was removed.

RNA Extraction

Brain tissue was processed with an RNeasy Plus Universal Mini Kit (Qiagen, catalog #73404) following protocol from the manufacturer with minor alterations. For tissue disruption and homogenization, step 3a. (RNeasy Plus Universal Handbook) of the protocol was followed without using a TissueRuptor. Using 950 -mL of the QIAzol Lysis Reagent provided in the kit and RNase-free, autoclaved pellet pestles (Fisher Scientific, #12-141-364) in 1.5mL RNase-free micro-centrifuge tubes, the tissue was mashed for 2 min until dissolved. The protocol was then followed from step 4 to completion. Extracted total RNA samples were analyzed with the Fragment Analyzer (Advanced Analytical, Ankeny, IA) to verify the RNA integrity. The Fragment Analyzer provides a RNA Quality Indicator (RQI) value. These samples had an RQI ≥8 indicating that the RNA was not degraded.

1-µg of RNA from each sample was prepared for sequencing by using the TruSeq Stranded RNA LT Kit with Ribo-Zero Gold (Illumina, #15032615) following the Low Sample Protocol of the TruSeq RNA Sample Prep Guide (Illumina, #15031048 Rev E). DNA template libraries were normalized and then diluted to 9 -pM quantities. Samples were sequenced using the MiSeq v2 (Illumina, San Diego, CA) sequencing platform. Three sequencing runs, using the MiSeq Reagent Kit v3, 150 cycle, (Illumina, #15043762) were performed to generate sufficient numbers of reads. Data were exported as FASTQ files. All RNA extraction and DNA template library construction was performed by the Molecular Research Core Facility at Idaho State University.

Genetic Analysis

Trinity (version 2.0.5) a de novo transcript assembler, was used to reconstruct the transciptome from the RNA-seq data. We used BFC to correct for sequencing error within the Illumina sequence {Heng 2015}. Blastx was used to translate transcripts into amino acid sequences {Altschul 1990} and then compared with the Swiss.prot database for annotation {Bairoch 2004}. Annotation was created using human homologs. Researchers have determined conserved sequences of genome in *Xenopus tropicalis* are comparable to 80% of human genes {Gilbert 2000}. For this reason we felt it was acceptable to annotate based on the human genome.

A gene set-based analysis was used to characterize differential expression at the three stages of development using sets of functionally related genes. Variations within expression of gene sets was determine with the R package edgeR {Chen 2010}, version 3.12.0. In particular, the ROAST, rotational gene set testing, was used {Wu 2010}. ROAST is intended for microarray data and is effective on small sample sizes; the number of rotations is independent of sample size {Wu 2010}. ROAST creates a test statistic that looks at the overall level of expression compared to a distribution curve created through a random rotational system. If the majority of genes within a set are above a specific threshold determined by the software, this gene set is considered to be up-regulated. If the majority is below another specific threshold, this gene set is considered to be down-regulated. Gene set testing determines a single p-value for a set instead of individual p-values for all genes within the set {Wu 2010}. This p-value is calculated through the combination of individual p-values for each gene within the set. A p-value cut off of 0.05 was selected to align with significance values used in other gene set analysis {Irizarry 2009}. In addition to the use of p-values in determining

significance, a false discover rate (FDR) test was also used to filter for significance {Subramanian 2005}. In particular it controls for the proportion of false positives {Subramanian 2005}. This can be the result of overlapping genes across several gene sets {Subramanian 2005}. An FDR value of <0.25 was selected as this is traditionally used in gene set analysis. And FDR of 25% indicates that results are likely to be valid in 75% of all cases. This has been deemed an appropriate measure in exploratory discovery (as with this study) in which further hypothesis will be explored later {MSigDB}.We obtained all gene sets of interest from the Molecular Signature Databases of the Broad Institute {MSigDB: Subramanian 2005}.

RESULTS

We were able to annotate 31,789 distinct mRNA and 53,102 transcripts using human homology. The expression of 11,111 genes and their various isoforms was determined. Of the 54 gene sets compared across the three developmental stages (as described in Table 1 and 2), none were observed to be significantly enriched between stage one and stage two tadpoles. We discovered 29 gene sets to be significantly enriched with a p-value <0.05 and FDR <0.25 between samples collected from stage one tadpoles and stage three adult frogs (Table 3). Of these 29 gene sets, 25 gene sets were significantly up-regulated in the adult frog and 4 gene sets were significantly down-regulated. A total of 24 gene sets were significantly up-regulated ($p<0.05$ and $FDR<0.25$) and 5 gene sets were significantly down-regulated ($p<0.05$ and $FDR<0.25$) between samples collected from stage two tadpoles, and stage three frogs (Table 4).

DISCUSSION

Nervous System

There were no gene sets related to the nervous system significantly down-regulated when comparing between any of the life stages. In comparison between stage one tadpoles and stage three adults, and stage two tadpoles and stage three adults, seven gene sets were significantly up-regulated in the adult stage. The genes involved in *peripheral nervous system development*, GO:0007422, *axonogenesis*, GO:0007409, *neurogenesis*, GO: 0022008, *neurite* and *neuron development*, GO:0031175 and GO:0048666, *neuron projection*, GO:0043005, and *neuron differentiation* GO:0030182 were all up-regulated in stage three adult frogs when compared with the both stages of tadpoles. Neurulation in amphibians occurs early in development, from Nieuwkoop and Faber stage 13 to Nieuwkoop and Faber stage 16 tadpoles, prior to the development of the tail bud {Gosner 1960}. Throughout the tadpole life stage, neuron development remains fairly stable {Duellman 1986}. Major reorganization of the nervous system is caused by the increase in thyroid hormone during metamorphic climax {Duellman 1986}. During metamorphic climax, degeneration of ganglia throughout the body especially in the tail is noted {Duellman 1986}. Mauthner cells and their long axons, special adaptations for aquatic life, degrade roughly two months after metamorphosis has been completed {Moulton 1968}. If reconstruction of the nervous system were to occur before entering into the metamorphosis, up-regulation would have been noted in stage one animals. With neurulation in amphibians occurring early in life, prior to the larval tadpole stage, a potential enrichment of neuron development could possibly be seen during this

embryonic stage, then again in the adult form. This would need to be further studied by means of additional experiments.

The gene set for *central nervous system development*, GO:0007417, and *brain development*, GO :0007420, were not enriched in any of the life stages. This indicates these gene sets were not enriched in either direction in our three stages of animals. This does not mean that these genes were not active during the various stages; however it may suggest that they are most highly expressed during the embryonic stage. This suggests the major development and differentiation of the central nervous system development occurs early in life prior to entering the larval life stage as a tadpole {Jacobson 1983}. *Neuron apoptosis*, GO: 0051402, was also not enriched in any stage of life. This finding is contrary to what would be expected. Thyroid hormone, found to cause the changes seen during metamorphosis, has been noted to cause cell death in amphibian brains {Tomei 1991}. As stated previously, neuronal cell death would be expected to be up-regulated during stage two and stage three animals. It has been suggested by White and Nicholl that genes associated with cell death, including neuron apoptosis, are always active, however suppressed during cell growth cycle. In frogs this suppression is caused by prolactin {White 1978}. A study has found that genes such as *ced*-9 are responsible for preventing the death of cells during development in *Caenorhabditis elegans* {Hengartner 1992}. It is possible that individual genes in the *neuron apoptosis* gene set are significantly enriched but the gene set as a whole is not. While not statistically significantly our finding indicated that the gene set would be up-regulated, with 38.4% of the genes found within the set becoming up-regulated in stage three in comparison with stage one. It is also possible that the *neuron apoptosis* gene set would have been significantly up-regulated in

stage two animals in tissue had been extracted later. Apoptosis is exhibited in the brain in Taylor and Kollros (TK) stage 21 *Rana pipiens* and Nieuwkoop and Faber (NF) stage 61 *Xenopus laevis* {Ishizuya-Oka 2010}. These stages are both after the forelimbs have developed {Ishizuya-Oka 2010}.

Tissue and Organ Development

Of the 17 gene sets related to tissue and organ development studied, five gene sets were significantly up-regulated when comparing adult stage three to stage one and stage two tadpoles; *anatomical structure development* (GO:0048856), *anatomical structure morphogenesis* (GO:0009653), *multicellular organismal development* (GO:0007275), *organ development* (GO:0048513), and *system development* (GO:0048731). These results are not what would be expected. The majority of organ modification occurs during the process of metamorphosis. For this reason it would have been expected that gene sets would have been up-regulated in stage two animals when compared to stage one and three. It is possible up-regulation of these gene sets would have occurred in stage two animals when compared to stage one animals if tissue had been extracted at a later time. Traditionally an animal is not considered to enter metamorphic climax until the forelimbs protrude {Ishizuya-Oka 2010}. This is around Nieuwkoop and Faber stage 58 in *Xenopus* and Taylor and Kollros stage 19-20 in *Rana* {Ishizuya-Oka 2010}. Around NF stage 60 and TK stage 21, major reorganization of organs such as the intestines, pancreas, and spleen occur via apoptosis {Ishizuya-Oka 2010}. For this reason I believe tissue samples were extracted too early to see changes in gene set expression between our stage one and stage two animals.

Heart development gene set GO:0007507, was down-regulated when comparing across stage three adult to stage two tadpole but not to stage one tadpoles. Amphibian hearts undergo dramatic morphological changes during metamorphosis. Tadpole hearts consist of two chambers, an atrium and ventricle, and a single loop system {Matthews 2016}. As the tadpole transitions from the use of gills to lungs, rearrangement of the circulatory system occurs to accommodate for the newly developed lungs. The heart of adult frogs consists of three chambers, two atria and one ventricle {Matthews 2016}. Blood will flow into the right atrium from sinus venous and into the left atrium from the lung {Matthews 2016}. Contraction of both atria will force blood into the ventricle {Matthews 2016}. Changes in arterial arches occur during the metamorphic transition. Tadpoles will have six atrial arches, which degrade until the existence of only three arches in adult frogs {Matthews 2016}. The down-regulation of the GO for heart development in adult frogs versus stage two tadpoles, but not stage one tadpoles suggests these transitions occur after the metamorphic process has been entered, and before metamorphic climax and adult stage has been reached.

It is important to note that the gene sets *epidermis development*, *gland development*, *skeletal development*, *skeletal muscle development*, *tissue development*, *tube development* and *tube morphogenesis*, and *vasculature development* showed no significant enrichment (either up or down) in any stage animal we studied. It is possible that these genes are only significant in the embryologic phase to create their relative structures. Then, upon entering the larval stage and metamorphosis are only modified through other genes. Last, as stated previously, it is possible these gene sets would have

been significantly up-regulated in our stage two animals if tissue had be extracted after the fore-limbs emerged.

Growth and its Regulation

Comparison of stage three to stage one and stage two animals yielded *developmental maturation* and *growth factor activity*, to being up-regulated in the later stage. Additionally the *negative regulation of growth, regulation of anatomical structure morphogenesi*s, and *regulation of organelle organization and biogenesis* were all up-regulated in adult frogs. Increase in thyroid hormone and drop in plasma prolactin levels trigger the initiation of metamorphosis {Duellman 1986}. Upon reaching metamorphic climax thyroid hormone levels rapidly return to a normal range comparable to pre-metamorphic levels {Duellman 1986}. In contrast prolactin levels continually drop until one last surge during metamorphic climax. They then remain suppressed throughout the remainder of life {Duellman 1986}. Prolactin prevents commencement of the developmental process, but its absence in the adult stage cannot explain the increase expression of regulation genes {Duellman 1986}. The removal of stimulus from the decrease in thyroid hormones may trigger the expression of many of these gene sets, in particular developmental maturation {Duellman 1986}. Corticosterone (another hormone) levels begin low in the body and gradually increase throughout metamorphosis. This hormone acts collectively with thyroid hormone to accelerate the metamorphic process {Bonette 2010: Duellman 1986}. Corticosteroids increase the mRNA levels of the thyroid receptor gene {Bonette 2010}. Kikuyama's laboratory has also shown corticosteroids increase the nuclear binding capacity of thyroid hormone {Kikuyama 1983}. There are two types of corticosteroid receptors; mineralocorticoid receptors and

glucocorticoid receptors {Kulkarni 2014}. When corticosteroids bind with their corresponding receptor, the complex will translocate into the nucleus where it will bind to mineralocorticoid or glucocorticoid response elements in promoter regions of various genes {Kulkarni 2014}.

Response to Stimuli

When comparing adult frogs to stage one and stage two tadpoles, four similar GO gene sets were significantly up-regulated; *response to abiotic stimulus* (GO: 0009628), *response to endogenous stimulus* (GO:0009719), *response to external stimulus* (GO:0009605), and *response to oxidative stress* (GO:0006950) {MSigDB}. Moreover *response to stress* (GO:0006950) was up-regulated in stage three adults in comparison to stage one tadpoles {MSigDB}.

During the metamorphic process the pancreas decreases to upwards of 80% of its original size {Duellman 1986: Esther 2009}. Upon the completion of metamorphosis the pancreas increases back to its original size or slightly larger {Duellman 1986: Esther 2009}. The pancreas is one of the endocrine glands producing hormones to trigger cellular response within the body {Duellman 1986: Esther 2009}. The gland does not become fully functional until it has been converted during the metamorphic process {Duellman 1986}. Conversion of this and other endocrine organs, as well as the increase in hormone receptor distribution within the adult frog could potentially explain the increase response to endogenous stimulus.

Genes within *response to external stimulus* include genes involved in response to visual stimulation {MSigDB}. During the metamorphic process photopigments in the eye are converted from the purple photosensitive pigment porphyropsin to roughly 70%

rhodopsin, a red-photosensitive pigment {Reuther 1971}. The upper part of the retina contains the rhodopsin pigment while the bottom half will contain the porphyropsin {Reuther 1971}. This is because bullfrog traditionally will sit with half their eye above water. The different photoreceptors are believed to aid in visual perception in the aquatic and terrestrial environments {Duellman 1986}. In addition to changes in the pigment, the eye increases in size and the internal and external corneas fuse to form a single adult cornea. Those changes all contribute to an increase in visual sensitivity and response to visual cues, necessary for the capturing of prey {Duellman 1986}. Changes to the olfactory system also occur during metamorphosis. The development of Jacobson's organ floor of the nasal sac and the differentiation of epidermal cells of the nasal chamber increase the functionality of the olfactory system. In addition to responding to the signals for food location, frogs will often use these cues for orientation {Madison 1977: Martof 1962}. These changes in sensory systems would also contribute to the increase in the gene set *response to abiotic stimulus*, as all of these allow for heightened awareness of the environmental conditions.

Response to UV (GO:0009411) was significantly down-regulated in adult frogs compared with tadpoles. It has been shown that several different species of tadpoles inhabiting either warm or cold ponds exhibit positive phototaxis {Ashby 1969: Beiswenger 1977: Mullally 1953}. When light intensity decreases, particularly at night, tadpoles will move towards open waters {Duellman 1986}. Early stage tadpoles respond most strongly towards green light {Liebmann 1968}. As development occurs tadpoles become preferential toward blue and green light, until the emergence of forelimbs, at which point tadpoles only respond well to blue light {Liebmann 1968}. This behavioral

pattern is likely a defense mechanism while feeding. Upon the completion of metamorphosis, and presentation of a more adaptive visual ability, that behavior pattern is no longer necessary and these genes become down-regulated in the adult stage.

Respiratory Patterns

Both the set *glutamate signaling pathway* (GO:0007215) and *glutamate receptor activity* (GO:0008066), were significantly down-regulated when comparing adult frogs to stage one and stage two tadpoles. The most common neurotransmitter in the brains of vertebrates is glutamate {Danbolt}. This neurotransmitter is responsible for excitatory signaling in the central nervous system {Danbolt}. Glutamate helps to produce and maintain the neural synapse necessary for synaptic plasticity {Danbolt}. This plasticity is the ability for a neuron to alter its structure and connections {Danbolt}. Glutamate is responsible for cognition, memory, and learning {Danbolt}.

Glutamate transmission has been linked with the development of breathing cycles within various vertebrates such turtles, birds, and frogs {Chen 2008: Johnson 1998: Vincen-Brown 2015}. Glutamate allows for lung burst activity in pre-metamorphic and post-metamorphic frogs during both aquatic and terrestrial periods of life {Chen 2008}. Such activity is accomplished through the stimulation of non-NMDA receptors {Chen 2008}. Changes in neurotransmitters controlling lung burst activity occur throughout development. GABA and glycine receptors play a more substantial role during adult life than pre-metamorphic stages {Galante 1996}. Our results indicate the rate of expression of glutamate receptors and glutamate signaling pathways are down-regulated during the adult stage of life. The change in genes associated with glutamate pathways and receptors

in pre-metamorphic tadpoles as compared to adult frogs align with the known role of glutamate in the generation of lung bursts during metamorphosis {Chen 2008}.

The set GO: 0007585 contain genes for *respiratory gaseous exchange* {MSigDB}. This class of genes was identified as being significantly enriched through up-regulation in the adult frog when compared to stage one and stage two animals. Genes occurring in the set GO:0007585 include those for regulation of respiration and proteins aiding in respiration. Frog tadpoles can respire via brachia, cutaneous, and gills {Duellman 1994}. Once reaching adult stages, frogs respire through their lungs, skin, and buccopharyngeal region {Duellman 1994}. Adult frogs rely most heavily upon the uptake of oxygen through lungs and the buccopharyngeal mucosa, because the absorption of oxygen through the skin is dependent on capillary proximity {Duellman 1994}. Variation in rates at different developmental stages has been correlated with the difference in body size of the animal {Duellman 1994}. An increased rate of oxygen consumption is noted with an increase in size {Duellman 1994}. Our results are consistent with these previous findings. Upon entering metamorphosis, a period characterized by dramatic increase in body size, those genes associated with respiratory gaseous exchange are up-regulated.

Feeding and Digestion

We determined that the set *feeding behavior* (GO:0007631), was significantly down-regulated when comparing stage three adult frogs with stage one and stage two tadpoles. All genes within this group are classified as genes associated with the intake of food {MSigDB}. Some of the genes included in this set are those for positive and negative regulation of eating and feeding behavior (e.g. neuropeptides S-receptor, and insulin-like peptide INSL5), suckling behavior in mammals (hairy and enhancer of split-

related protein, HELT), and conditioned taste aversion (mGluR7) {Carbon 2009}. This down-regulation of feeding genes aligns with the observed differences in feeding habits between tadpoles and adult frogs. Frog tadpoles are considered continuous feeders, based on research findings noting the digestive tracts filled with ingested material {Jenssen 1967}. Studies on the digestive tract during different metamorphic stages concluded that tadpoles fast during the metamorphic process {Jenssen 1967}. Fasting typically begins upon the appearance of forelimbs {Jenssen 1967}. Behavioral studies indicate that juvenile green frogs, *Rana clamitans*, exhibit a higher frequency of feeding than adult frogs {Jenseen 1966}. Potential explanation of this change in feeding habits, noted at different ages, could be related to sexual maturity. Adult frogs may unintentionally limit their available food source during mating seasons by restricting themselves to breeding sites {Jenseen 1966}. Although feeding habits were down-regulated, the gene set *digestion* (GO:0007586) was not significantly enriched in either direction. This gene sets contains genes involved in coding of digestive enzymes, for example gastricsin precursor (PGC) and pancreatic alpha amylase (AMY2A). This outcome indicates that despite drastic changes occurring to the digestive tract during the metamorphic process, it is possible that coding for enzymes necessary in the digestion of various materials remain constant throughout life.

CONCLUSION

The results of our gene set testing have implications for the genetic alterations occurring during the metamorphic process in the American bullfrog. This is the first study of gene expression in the bullfrog between larval tadpoles and the adult frog. It

allows us to create a basis for alterations occurring to genetic expression contributing to the changes noted during metamorphosis. We do have some cautions regarding our results.

Because of the staging type used in this study, we cannot exclude the possibility that stage two animals had not yet entered into metamorphosis and in turn could essentially be considered in the same stage as stage one tadpoles. This could explain the reason we observed no significant changes in gene set expression between stage one and stage two samples. In the future, therefore, tissue extraction from different stages should align more closely with the traditional Taylor and Kollros method. An increase in replicates for each stage would also strengthen any conclusions reached through this study. Annotation of our transcriptome was created using homologs from the human genome. The availability of a whole-genome sequence for the American bullfrog would increase the number of identified transcripts used for comparison across stages. Last, we must note that it may be beneficial to study the expression of certain gene sets in more appropriate tissue. For example the gene set *digestion* or *tube morphogenesis* may be more relevant to expression within tissue extracted from the digestive tract rather than that of the brainstem.

Despite these caveats, our gene set analysis revealed some interesting patterns. For example a decrease in expression of genes associated with glutamate signaling and glutamate receptors activity was noted in stage three adult frogs. This is also true with feeding behaviors. The majority of gene sets found to be up-regulated in the adult frog were associated with general organism development and morphogenesis, as well as neuron development and differentiation. Possibly the most interesting finding was the up-

regulation of the gene set *heart development* in stage two tadpoles. This work establishes a basis for future investigations of American bullfrog morphogenesis. Another possible next step would be to test for similar patterns in expression in other amphibian species known to undergo metamorphosis. Additionally, through the establishment of expression profiles in normal contaminant-free environments we can begin to create comparisons of expressions in tadpoles and frogs living in polluted environments. This would lead to understanding the effects of pollutants on the metamorphic process.

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Table One:

List of the 54 gene ontology gene sets hypothesized to be enriched during the metamorphic process of the bullfrog.

GO Symbol	GO Description	Direction
GO related to the nervous system		
GO:0007409	Axonogenesis	(+)
GO:0007420	Brain Development	(+)
GO:0007417	Central Nervous System Development	(+)
GO:0031175	Neurite Development	(+)
GO:0022008	Neurogenesis	(+)
GO:0051402	Neuron Apoptosis	(-)
GO:0048666	Neuron Development	(+)
GO:0030182	Neuron Differentiation	(+)
GO:0043005	Neuron Projection	(+)
GO:0007422	Peripheral Nervous System Development	(+)
GO related to tissue/organ development		
GO:0048856	Anatomical Structure Development	(+)
GO:0048646	Anatomical Structure Formation	(+)
GO:0009653	Anatomical Structure Morphogenesis	(+)
GO:0008544	Epidermis Development	(+)
GO:0048732	Gland Development	(+)
GO:0007507	Heart Development	(+)
GO:0007275	Multicellular Organismal Development	(+)
GO:0048513	Organ Development	(+)
GO:0009887	Organ Morphogenesis	(+)
GO:0001501	Skeletal Development	(+)
GO:0007519	Skeletal Muscle Development	(+)
GO:0048731	System Development	(+)
GO:0009888	Tissue Development	(+)
GO:0048729	Tissue Morphogenesis	(+)
GO:0035295	Tube Development	(+)
GO:0035239	Tube Morphogenesis	(+)
GO:0001944	Vasculature Development	(+)
GO related to growth		
GO:0048589	Developmental Growth	(+)
GO:0021700	Developmental Maturation	(+)
GO:0040007	Growth	(+)
GO:0008083	Growth Factor Activity	(+)

GO related to regulation of growth/development		
GO:0045926	Negative Regulation of Growth	(-)
GO:0050793	Positive Regulation of Development Process	(+)
GO:0009653	Regulation of Anatomical Structure Morphogenesis	(+)
GO:0022603	Regulation of Development Process	(+)
GO:0050767	Regulation of Neurogenesis	(+)
GO:0033043	Regulation of Organelle Organization and Biogenesis	(+)
GO related to response to stimuli		
GO:0009628	Response To Abiotic Stimulus	(+)
GO:0009607	Response To Biotic Stimulus	(+)
GO:0009719	Response To Endogenous Stimulus	(+)
GO:0009605	Response To External Stimulus	(+)
GO:0009408	Response To Heat	(+)
GO:0009416	Response To Light Stimulation	(+)
GO:0006979	Response To Oxidative Stress	(+)
GO:0006950	Response To Stress	(+)
GO:0009266	Response To Temperature Stimulus	(+)
GO:0009411	Response To UV	(+)
GO related to glutamate		
GO:0008066	Glutamate Receptor Activity	(+)
GO:0007215	Glutamate Signaling Pathway	(+)
Other GO groups		
GO:0007586	Digestion	(-)
GO:0007631	Feeding Behavior	(-)
GO:0042592	Homeostatic Process	(+)
GO:0045271	Respiratory Chain Complex I	(+)
GO:0007585	Respiratory Gaseous Exchange	(+)

A complete list of the 54 GO sets hypothesized to be enriched during the metamorphic process in the American bullfrog. Direction indicated the direction of predicted change in gene expression; (+) indicated gene sets will be up-regulated in later stages of development, (-) indicated genes sets will be down-regulated in the later stages of development.

Table Two: A description of the functionality of each gene set explored.

GO related to the nervous system		
GO:0007409	Axonogenesis	The creation of an axon, the part of a neuron that carries efferent action potentials.
GO:0007420	Brain Development	The process of developing the brain from an immature to mature state.
GO:0007417	Central Nervous System Development	The creation of the brain, spinal cord, and spinal nerves over time.
GO:0031175	Neurite Development	The development of any part extending from a neural cell, such as axons and dendrites.
GO:0022008	Neurogenesis	The development of cells within the nervous system.
GO:0051402	Neuron Apoptosis	The process of apoptosis, or programmed death, of neurons.
GO:0048666	Neuron Development	The progression of a neuron over time to a mature state.
GO:0030182	Neuron Differentiation	The specialization of a neuron.
GO:0043005	Neuron Projection	The process of extending the nerve cell through axons and dendrites.
GO:0007422	Peripheral Nervous System Development	The formation of the network of nerves connecting sensory organs, muscles, and other parts of the body to the central nervous system.
GO related to tissue/organ development		
GO:0048856	Anatomical Structure Development	Genes for the progression of an anatomical structure from an initial condition to maturation.
GO:0048646	Anatomical Structure Formation	Genes associated with the initial formation of an anatomical structure.
GO:0009653	Anatomical Structure Morphogenesis	Pertaining to the creation of anatomical structures.
GO:0008544	Epidermis Development	All genes involved in the maturation of epidermis.
GO:0048732	Gland Development	The development of an organ whose specific function is secretion.

GO:0007507	Heart Development	Genes involved in the progressive development of the heart.
GO:0007275	Multicellular Organismal Development	Genes involved in the process of progressing from an initial state to a mature state.
GO:0048513	Organ Development	The development of a tissue or tissues that work together for a function.
GO:0009887	Organ Morphogenesis	The generation and organization of an organ.
GO:0001501	Skeletal Development	Genes whose outcome is for the development of the skeleton.
GO:0007519	Skeletal Muscle Development	The development of adult muscle through the fusion of myoblasts, formation of myofibrils, and development of neuromuscular junctions.
GO:0048731	System Development	The process of creating an organismal system over time.
GO:0009888	Tissue Development	The formation of tissue over time.
GO:0048729	Tissue Morphogenesis	The generation and organization of tissue.
GO:0035295	Tube Development	The process of producing a tube over time. These tubes are used for transport of gases, liquids, and cells. They can include the gastrointestinal tract, urinary tract, vascular system and more.
GO:0035239	Tube Morphogenesis	The generation and organization of tubes.
GO:0001944	Vasculature Development	The creation of a mature vasculature system.
GO related to growth		
GO:0048589	Developmental Growth	The increase in mass of an organism with time until a mature state is reached.
GO:0021700	Developmental Maturation	The process of cells, cellular components, and anatomical structures reaching full functionality.
GO:0040007	Growth	The increase in size or mass of the entire organism.

GO:0008083	Growth Factor Activity	The stimulation of a cell to grow or proliferate.
GO related to regulation of growth/development		
GO:0045926	Negative Regulation of Growth	The act of preventing, reducing, or stopping the extent of growth.
GO:0050793	Positive Regulation of Development Process	The process of activating or increasing the rate of development to a mature state.
GO:0009653	Regulation of Anatomical Structure Morphogenesis	Modulation of frequency and rate of morphogenesis in anatomic structures.
GO:0022603	Regulation of Development Process	Anything regulating the rate and extent of development from and initial to mature state.
GO:0050767	Regulation of Neurogenesis	The regulation of rate and extent of neuron origin and formation.
GO:0033043	Regulation of Organelle Organization and Biogenesis	The regulation of processes involved in arrangement or an organelle.
GO related to response to stimuli		
GO:0009628	Response To Abiotic Stimulus	Change in cell or organism as a result of non-living stimulus
GO:0009607	Response To Biotic Stimulus	Change caused by a stimulus produced by a living organism.
GO:0009719	Response To Endogenous Stimulus	Change in activity because of endogenous stimulus.
GO:0009605	Response To External Stimulus	Change in an organism or its cells caused by external stimulus.
GO:0009408	Response To Heat	A temperature stimulus above the optimal temperature triggering a change in state.
GO:0009416	Response To Light Stimulation	Change in state caused by electromagnetic radiation of infrared, visible, or ultraviolet light.
GO:0006979	Response To Oxidative Stress	Change caused by oxidative stress. This can be from exposure to reactive oxygen species like superoxide anions.
GO:0006950	Response To Stress	A stimulus indicating the organism is under stress, likely from exogenous factors, causing a change in state.

GO:0009266	Response To Temperature Stimulus	A change in temperature causing the change in overall state or activity of organism or cells.
GO:0009411	Response To UV	Change caused by UV light stimulus.
GO related to glutamate		
GO:0008066	Glutamate Receptor Activity	Genes that cause a change in cell activity by combination with glutamate.
GO:0007215	Glutamate Signaling Pathway	Molecular signaling generated by the binding of glutamate to cell surface receptors.
Other GO groups		
GO:0007586	Digestion	All elements (i.e. physical, chemical, biochemical) involved in breaking down ingested nutrients from absorption.
GO:0007631	Feeding Behavior	Genes associated with the behavior of in-taking food.
GO:0042592	Homeostatic Process	The processes involved in maintaining internal equilibrium.
GO:0045271	Respiratory Chain Complex I	The first enzyme found in the respiratory chain at which NADH enters into the complex.
GO:0007585	Respiratory Gaseous Exchange	The process of exchanging gas between and organism and the environment.

A description of the role of genes found in each GO gene set. All gene sets were selected from the Molecular Signature Databases of the Broad Institute {MSigDB: Subramanian 2005}.

Table Three:

ROAST gene set analysis for differential expression of GO terms between stage one and stage three for the American bullfrog.

GO term	GO definition	Number of Genes	Prop Down	Prop Up	Direction	P value	FDR
Gene sets up-regulated:							
GO related to the nervous system							
GO:0007409	Axon-ogenesis	579	0.2815199	0.39551	Up	0.0004	0.00564546
GO:0031175	Neurite Development	820	0.2890244	0.378049	Up	0.001	0.00564546
GO:0022008	Neurogenesis	1216	0.3149671	0.355263	Up	0.0024	0.00882
GO:0048666	Neuron Development	900	0.2955556	0.371111	Up	0.0011	0.00564546
GO:0030182	Neuron Differentiation	1073	0.3122088	0.357875	Up	0.0052	0.01463684
GO:0043005	Neuron Projection	698	0.2965616	0.401146	Up	0.0041	0.01275
GO:0007422	Peripheral Nervous System Development	50	0.18	0.54	Up	0.0001	0.00135
GO related to tissue/organ development							
GO:0048856	Anatomical Structure Development	3246	0.3065311	0.327172	Up	0.0006	0.00564546
GO:0009653	Anatomical Structure Morph-ogenesis	1847	0.302653	0.336762	Up	0.0021	0.00851539
GO:0007275	Multicellular Organismal Development	3093	0.3145813	0.325574	Up	0.0012	0.00564546
GO:0048513	Organ Development	1864	0.3154506	0.314378	Up	0.0234	0.04664483
GO:0048731	System Development	2789	0.3087128	0.333094	Up	0.0001	0.00135
GO related to growth							
GO:0021700	Develop-mental Maturation	154	0.2987013	0.383117	Up	0.0008	0.00564546

GO:0008083	Growth Factor Activity	60	0.216666 7	0.3166 67	Up	0.0098	0.021 9375
GO related to regulation of growth/development							
GO:0045926	Negative Regulation of Growth	128	0.320312 5	0.3437 5	Up	0.0043	0.012 75
GO:0022603	Regulation of Anatomical Structure Morphogenes is	578	0.316609	0.3408 3	Up	0.0067	0.016 32273
GO:0033043	Regulation of Organelle Organization and Biogenesis	673	0.280832 1	0.3224 37	Up	0.0042	0.012 75
GO related to response to stimuli							
GO:0009628	Response to Abiotic Stimulus	717	0.295676 4	0.3221 76	Up	0.0057	0.015 255
GO:0009719	Response to Endogenous Stimulus	1083	0.273314 9	0.3471 84	Up	0.0007	0.005 64546
GO:0009605	Response to External Stimulus	1498	0.261014 7	0.3578 1	Up	0.0009	0.005 64546
GO:0006979	Response to Oxidative Stress	256	0.238281 3	0.3437 5	Up	0.0244	0.046 64483
GO:0006950	Response to Stress	2403	0.285060 3	0.3179 36	Up	0.0251	0.046 64483
Other GO groups							
GO:0042592	Homeostatic Process	961	0.281997 9	0.3309 05	Up	0.0064	0.016 32273
GO:0045271	Respiratory Chain Complex I	38	0.026315 8	0.6578 95	Up	0.0122	0.025 23462
GO:0007585	Respiratory Gaseous Exchange	50	0.18	0.38	Up	0.0012	0.005 64546
Gene sets down-regulated:							
GO related to stimuli							
GO:0009411	Response to UV	83	0.337349 4	0.1445 78	Down	0.0025	0.008 82

GO related to respiratory and feeding function:

GO:0008066	Glutamate Receptor Activity	26	0.5769231	0.115385	Down	0.0091	0.021 24783
GO:0007215	Glutamate Signaling Pathway	65	0.5076923	0.138462	Down	0.0107	0.023 004
GO:0007631	Feeding Behavior	65	0.4307692	0.184615	Down	0.0021	0.008 51539

Results of the 29 significantly enriched GO terms between stage one and stage three animals, tested for using ROAST. Number of genes refers to the number of genes in the set. PropDown and PropUp indicated the proportion of genes within the gene set that decreased or increased with an absolute change greater than $\sqrt{2}$. FDR represents the false discovery rate.

Table Four: ROAST gene set analysis for differential expression of GO terms between stage two and stage three for the American bullfrog.

GO term	GO definition	Number of Genes	Prop Down	Prop Up	Direction	P value	FDR
Gene sets up-regulated:							
GO related to the nervous system							
GO:0007409	Axono-genesis	579	0.26943005	0.373057	Up	0.0003	0.00405
GO:0031175	Neurite Development	820	0.27804878	0.3585366	Up	0.0005	0.00405
GO:0022008	Neurogenesis	1216	0.30263158	0.3371711	Up	0.003	0.0122539
GO:0048666	Neuron Development	900	0.28	0.3522222	Up	0.0011	0.0070875
GO:0030182	Neuron Differentiation	1073	0.30195713	0.3364399	Up	0.0067	0.0211235
GO:0043005	Neuron Projection	698	0.29799427	0.3925501	Up	0.0039	0.01485
GO:0007422	Peripheral Nervous System Development	50	0.14	0.48	Up	0.0001	0.0027
GO related to tissue/organ development							
GO:0048856	Anatomical Structure Development	3246	0.30067776	0.3142329	Up	0.0005	0.00405
GO:0009653	Anatomical Structure Morphogenesis	1847	0.30157011	0.3188955	Up	0.0059	0.0197438
GO:0007275	Multicellular Organismal Development	3093	0.30746848	0.3152279	Up	0.0022	0.0122539
GO:0048513	Organ Development	1864	0.31276824	0.3025751	Up	0.0426	0.079231
GO:0048731	System Development	2789	0.30225887	0.320545	Up	0.0004	0.00405
GO related to growth							
GO:0008083	Growth Factor Activity	60	0.25	0.2833333	Up	0.0193	0.04158

GO:0021700	Developmental Maturation	154	0.25974026	0.3831169	Up	0.0005	0.00405
GO related to regulation of growth/development							
GO:0045926	Negative Regulation of Growth	128	0.359375	0.3515625	Up	0.0134	0.0327682
GO:0022603	Regulation of Anatomical Structure Morphogenesis	578	0.32179931	0.3148789	Up	0.024	0.0497423
GO:0033043	Regulation of Organelle Organization and Biogenesis	673	0.29420505	0.3060921	Up	0.0043	0.0153
GO related to response to stimuli							
GO:0009628	Response to Abiotic Stimulus	717	0.29428173	0.2956764	Up	0.0079	0.021195
GO:0009719	Response to Endogenous Stimulus	1083	0.26869806	0.3379501	Up	0.001	0.0070875
GO:0009605	Response to External Stimulus	1498	0.25967957	0.3371162	Up	0.0024	0.0122539
GO:0006979	Response to Oxidative Stress	256	0.2109375	0.3320312	Up	0.0337	0.0673
Other GO groups							
GO:0042592	Homeostatic Process	961	0.27991675	0.3194589	Up	0.0074	0.021195
GO:0045271	Respiratory Chain Complex I	38	0.02631579	0.5526316	Up	0.0183	0.0410625
GO:0007585	Respiratory Gaseous Exchange	50	0.16	0.34	Up	0.0026	0.0122539
Gene sets down-regulated:							
GO related to tissue/organ development							
GO:0007507	Heart Development	332	0.35240964	0.253012	Down	0.039	0.0751179
GO related to response to stimuli							
GO:0009411	Response to UV	83	0.37349398	0.1686747	Down	0.0077	0.021195

GO related to glutamate							
GO:0008066	Glutamate Receptor Activity	26	0.5384 6154	0.11538 46	Down	0.0091	0.0232 714
GO:0007215	Glutamate Signaling Pathway	65	0.4923 0769	0.15384 62	Down	0.0146	0.0341 609
Other GO groups							
GO:0007631	Feeding Behavior	65	0.4769 2308	0.21538 46	Down	0.0029	0.0122 539

The results of ROAST gene set analysis between stage two and stage three bullfrogs representing the 29 significantly enriched gene sets. The GO term and definition are those retrieved from the MSigDB [31]. The number of genes refers to the number of genes found in that specific gene set. PropDown and PropUp indicated the proportion of genes in the set that are changed with a fold greater than$\sqrt{2}$. FDR represents the false discovery rate.